AWARD NUMBER: W81XWH-15-2-0046

TITLE: VIPER: Chronic Pain after Amputation: Inflammatory Mechanisms, Novel Analgesic Pathways, and Improved Patient Safety

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CONTRACTING ORGANIZATION: Duke University
Durham NC 27705-4677

REPORT DATE: October 2016

TYPE OF REPORT: Annual

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
Oct 2016	Annual	15 Sep 2015 - 14 Sep 2016

4. TITLE AND SUBTITLE

VIPER: Chronic Pain after Amputation: Inflammatory Mechanisms, Novel Analgesic Pathways, and Improved Patient Safety

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-15-2-0046

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

Duke University
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Durham, NC 27705

8. PERFORMING ORGANIZATION REPORT NUMBER

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

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

10. SPONSOR/MONITOR'S ACRONYM(S)

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

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Chronic pain is a significant problem after nerve injury from trauma or surgery. Current therapies and attempts at prevention have proven largely ineffective. Through analysis of data obtained in the Molecular Signatures of Chronic Pain Subtypes study termed Veterans Integrated Pain Evaluation Research (VIPER) (W81XWH-11-2-0003) we have discovered two novel pain pathways with potential therapeutic relevance (Wnt and TGR5). In addition, we recognize that improving the safety and efficacy of existing therapies must continue to be a priority and plan to use the large pharmacogenomic database at Vanderbilt University to identify patients at risk for adverse opioid related events. The current proposal intends to study the contribution of non-neuronal immune cells (macrophages) to chronic pain while also evaluating novel analgesics in relevant animal models. The current proposal also attempts to determine the optimal patient population for opioid therapy while identifying those patients at greatest risk from opioids.

15. SUBJECT TERMS

Post-amputation pain, Phantom limb pain, Residual limb pain, neuropathic pain, novel analgesics, opioid related adverse events.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	42	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION:
Chronic pain is a significant problem after nerve injury from trauma or surgery. Current therapies and attempts at prevention have proven largely ineffective. Through analysis of data obtained in the Molecular Signatures of Chronic Pain Subtypes study termed Veterans Integrated Pain Evaluation Research (VIPER) (W81XWH-11-2-0003) we have discovered two novel pain pathways with potential therapeutic relevance (Wnt and TGR5). In addition, we recognize that improving the safety and efficacy of existing therapies must continue to be a priority and plan to use the large pharmacogenomic database at Vanderbilt University to identify patients at risk for adverse opioid related events. The current proposal intends to study the contribution of non-neuronal immune cells (macrophages) to chronic pain while also evaluating novel analgesics in relevant animal models. The current proposal also attempts to determine the optimal patient population for opioid therapy while identifying those patients at greatest risk from opioids.

KEYWORDS:
Post-amputation pain, Phantom limb pain, Residual limb pain, neuropathic pain, novel analgesics, opioid related adverse events.

ACCOMPLISHMENTS:
What were the major goals of the project?

Goal 1: Characterize the role of Wnt signaling in macrophage polarization, mouse nerve injury models and human neuroinflammation.

Major Task 1: Characterize macrophage polarization changes after Wnt signaling modification in mouse macrophage cell culture – 0% complete

Major Task 2: Determine the specific wnt pathway responsible for prevention of mechanical allodynia in a mouse model of peripheral nerve injury and correlate this with macrophage polarization state and IL-6 to IL-10 ratio – 20% complete. We have collected plasma samples from mice with SNI and will now perform ELISA for IL-6 and IL-10 both at Duke and UHSHS.

Major Task 3: Characterize wnt pathway expression and DNA methylation changes in humans before and after amputation and determine the role of cytokine ratio measurement in prediction of pain phenotype - 30% complete

We have completed initial qPCR analysis of VIPER patient plasma looking at a number of wnt pathway constituents. This work was done by the lab at UHSHS and the results are displayed in Table 1 below. In this patient population there was a significant difference in the CTNNB1 gene with upregulation apparent in patients with pain. Two other wnt pathway constituents had differences between case and control that almost reached significance at the time of enrollment which was 3-18 months after amputation. The presence of one significant and a number of almost significant changes in wnt pathway constituents is exciting and, frankly, somewhat unexpected since the phenotype is already present at the collection time point. We will continue this experiment with VIPER valproate patient plasma using presurgical samples (and the knowledge of who goes on to develop chronic pain) to determine if wnt pathway

expression at the time of injury correlates with propensity to develop chronic pain in the future.

Table 1: Expression analysis of selected wnt pathway constituents in patients with and without residual limb pain enrolled in the Veterans Integrated Pain Evaluation Research (VIPER) study.

wnt pathway gene	Case	Control	p-value
APC	0.928	1.018	0.11
CTNNB1	0.9	1.018	0.05
FZD1	0.884	1.024	0.06
FZD3	1.183	1.081	0.25
LRP-3	1.152	1.118	0.4
LRP6-3	1.579	1.267	0.08

Goal 2: Determine the role of TGR5 in astrocyte activation and treatment of mechanical allodynia in a mouse model of neuropathic pain.

Major Task 1: Determine role of TGR5 signaling in astrocyte activation – 30% complete

Astrocyte culture conditions have been worked out. Initial experiment looking at astrocyte activation using the TGR5 agonists deoxycholate and OA did not show differences in activation as our initial experiments did. We will continue this Task and perform using our Roche agonist.

Major Task 2: Determine the role of TGR5 signaling in treating mechanical allodynia in a mouse peripheral nerve injury model - 100% complete

The TGR5 agonist (deoxycholate) reduces baseline mechanical sensitivity in C57Bl6 mice (Figure 1)

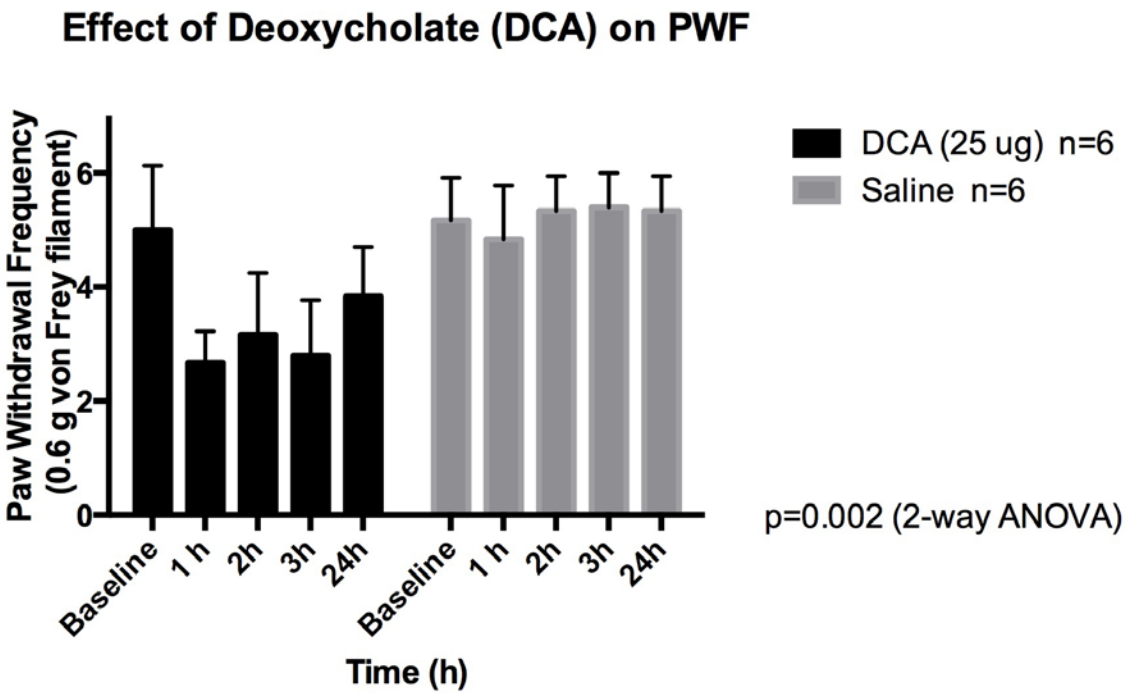


Figure 1: DCA reduces mechanical sensitivity at baseline

We also found that the TGR5 agonist deoxycholate reduces mechanical allodynia in a peripheral nerve injury mouse model most dramatically at 21 days after injury suggesting that the effect is occurring through an astrocyte activation pathway as astrocyte activation occurs in the late stages of neuropathic pain transition (Figure 2)

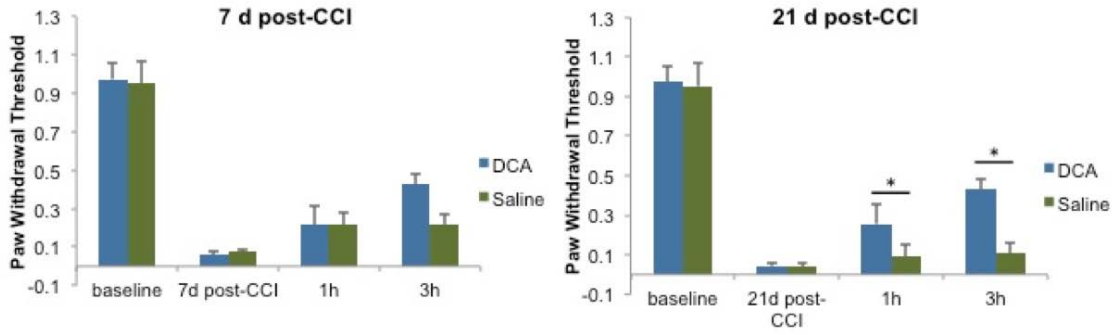


Figure 2: DCA reduces mechanical allodynia in a CCI model of peripheral nerve injury

Most excitingly, the orally available TGR5 agonist obtained from Roche decreases mechanical allodynia after SNI peripheral nerve injury at 7 days after injury (Figure 3)

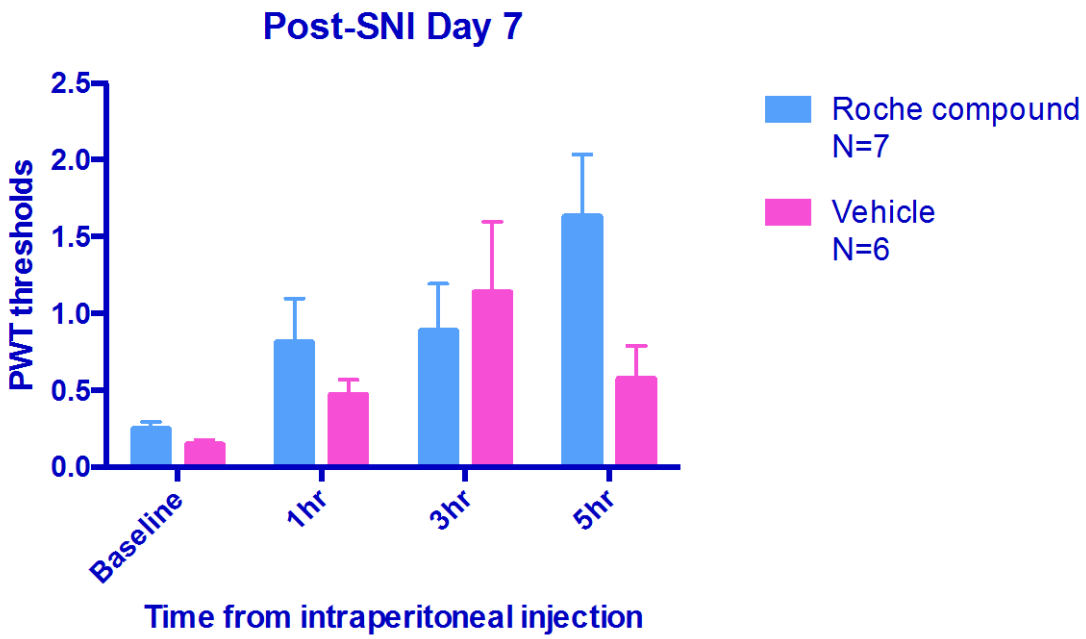


Figure 3: Paw withdrawal threshold is increased (allodynia relieved) by treatment with the Roche TGR5 agonist at day 7 after SNI surgery at the 5hr time point.

Goal 3: Use existing data from the Vanderbilt EMR and genotyping repositories to look for associations between genetic variants and pain phenotypes

Major Task 1: Preliminary analyses conducted to confirm the precise numbers of patients for whom there are sufficient data available. Validation of previously published genotype-phenotype associations – 30% complete

With the Vanderbilt team, we have begun to tackle the most difficult part of this aim which is to identify patients who have had opioid related adverse events in hospital. Once these patients are identified, SNPs associated with risk can be defined using the existing dataset. Below is a brief overview of our selection criteria. This will be expanded upon in the next two quarterly reports. I also have a meeting set up with Vanderbilt in January to discuss patient selection for opioid adverse events.

Outcome =

PRIMARY OUTCOME = MAJOR Adverse events PROBABLE = A + B

SECONDARY OUTCOME = MAJOR Adverse events POSSIBLE = B

A= MAJOR ADVERSE OUTCOME OCCURRED = {DEATH OR CARDIAC/ RESPIRATORY ARREST OR ICU ADMISSION OR RESPIRATORY SUPPORT OR COMPLICATIONS OF SURGICAL AND MEDICAL CARE NOT ELSEWHERE}

Death => (based on UB-04 Discharge Status Code).

Cardiac/Respiratory Arrest => ICD-9 Diagnoses Codes 427.5 / 799.0 / 799.1 OR ICD-9 Procedure Codes 99.60/ 99.62 / 93.93 OR CPT Code 92950 (CPR) / or ICD9 V Code V 12.53

ICU Admission = Charges for ICU Admission

RESPIRATORY SUPPORT = INTUBATION (96.04) OR NON-INVASIVE VENTILATION 93.9x OR MECHANICAL VENTILATION 96.7x
OR COMPLICATIONS NOT ELSEWHERE CLASSIFIED (ICD-9 code 997.3 respiratory complications; 997.01 neurologic complications) OR Hypercapnia (786.09) OR Acute respiratory failure (518.81, 518.82, 518.83, 519.8) OR Apnea (786.03) OR Resp Distress (786.09)

B = OPIOID OVERDOSE MAY HAVE OCCURRED = {USE OF OPIOID REVERSAL OR DIAGNOSIS OF POISONING}
USE OF OPIOID REVERSAL = CHARGE FOR NALOXONE OR ICD-9 code for poisoning by sedatives and hypnotics 967.X OR central nervous system depressants ICD-9 code 968.X OR psychotropic agents 969.X

Major Task 2: Discovery and validation of novel exomic variants associated with opioid adverse drug events – 0% complete

What was accomplished under these goals?

Overall Progress
We are progressing as expected with the organization, experiments and logistics of this project. Following is a detailed list outlining accomplishments for this quarter.

- In late 2015, Dr. Van de Ven and Dr. Buchheit traveled to Vanderbilt University to meet with Dr. Shaw, Dr. Walsh and Dr. Bruehl over the course of two days to design the algorithms necessary to accurately identify opioid related adverse events from the Vanderbilt database. During our more recent visit to Vanderbilt, we also collaborated on a VIPER cytokine analysis paper that was accepted by the journal PAIN this month.
- In our Duke laboratory, we completed the Material Transfer Agreement and received the Roche TGR5 agonist necessary to complete specific aim 2. We have completed study of the effect of this agonist, with intraperitoneal injection, on the baseline paw sensitivity of treated mice.
- In our Duke laboratory, we have reproduced the decrease in baseline mechanical allodynia seen with deoxycholate (A constitutive TGR5 agonist) treatment in mouse paw. See figure above.
- We have found that TGR5 agonist given intraperitoneally reduced mechanical allodynia on day 7 after injury. See figure above.
- We have begun preliminary work with one of the Duke Flow Cytometry facilities to ensure the experimental parameters to complete flow sorting of macrophages are correct.
- UHSHS has been sent the cDNA samples from VIPER study patients for wnt pathway analysis. Because the amount of cDNA from these patients was limited, we decided to save the wnt arrays for the VIPER Valproate samples and to perform targeted wnt pathway analysis on the VIPER samples. They have completed initial qPCR analysis of a number of wnt pathway constituents,

What opportunities for training and professional development has the project provided?
Nothing to Report

How were the results disseminated to communities of interest?
Though we are still in the beginning stages of this work, we have had some interesting results that have been presented in poster and abstract form at our departmental research retreat.

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

Description of work to be performed/completed during the next reporting period
During the next three months, we expect:

- Final ACURO animal protocol approval
- Complete quantitative PCR analysis of the first round of human amputee blood samples to evaluate wnt pathway gene expression changes
- Begin ELISA based analysis of macrophage and astrocyte cultures to determine the effects of TGR5 agonists and wnt agonists on these important cell types.

IMPACT:
Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
Pain medicine is limited by the limited number of new analgesics and adverse effects of opioids. Over the past year we have confirmed that TGR5 is important in both inflammatory pain and neuropathic pain and that a TGR5 agonist reduces the sensitivity in an animal pain model. There is a long road before therapies like this can be used in humans but we are taking the first steps.

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?
We are still early in this project and results have not been published although there is preliminary evidence that TGR5 agonists are able to treat mechanical allodynia in mice.

CHANGES/PROBLEMS:
Nothing to report

Changes in approach and reasons for change
There were no significant changes in approach. Minor changes include the method of delivery of TGR5 agonists. We have decided to use intraperitoneal dosing for two reasons: 1) mice treated intrathecally were behaving strangely and we were concerned the solvent needed to dissolve the TGR5 was causing neurotoxicity 2) the intraperitoneal approach (equivalent to IV in humans) will be a more convenient treatment site when these therapies are eventually tried in human trials.

Other minor changes include additional validation of flow cytometry results using qPCR of known targets that distinguish M1 from M2 phenotype in macrophages.

Actual or anticipated problems or delays and actions or plans to resolve them
ACURO approval was delayed, but this did not delay completion of animal work as we used other funding sources to continue our IACUC approved protocols

Astrocyte culture was unsuccessful for the first 2 attempts. The problem turned out to be a simple error in that the growth media being used did not contain enough glucose. This problem has been resolved and astrocyte cultures are now growing, but ELISA work has not yet begun.

Changes that had a significant impact on expenditures
No significant changes on expenditures

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
No changes or deviations

PRODUCTS:
Published
Chamessian A*, Van de Ven TJ*, Buchheit T, Hsia H, McDuffie M, Gamazon ER, Walsh C, Bruehl S, Buckenmaier C, Shaw A. Differential Expression of Systemic Inflammatory Mediators in Amputees with Chronic Residual Limb Pain. Pain. Publish Ahead of Print, 23 September 2016, 10.1097/j.pain.000000000000072. *Co-first authors.

Kent ML, Hsia HJ, Van de Ven TJ, Buchheit TE. Perioperative Pain Management Strategies for Amputation: A Topical Review. Pain Med. 2016 Jul 8. pii: pnw110. [Epub ahead of print]. PubMed PMID: 27402960

Books or other non-periodical, one-time publications.
Nothing to report

Other publications, conference papers, and presentations.
Submitted:
Chamessian A, Qadri Y, Cummins M, Berta T, Hendrickson M, Buchheit T, Van de Ven T, “5-hydroxymethylcytosine (5hmC) and Ten-eleven translocation 1-3 (TET1-3) proteins in the dorsal root ganglia: expression and dynamic regulation in neuropathic pain.” Submitted, Brain Research, July 2016.

Website(s) or other Internet site(s)
Nothing to report

Technologies or techniques
Nothing to report

Other Products
Nothing to report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS
What individuals have worked on the project?

Name: Thomas Van de Ven
Project Role: Principal Investigator
Nearest person month worked: 4.58
Contribution to Project: Coordinates all aspects of the project and assumes overall responsibility for its success.

Name: Ru-Rong Ji
Project Role: Co Investigator

Nearest person month worked: 0.48
Contribution to Project: He is responsible for interpreting and troubleshooting the proposed animal behavioral testing and cell culture experiments and his lab provides deep expertise in all experimental procedures

Name: Alexander Chamessian
Project Role: Graduate Student
Nearest person month worked: 12
Contribution to Project: He is responsible, along with Dr. Van de Ven, for completion of all animal behavior and cell culture experiments.

Name: Rachel Morales
Project Role: Program Manager
Nearest person month worked: 1.80
Contribution to Project: Overall project manager for all aspects of the proposal, including coordination of the biological samples, shipment of samples between sites and data organization, and ensures that the supplies are ordered and available

Name: Thomas Buchheit
Project Role: Co Investigator
Nearest person month worked: 0.24
Contribution to Project: Works closely with Dr. Van de Ven on all aspects of the project
Funding Support: Other resources

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: Vanderbilt University Medical Center
Location of Organization: 1161 21st Avenue South, Nashville, TN 37232-2520
Partner's contribution to the project: Collaborated in the research

Organization Name: Henry M. Jackson Foundation for the Advancement of Military Medicine Inc.
Location of Organization: 6720 A Rockledge Drive, Bethesda, MD 20817
Partner's contribution to the project: Collaborated in the research

SPECIAL REPORTING REQUIREMENTS
QUAD CHARTS:
Attached

APPENDICES:
Attachment 1- Quad Chart
Attachment 2- Manuscript

VIPER II: Chronic Pain After Amputation: Inflammatory Mechanisms, Novel Analgesic Pathways, and Improved Patient Safety.

PI: Van de Ven, Thomas **Org:** Duke University **Award Amount:** $1,500,000

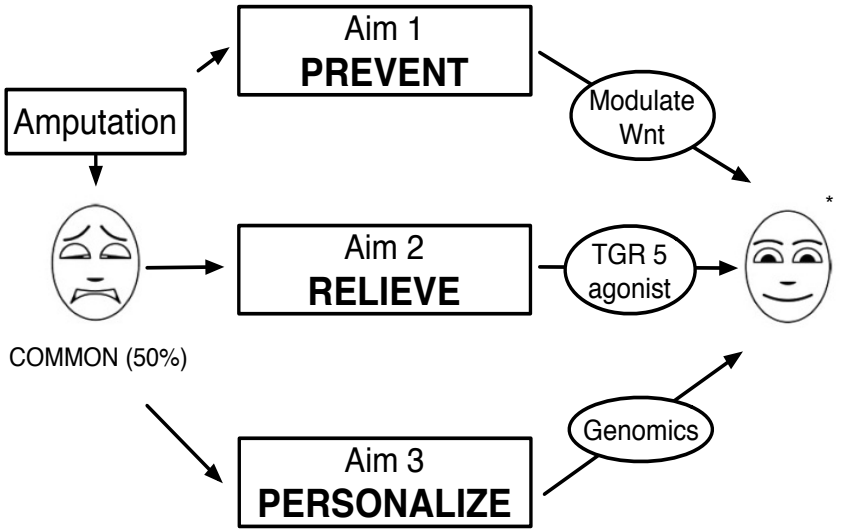
Study Aims

Problem: Current therapies for residual limb pain are ineffective or produce significant side effects.

Hypotheses: 1) Biomarkers found in the Veterans Integrated Pain Evaluation Research Study (VIPER) will lead to novel analgesics. 2) Pharmacogenomic profiling will improve the safety and effectiveness of current analgesics.

Approach

Convergent analysis of VIPER data show both the TGR5 and Wnt pathways to be important in chronic residual limb pain. We will:
1) Define the role of Wnt signaling in inflammation and mechanical allodynia after nerve injury using cell culture and animal models.
2) Test the effectiveness of TGR5 pathway agonists for the treatment of allodynia after nerve injury using animal models.
3) Use human pharmacogenomic predictors to improve the safety and effectiveness of current opioid treatments.



Timeline and Cost

Activities CY	15	16	17	18
Aim 1: Wnt - Cell culture, animal behavioral testing and cytokine measurement.				
Aim 2: TGR5 – Animal behavioral testing and cell culture experiments				
Aim 3: Pharmacogenomic analysis				
Reports (▮) and Manuscripts (♦)			▮♦	▮♦♦▮
Estimated Total Budget ($K)	$200K	$500K	$500K	$300K

Goals/Milestones

CY15 Goals
- ✓ Begin macrophage polarization and astrocyte activation experiments
- ✓ Begin designing data capture for pharmacogenomic analyses
- ✓ Begin animal behavioral TGR5 experiments

CY16 Goals
- ✓ Begin wnt pathway human gene expression analysis
- ✓ Complete cell culture experiments
- ❑ Complete pharmacogenomic validation experiments
- ❑ Begin pharmacogenomic discovery experiments

CY17 Goals
- ❑ Complete ELISA and flow cytometry experiments
- ❑ Continue animal behavioral testing
- ❑ Continue pharmacogenomic discovery experiments
- ❑ Complete one manuscript

CY18 Goals
- ❑ Complete all experiments
- ❑ Complete two manuscripts
- ❑ Develop follow-on studies and apply for follow-on funding

PAIN

Differential Expression of Systemic Inflammatory Mediators in Amputees with Chronic Residual Limb Pain

--Manuscript Draft--

Manuscript Number:	
Article Type:	Research Paper
Section/Category:	Clinical Science
Keywords:	residual limb pain, chronic post surgical pain, neuropathic pain, inflammatory markers, inflammation
Corresponding Author:	Thomas Van de Ven, M.D., Ph.D. Duke University Medical Center and Durham VA Medical Center Durham, NC UNITED STATES
First Author:	Alexander Chamessian, B.S.
Order of Authors:	Alexander Chamessian, B.S.
	Thomas Van de Ven, M.D., Ph.D.
	Thomas Buchheit, M.D.
	Hung-Lun Hsia, M.D.
	Mary McDuffie, RN BSN CCRP
	Eric R Gamazon, Ph.D.
	Colin G Walsh, M.D.
	Stephen Bruehl, Ph.D.
	Chester 'Trip' Buckenmaier III, MD
	Andrew D Shaw, MB, FRCA, FFICM, FCCM
Abstract:	Chronic post-surgical pain impacts the majority of amputees, with more than half experiencing neuralgic residual limb pain. The transition from normal acute post-amputation pain to chronic residual limb pain likely involves both peripheral and central inflammatory mechanisms. As part of the Veterans Integrated Pain Evaluation Research (VIPER) study, we investigated links between systemic inflammatory mediator levels and chronic residual limb pain. Subjects included 36 recent active duty military traumatic amputees with chronic residual limb pain and 40 without clinically significant pain. Blood samples were obtained and plasma concentrations of an array of inflammatory mediators were analyzed. Residual limb pain intensity and pain catastrophizing were assessed to examine associations with inflammatory mediators. Pro-inflammatory mediators including TNF-α, TNF-β, IL-8, ICAM-1, Tie2, CRP, and SAA were elevated in patients with chronic residual limb pain. Across all patients, residual limb pain intensity was associated positively with levels of several pro-inflammatory mediators (IL-8, TNF-α, IL-12, TNF-β, PIGF, Tie2, SAA and ICAM-1), and inversely with concentrations of the anti-inflammatory mediator IL-13, as well as IL-2 and Eotaxin-3. Pain catastrophizing correlated positively with IL-8, IL-12, TNF-β, PIGF, and ICAM-1, and inversely with IL-13. Significant associations between catastrophizing and residual limb pain intensity were partially mediated by TNF-α, TNF-β, SAA, and ICAM-1 levels. Results suggest that chronic post-amputation residual limb pain is associated with excessive inflammatory response to injury or to inadequate resolution of the post-injury inflammatory state. Impact of pain catastrophizing on residual limb pain may be due in part to common underlying inflammatory mechanisms.

DukeMedicine

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Cover Letter

Manuscript Title: Differential Expression of Systemic Inflammatory Mediators in Amputees with Chronic Residual Limb Pain

Dear editorial staff of Pain,

Thank you for considering publication of our study examining differences in plasma inflammatory markers in amputees with and without residual limb pain more than three months after amputation. We believe this data provides support for the hypothesis that chronic neuropathic pain is a result of a prolonged neuroinflammatory response by showing that amputees with chronic residual limb pain have an overall pro-inflammatory plasma signature even after wound healing is complete.

- This contribution represents original work that has not been previously published or submitted for publication elsewhere. It has been read and approved by all authors.
- There are no author conflicts of interest to report.
- This work was completed with funding support from
    - Congressionally Directed Medical Research Programs and the Department of Defense award# DM102142, W81XWH-12-2-0129 and W81XWH-15-2-0046
    - T32 NIH grant# 2T32GM008600
    - Reflex Sympathetic Dystrophy Syndrome Association grant

Sincerely,

Thomas Van de Ven, MD,PhD

Abstract

Chronic post-surgical pain impacts the majority of amputees, with more than half experiencing neuralgic residual limb pain. The transition from normal acute post-amputation pain to chronic residual limb pain likely involves both peripheral and central inflammatory mechanisms. As part of the Veterans Integrated Pain Evaluation Research (VIPER) study, we investigated links between systemic inflammatory mediator levels and chronic residual limb pain. Subjects included 36 recent active duty military traumatic amputees with chronic residual limb pain and 40 without clinically significant pain. Blood samples were obtained and plasma concentrations of an array of inflammatory mediators were analyzed. Residual limb pain intensity and pain catastrophizing were assessed to examine associations with inflammatory mediators. Pro-inflammatory mediators including TNF-α, TNF-β, IL-8, ICAM-1, Tie2, CRP, and SAA were elevated in patients with chronic residual limb pain. Across all patients, residual limb pain intensity was associated positively with levels of several pro-inflammatory mediators (IL-8, TNF-α, IL-12, TNF-β, PIGF, Tie2, SAA and ICAM-1), and inversely with concentrations of the anti-inflammatory mediator IL-13, as well as IL-2 and Eotaxin-3. Pain catastrophizing correlated positively with IL-8, IL-12, TNF-β, PIGF, and ICAM-1, and inversely with IL-13. Significant associations between catastrophizing and residual limb pain intensity were partially mediated by TNF-α, TNF-β, SAA, and ICAM-1 levels. Results suggest that chronic post-amputation residual limb pain is associated with excessive inflammatory response to injury or to inadequate resolution of the post-injury inflammatory state. The impact of pain catastrophizing on residual limb pain may be due in part to common underlying inflammatory mechanisms.

Differential Expression of Systemic Inflammatory Mediators in Amputees with Chronic Residual Limb Pain

Alexander Chamessian[5], Thomas Van de Ven*[1,6], Thomas Buchheit[1,6], Hung-Lun Hsia[1,6], Mary McDuffie[7], Eric Gamazon[3], Colin Walsh[4,8], Stephen Bruehl[2], Chester 'Trip' Buckenmaier III[7,9], Andrew Shaw [2]

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All the authors have no financial interests in this study.

Number of figures: 2

Number of Tables: 3

Number of words in text: 3428

Number of references: 36

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Disclaimer: The views expressed in this publication are those of the authors, and do not necessarily reflect the official policy of the Department of the Army, the Department of Defense, or the United States Government.

Abstract

Chronic post-surgical pain impacts the majority of amputees, with more than half experiencing neuralgic residual limb pain. The transition from normal acute post-amputation pain to chronic residual limb pain likely involves both peripheral and central inflammatory mechanisms. As part of the Veterans Integrated Pain Evaluation Research (VIPER) study, we investigated links between systemic inflammatory mediator levels and chronic residual limb pain. Subjects included 36 recent active duty military traumatic amputees with chronic residual limb pain and 40 without clinically significant pain. Blood samples were obtained and plasma concentrations of an array of inflammatory mediators were analyzed. Residual limb pain intensity and pain catastrophizing were assessed to examine associations with inflammatory mediators. Pro-inflammatory mediators including TNF-α, TNF-β, IL-8, ICAM-1, Tie2, CRP, and SAA were elevated in patients with chronic residual limb pain. Across all patients, residual limb pain intensity was associated positively with levels of several pro-inflammatory mediators (IL-8, TNF-α, IL-12, TNF-β, PIGF, Tie2, SAA and ICAM-1), and inversely with concentrations of the anti-inflammatory mediator IL-13, as well as IL-2 and Eotaxin-3. Pain catastrophizing correlated positively with IL-8, IL-12, TNF-β, PIGF, and ICAM-1, and inversely with IL-13. Significant associations between catastrophizing and residual limb pain intensity were partially mediated by TNF-α, TNF-β, SAA, and ICAM-1 levels. Results suggest that chronic post-amputation residual limb pain is associated with excessive inflammatory response to injury or to inadequate resolution of the post-injury inflammatory state. The impact of pain catastrophizing on residual limb pain may be due in part to common underlying inflammatory mechanisms.

Introduction

Chronic pain due to nerve trauma is a significant health problem. Among the various origins of such pain, limb amputation stands out as particularly important. In the United States alone, more than 100,000 patients per year undergo amputation due to trauma or medical conditions including diabetes and peripheral vascular disease and the incidence of long-term morbidity due to chronic pain in this population ranges from 50-80% [13,15,36].

Although the precise pathogenesis of chronic pain due to nerve trauma still remains elusive, great progress has been made in recent years in our understanding of this condition. [7,11,25]. There is now abundant evidence from preclinical animal models that the immune system plays a critical role in driving chronic pain[1,6,12,19]. Several human studies have also corroborated the important role of the immune system in various chronic pain states, with a particular focus on systemic inflammatory mediators such as cytokines, chemokines and related molecules. For example, in one study of patients with Complex Regional Pain Syndrome (CRPS), pro-inflammatory mediators such as tumor necrosis factor (TNF) and interleukin-(IL-)2 were found to be significantly elevated compared to controls, while anti-inflammatory mediators such as IL-4 and IL-10 exhibited the opposite trend[30]. Similarly, a study comparing patients with painful vs. non-painful peripheral neuropathies demonstrated that patients with painful neuropathy had elevated systemic TNF and IL-2 (both protein and mRNA) compared to their non-painful counterparts[31].

Given these past findings, we hypothesized that a systemic pro-inflammatory profile is also associated with chronic pain after nerve trauma due to amputation. To address this question, we examined blood samples collected from a cohort of recent active duty military post-traumatic amputees in the Veterans Integrated Pain Amputation Evaluation Research (VIPER) study [4] This study employed a case-control design, with amputees experiencing chronic residual limb pain classified as cases, and amputees with little or no pain designated as controls. Unique features of the VIPER study include the lack of significant co-morbidities in the otherwise young and healthy study cohort, as

well as the fact that both case and control groups experienced the same traumatic injury, minimizing the likelihood that injury status would confound results. The first aim of the present study was to investigate systemic inflammatory profiles in a subset of 76 patients from the VIPER cohort using multiplexed, high-sensitivity, electrochemiluminescent assays.

Pain catastrophizing shows ubiquitous associations with pain intensity across various chronic pain conditions, including phantom limb pain post-amputation [29,33]. However, little is known about links between catastrophizing and chronic post-amputation residual limb pain. While a few studies using evoked pain models have examined possible links between catastrophizing and inflammatory status[8,9]; this issue has received little study in the chronic pain setting [27] A second aim of this study was therefore to examine associations between catastrophizing, chronic post-amputation pain, and systemic inflammatory profiles.

Methods

Design

Data were obtained as part of a larger observational case-control study comparing young recent active duty military traumatic amputees with and without significant pain 3 to 18 months after injury. After enrollment, study subjects provided blood samples and psychometric data were collected. Patients were assigned case or control status based on average pain score over the week prior to enrollment (Figure 1). Blood plasma samples were then sent to the Duke Biomarker Core facility for inflammatory marker detection using the MesoScaleDiscovery System.

Subjects

All study procedures were approved by the Institutional Review Board of Walter Reed National Military Medical Center (WRNMMC). Subjects included 36 cases (as defined below) and 40 controls who had undergone post-traumatic amputations while on active duty. All potential subjects were being treated at WRNMMC and the clinical research was supervised through the Defense and Veterans Center for Integrative Pain Management (DVCIPM – DVCIPM.org), part of the Uniformed Services University.

Subjects were included if they were a military health care system beneficiary aged 18 years or older and undergoing treatment at WRNMMC with a diagnosis of post-injury amputation of all or part of one limb. Amputation injury must also have occurred between 3 and 18 months prior to enrollment. Patients were excluded if they were afflicted with severe traumatic brain injury, significant cognitive deficits, substantial hearing loss, spinal cord injury with permanent or persistent deficits, ongoing tissue damage that might cause pain, infection, heterotrophic ossification, poorly fitting prosthesis, or hip disarticulation.

We defined "Cases" as those with clinically significant pain, defined as an average pain score over the past week of greater than or equal to 3/10 on a numeric rating scale (NRS) (Figure 1). Those patients with clinically significant pain were further adjudicated into pain subtypes. Those subjects reporting no pain or pain less than 3/10 but greater than 0/10 were considered "Controls" (pain subtypes were not analyzed in the latter subgroup). This case/control methodology was chosen to facilitate the separate biomarker discovery and genomic analysis aims of the larger project.

Subject characteristics are summarized in Table 1. There were no significant differences between groups in subject age, BMI, ethnicity, smoking status, or time between injury and enrollment. Patients defined as cases reported significantly higher levels of pain catastrophizing. By study design, cases also had significantly higher average pain scores.

Procedures

After written informed consent was obtained, blood samples were obtained from each patient at one timepoint for subsequent analysis. For preparation of plasma, 6ml of blood was collected in EDTA-containing K2 tubes and inverted to mix. Tubes were then spun at 3,000g for 20 minutes at 4 degrees C. Plasma fraction was collected with a pipette and aliquoted into 1.5ml cryovials and stored at -20 degrees C for 24 hours and subsequently at -80 degrees C.

After blood sample collection, subjects completed the pain and psychometric measures described below.

Measures

Ratings of average pain intensity over the past week were provided by all subjects using the self-report version of the Leeds Assessment of Neuropathic Symptoms and Signs scale [2]. The S-LANSS is a validated measure of pain intensity and neuropathic pain characteristics. Pain intensity on the S-LANSS is rated on an 11-point numeric pain rating scale, anchored with "No Pain" and "Pain As Severe As It Could Be." Given the current hypotheses and to minimize the number of analyses conducted, data regarding neuropathic pain characteristics from the S-LANSS are not reported here. All subjects also completed the Pain Catastrophizing Scale (PCS), a widely-used and validated measure of pain catastrophizing [20,28] Focus in the current study was on overall level of catastrophizing as reflected in total PCS scores.

Inflammatory Mediator Assays

The Neuroinflammation Panel 1 by MesoScaleDiscovery (MSD #K15210D) was used to quantify 37 acute inflammatory and injury markers in human serum. These sandwich immunoassays consist of five microplates, each pre-coated with capture antibodies on 4 to 10 independent spots and are grouped based on optimal performance in a multiplex panel as follows: Proinflammatory Panel 1 (IFNγ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-13, and TNF-α), Cytokine Panel 1 (IL-1α, IL-5, IL-7, IL-12/IL-23p40, IL-15, IL-16, IL-17a, TNF-β, and VEGF), Chemokine Panel 1 (Eotaxin, MIP-1β, Eotaxin-3, TARC, IP-10, MIP-1α, MCP-1, MDC, and MCP-4), Angiogenesis Panel 1 (VEGF-c, VEGF-D, Tie-2, Flt-1, PIGF, and bFGF), and Vascular Injury Panel 2 (SAA, CRP, VCAM-1, and ICAM-1). Each of these panels is a V-plex assay indicating it is fully validated according to fit-for-purpose principles and the FDA's analytical validation guidelines, offering highly sensitivity and reproducible results from lot-to-lot. All assays were run according to the manufacturer and samples were run in duplicate. Values below LLOD were defined as ½ LLOD when determining significant differences in inflammatory mediator concentration between cases and controls.

Statistical Analysis

All analyses were conducted using IBM SPSS for Statistics version 23. Initial examination of the distributions of the inflammatory mediators indicated most were not normally distributed. Because of this, we used the nonparametric Mann-Whitney U test for evaluating differences in inflammatory mediators between groups (Case vs. Control) and used nonparametric correlations (Spearman's rho) for examining associations between residual limb pain levels, pain catastrophizing (PCS scores), and inflammatory mediators.

Because of the unique data available in this study, we evaluated a mediation model in which the association of catastrophizing with chronic post-amputation residual limb pain intensity was conveyed in part via indirect effects of inflammatory mediators. To evaluate this mediation model, the approach of Preacher and Hayes (2004) was used to test the significance of the indirect effects[23]. Custom SPSS dialogue (the Indirect Procedure; http://www.afhayes.com/spss-sas-and-mplus-macros-and-code.html#sobel) was used to test the significance of indirect effects in these models using bootstrapping procedures[23]. This bootstrap methodology tested each mediation model in a series of 1000 random subsamples repeatedly drawn from the full sample, generating 95% confidence intervals (bias corrected) around the indirect effect test statistic. If the 95% confidence interval for the indirect effect generated by the model did not include zero, this indicated that the hypothesized indirect (mediated) effect was significant at the $p<.05$ level. To minimize the risk of bias in estimation of indirect effects, inflammatory mediator values were normalized via log-transformations prior to conducting mediation analyses.

In preliminary analyses, time (in months) since amputation was examined as it related to the primary outcomes to determine whether it might confound primary analyses. Correlational analyses indicated that pain duration was not associated with either average residual limb pain intensity (Spearman's rho = -0.07, $\underline{p} = 0.572$) or catastrophizing scores on the PCS (Spearman's rho = -0.13, $\underline{p} = 0.274$), nor with most inflammatory mediator values. Exceptions to the latter were: IL-12 (Spearman's rho = -0.24, $\underline{p} = 0.035$), IL-15 (Spearman's rho = 0.26, $\underline{p} = 0.026$), IL-16 (Spearman's rho = -0.27, $\underline{p} = 0.022$), IL-1alpha (Spearman's rho = 0.27, $\underline{p} = 0.021$), and VCAM-1

(Spearman's rho = -0.23, p = 0.044). Because of the general absence of associations of pain duration with the key outcomes of interest, it was not included as a control variable in the analyses reported below.

Results

Multiple inflammatory mediators are upregulated in amputees with residual limb pain

To determine whether any differences in systemic inflammatory mediators were present between the case and control groups, we measured the levels of 37 inflammatory mediators in all 76 patients. Relative to patients defined as controls, patients defined as cases exhibited significantly higher levels of for TNF-α, TNF-β, IL-8, ICAM-1, Tie2, CRP, and SAA (Table 2). Each of the elevated markers have mainly pro-inflammatory properties. Descriptive statistics for all of the mediators tested are shown in Table 2.

Inflammatory mediators correlate with pain severity and catastrophizing in amputees with significant residual limb pain

A second aim of this study was to examine associations between systemic inflammatory mediators, post-amputation residual limb pain intensity, and pain catastrophizing. Ratings of average residual limb pain intensity and PCS scores were significantly correlated, $r(74) = 0.62$, $p<.001$. Table 3 summarizes associations between systemic inflammatory mediators and both residual limb pain intensity and PCS scores. Higher pain intensity was found to be associated with significantly higher levels of IL-8, IL-12, TNF-α, TNF-β, PIGF, Tie2, SAA, and ICAM-1, with inverse associations noted for IL-2, IL-13, and Eotaxin-3. Similarly, higher PCS scores were associated with significantly higher levels of IL-8, IL-12, TNF-β, PIGF, and ICAM-1, with an inverse association observed with IL-13. To address possible inflated type I error due to the number of inflammatory mediators examined, permutation testing (1000 permutations) was conducted to determine empirical probability values for the correlational analyses as a set. As indicated at the bottom of Table 3, set-wise associations with levels of

inflammatory mediators were highly significant for both average pain intensity and catastrophizing. These results indicate that the associations reported between these two variables and inflammatory status are unlikely to represent spurious findings.

Do Inflammatory Mediators Contribute to Associations between Pain Catastrophizing and Residual Limb Pain Intensity?

We considered the possibility that the positive association between pain catastrophizing levels (PCS) and residual limb pain intensity might be accounted for in part by indirect effects conveyed through systemic inflammatory mediators. While the only requirement for conducting analyses to test this model was that catastrophizing needed to be associated with pain intensity, we restricted our analyses to only those inflammatory mediators showing significant associations with the outcome of interest (residual limb pain intensity) to limit the number of analyses conducted.

Results using bootstrapped mediation tests indicated significant indirect effects between PCS scores and residual limb pain intensity via TNF-α (95% CI: 0.0004 – 0.0308), TNF-beta (95% CI: 0.0027 – 0.0309), SAA (95% CI: 0.0005 – 0.0351), and ICAM-1 (95% CI: 0.0011 – 0.0515). In each case, there were also significant direct effects of PCS scores on residual limb pain intensity independent of systemic inflammatory mediators (p's<.001). Overall, these results indicated that the positive association between pain catastrophizing and residual limb pain intensity was partially mediated by TNF-α, TNF-beta, SAA, and ICAM-1 levels in plasma (model summarized in Figure 2). Tests of indirect effects for other systemic inflammatory mediators showing associations with residual limb pain intensity in correlational analyses were all nonsignificant (p's > .05).

Discussion

In this study, we found elevated systemic levels of several pro-inflammatory mediators in amputees with residual limb pain (Cases) compared to those without clinically significant residual limb pain (Controls). Specifically, as might be expected given animal work and human findings in other chronic pain populations, the pro-

inflammatory mediators TNF-α, TNF-b, IL-8, CRP, SAA, Tie2 and ICAM-1 were significantly elevated in cases compared to controls.

Across both patient groups, intensity of residual limb pain was associated positively with levels of several pro-inflammatory mediators (IL-8, TNF-α, IL-12, TNF-β, PIGF, Tie2, SAA and ICAM-1), and inversely with concentrations of the anti-inflammatory mediator IL-13, as well as IL-2 and Eotaxin-3. Eotaxin-3, initially thought to have mainly pro-inflammatory properties through agonism of CCR3, more recently was found to be an antagonist for multiple CCR receptors whose blockade prevents chemotaxis [21]. Similarly, IL-2 was initially thought to be mainly pro-inflammatory, stimulating cytotoxic T-cells and NK cells, but was later found to be an important stimulator of T_{reg} cells[3]. Taken together, these findings demonstrate an overall pro-inflammatory signature in amputees with chronic pain. To the best of our knowledge, this is the first comprehensive study of systemic inflammatory mediators in human subjects with residual limb pain following amputation.

This study also appears to be the first to examine associations between post-amputation residual limb pain and levels of pain catastrophizing, a cognitive factor previously shown to exacerbate chronic pain intensity across a variety of other pain conditions[24,33]. Results, not surprisingly, indicated that elevated catastrophizing was strongly correlated with greater residual limb pain intensity. Although the causal direction of these effects cannot be ascertained due to the design of this study, the results nonetheless add to the existing literature by extending the apparent negative effects of pain catastrophizing into the post-amputation residual limb pain population.

Finally, the current findings to our knowledge are among the first to systematically examine possible links among catastrophizing, chronic pain intensity, and a comprehensive array of inflammatory mediators. While a limited number of studies have examined associations between catastrophizing and selected inflammatory mediators in the context of laboratory evoked pain stimuli, this issue has received little study in the chronic pain setting[8,9]. The present results revealed that in the context of post-amputation residual limb pain, elevated catastrophizing levels were associated with higher levels of IL-8, IL-12, TNF-β, PIGF, and ICAM-1, and lower levels of IL-13. These findings are consistent with generally pro-inflammatory influences of

catastrophizing. Interestingly, the strong positive association between catastrophizing and residual limb pain intensity was statistically mediated by TNF-α, TNF-β, SAA, and ICAM-1 levels. In other words, this study suggests the possibility that catastrophizing might be linked with an elevated pro-inflammatory profile, which in turn produces elevated chronic pain intensity. Definitive conclusions regarding this causal model must await replication using a design with evaluation of pain catastrophizing, inflammatory mediator levels, and chronic pain intensity over time. Extending these results to other chronic pain conditions would also be worthwhile.

Exaggerated pro-inflammatory response in amputees with chronic pain

Our results demonstrate that there is an exaggerated and enduring pro-inflammatory response in amputees with chronic residual limb pain compared to amputees without pain. This finding is consistent with a substantial body of preclinical evidence suggesting that several of the mediators that were associated with pain in this study may be implicated in the pathogenesis of neuropathic pain. Of these, TNF and IL-6 are the most studied, with these pro-algesic cytokines having pleiotropic effects on neurons and immune cells throughout the neuraxis following nerve injury[16,17,34,35]. IL-8, while not examined directly in nerve injury, has been shown to cause hyperalgesia when administered exogenously to rodents, and has been associated with widespread tenderness to palpation in a large clinical study of TMD sufferers[18,26]. IL-2 and IL-12, have both been shown to be pro-algesic in animal models, and in clinical studies of CRPS and painful small fiber neuropathy, IL-2 was shown to be elevated at the protein and mRNA level from blood samples[30,31].

In further support of an overall pro-inflammatory signature in post-amputation residual limb pain, we report for the first time that IL-13, a cytokine with known anti-inflammatory properties, was negatively correlated with pain. This cytokine is closely related to the anti-inflammatory cytokine IL-4, which has been shown to attenuate neuropathic pain behaviors in animal models of nerve injury [14,32], and has been associated differentially with painless neuropathy in humans [31]. Indeed, the type II IL-4 receptor is the main functional receptor for IL-13, demonstrating the overlapping biological roles of these two cytokines[10]. Given these similarities, one might predict

that IL-13 would also play a role in the modulation of neuropathic pain. Our finding that IL-13 is inversely correlated with average pain scores with moderate effect size and high statistical significance suggests that this may indeed be the case.

Our unbiased analysis additionally allowed us to discover several other mediators not previously associated with post-amputation residual limb pain in clinical or preclinical models. For example, we found that the pro-inflammatory cytokine, TNF-β, which bears many biological similarities to TNF-α, was elevated in the cases and positively associated with limb pain intensity. Given the recognized importance of TNF-α in neuropathic pain, it is likely that TNF-b, which shares the same receptor with TNF-α, may also an important player in the pathogenesis of pain after nerve injury. Other novel associations revealed in our study include the cell adhesion molecule ICAM-1, the pro-angiogenic factors Tie2 and PlGF, the chemokine Eotaxin-3, and the acute phase reactants CRP and SAA, all of which further support the notion of a persistent pro-inflammatory state in amputees with pain.

Of particular interest is CRP, an acute phase reactant that is widely used in clinical practice as a general marker of inflammation. The elevation of CRP in amputees with chronic residual limb pain is consistent with a systemic pro-inflammatory state in this group. Recent work in a large community-based sample of women has reported small but significant positive associations between CRP levels and persistently elevated bodily pain, further supporting the pain relevance of CRP elevations [5]. Of note, CRP is an important risk factor for cardiovascular morbidity, with a value greater than 3 mg/L considered high risk [22]. The median CRP value of ~ 4 mg/L in the case group in the current study suggests these individuals could be at high risk for future cardiovascular disease.

Limitations

As a cross-sectional, observational study, this trial was not designed to determine causation with regards to any of the inflammatory mediators that were measured. Furthermore, since we only gathered information on this patient cohort at a single time point, several months after they had suffered injury and experienced chronic pain, we could not examine the temporal dynamics of the inflammatory mediators under study.

Thus, the possibility that the differential mediator profiles in these patients may have been present before injury cannot be excluded. Finally, the conclusions from this study arise from a modest sized cohort of primarily young and male military veterans (n=76), potentially limiting the generalizability of our findings. Given the modest sample size and the relatively large number of associations examined, concerns might be raised as to whether the effects reported might simply be due to inflated Type I error. Results of permutation testing indicating highly significant set-wise correlations between levels of all inflammatory mediators and both residual limb pain intensity and catastrophizing levels argues against our reported findings being spurious.

Conclusion

Amputees suffering from residual limb pain exhibit an overall pro-inflammatory signature when compared with amputees without significant pain. A pro-inflammatory profile is associated with both greater pain intensity and higher pain catastrophizing levels. These results generate intriguing hypotheses regarding the links between causation and resolution of the inflammatory state and chronic pain following nerve trauma. The mediators measured here may have utility as potential biomarkers of nerve injury-induced pain.

Acknowledgements

Grant support from Congressionally Directed Medical Research Programs and the Department of Defense awards MR130082, W81XWH-12-2-0129 and W81XWH-15-2-0046. Partial support was also provided by NIH grant 2T32GM008600 and a grant from the Reflex Sympathetic Dystrophy Syndrome Association. Authors have no financial conflicts of interest to report.

References

[1] Austin PJ, Moalem-Taylor G. The neuro-immune balance in neuropathic pain: Involvement of inflammatory immune cells, immune-like glial cells and cytokines. J. Neuroimmunol. 2010;229:26–50.

[2] Bennett MI, Smith BH, Torrance N, Potter J. The S-LANSS score for identifying pain of predominantly neuropathic origin: validation for use in clinical and postal research. The Journal of Pain 2005;6:149–158.

[3] Boyman O, Sprent J. The role of interleukin-2 during homeostasis and activation of the immune system. Nat Rev Immunol 2012;12:180–190.

[4] Buchheit T, Van de Ven T, John Hsia HL, McDuffie M, MacLeod DB, White W, Chamessian A, Keefe FJ, Buckenmaier CT, Shaw AD. Pain Phenotypes and Associated Clinical Risk Factors Following Traumatic Amputation: Results from Veterans Integrated Pain Evaluation Research (VIPER). Pain Medicine 2015:n/a–n/a.

[5] Burns JW, Quartana PJ, Bruehl S, Janssen I, Dugan SA, Appelhans B, Matthews KA, Kravitz HM. Chronic pain, body mass index and cardiovascular disease risk factors: tests of moderation, unique and shared relationships in the Study of Women's Health Across the Nation (SWAN). J Behav Med 2015;38:372–383.

[6] Calvo M, Dawes JM, Bennett DLH. The role of the immune system in the generation of neuropathic pain. Lancet Neurol 2012;11:629–642.

[7] Cohen SP, Mao J. Neuropathic pain: mechanisms and their clinical implications. BMJ 2014;348:f7656–f7656.

[8] Cruz-Almeida Y, King CD, Wallet SM, Riley JL. Immune biomarker response depends on choice of experimental pain stimulus in healthy adults: a preliminary study. Pain Res Treat 2012;2012:538739–7.

[9] Edwards RR, Kronfli T, Haythornthwaite JA, Smith MT, McGuire L, Page GG. Association of catastrophizing with interleukin-6 responses to acute pain. PAIN 2008;140:135–144.

[10] Gordon S, Martinez FO. Alternative activation of macrophages: mechanism and functions. Immunity 2010.

[11] Hehn von CA, Baron R, Woolf CJ. Deconstructing the Neuropathic Pain Phenotype to Reveal Neural Mechanisms. Neuron 2012;73:638–652.

[12] Ji R-R, Xu Z-Z, Gao Y-J. Emerging targets in neuroinflammation- driven chronic pain. Nat Rev Drug Discov 2014:1–16.

[13] Kehlet H, Jensen TS, Woolf CJ. Persistent postsurgical pain: risk factors and

prevention. The Lancet 2006;367:1618–1625.

[14] Kiguchi N, Kobayashi Y, Saika F, Sakaguchi H, Maeda T, Kishioka S. Peripheral interleukin-4 ameliorates inflammatory macrophage-depend... - PubMed - NCBI. PAIN 2015:1.

[15] Kooijman CM, Dijkstra PU, Geertzen JHB, Elzinga A, van der Schans CP. Phantom pain and phantom sensations in upper limb amputees: an epidemiological study. PAIN 2000;87:33–41.

[16] Leung L, Cahill CM. TNF-alpha and neuropathic pain--a review. J Neuroinflammation 2010;7:27.

[17] Lindenlaub T, Teuteberg P, Hartung T, Sommer C. Effects of neutralizing antibodies to TNF-alpha on pain-related behavior and nerve regeneration in mice with chronic constriction injury. Brain Research 2000;866:15–22.

[18] Lorenzetti BB, Poole S, Veiga FH, Cunha FQ, Ferreira SH. Cytokine-mediated inflammatory hyperalgesia limited by interleukin-13. European Cytokine Network 2001;12:260–267.

[19] Marchand F, Perretti M, McMahon SB. Role of the Immune system in chronic pain. Nat Rev Neurosci 2005;6:521–532.

[20] Osman A, Barrios FX, Gutierrez PM, Kopper BA, Merrifield T, Grittmann L. The Pain Catastrophizing Scale: further psychometric evaluation with adult samples. J Behav Med 2000;23:351–365.

[21] Petkovic V, Moghini C, Paoletti S, Uguccioni M. Eotaxin-3/CCL26 is a natural antagonist for CC chemokine receptors 1 and 5 A human chemokine with a regulatory role. Journal of Biological … 2004.

[22] Pfützner A, Forst T. High-sensitivity C-reactive protein as cardiovascular risk marker in patients with diabetes mellitus. - PubMed - NCBI. Diabetes Technology & Therapeutics 2006;8:28–36.

[23] Preacher KJ, Hayes AF. SPSS and SAS procedures for estimating indirect effects in simple mediation models. Behavior Research Methods, Instruments, & Computers 2004;36:717–731.

[24] Richardson C, Glenn S, Horgan M, Nurmikko T. A prospective study of factors associated with the presence of phantom limb pain six months after major lower limb amputation in patients with peripheral vascular disease. The Journal of Pain 2007;8:793–801.

[25] Scholz J, Woolf CJ. The neuropathic pain triad: neurons, immune cells and glia. Nat Neurosci 2007;10:1361–1368.

[26] Slade GD, Conrad MS, Diatchenko L, Rashid NU, Zhong S, Smith S, Rhodes J, Medvedev A, Makarov S, Maixner W, Nackley AG. Cytokine biomarkers and chronic pain: Association of genes, transcription, and circulating proteins with temporomandibular disorders and widespread palpation tenderness. PAIN 2011;152:2802–2812.

[27] Sturgeon JA. Psychological therapies for the management of chronic pain. Psychol Res Behav Manag 2014;7:115–124.

[28] Sullivan MJL, Bishop SR, Pivik J. The Pain Catastrophizing Scale: Development and validation. Psychological Assessment 1995;7:524–532.

[29] Theunissen M, Peters ML, Bruce J, Gramke H-F, Marcus MA. Preoperative anxiety and catastrophizing: a systematic review and meta-analysis of the association with chronic postsurgical pain. The Clinical Journal of Pain 2012;28:819–841.

[30] Uçeyler N, Eberle T, Rolke R, Birklein F, Sommer C. Differential expression patterns of cytokines in complex regional pain syndrome. PAIN 2007;132:195–205.

[31] Uçeyler N, Rogausch JP, Toyka KV, Sommer C. Differential expression of cytokines in painful and painless neuropathies. Neurology 2007;69:42–49.

[32] Uçeyler N, Topuzoğlu T, Schießer P, Hahnenkamp S, Sommer C. IL-4 Deficiency Is Associated with Mechanical Hypersensitivity in Mice. PLoS ONE 2011;6:e28205.

[33] Vase L, Nikolajsen L, Christensen B, Egsgaard LL, Arendt-Nielsen L, Svensson P, Staehelin Jensen T. Cognitive-emotional sensitization contributes to wind-up-like pain in phantom limb pain patients. PAIN 2011;152:157–162.

[34] Wei X-H, Na X-D, Liao G-J, Chen Q-Y, Cui Y, Chen F-Y, Li Y-Y, Zang Y, Liu X-G. The up-regulation of IL-6 in DRG and spinal dorsal horn contributes to neuropathic pain following L5 ventral root transection. Experimental Neurology 2013;241:159–168.

[35] Zhang L, Berta T, Xu Z-Z, Liu T, Park JY, Ji R-R. TNF-α contributes to spinal cord synaptic plasticity and inflammatory pain: distinct role of TNF receptor subtypes 1 and 2. PAIN 2011;152:419–427.

[36] Ziegler-Graham K, MacKenzie EJ, Ephraim PL, Travison TG, Brookmeyer R. Estimating the prevalence of limb loss in the United States: 2005 to 2050. Archives of Physical Medicine and Rehabilitation 2008;89:422–429.

Figure 1. VIPER study flow diagram defining inclusion/exclusion criteria and patient adjudication results.

Figure 2. Model in which effects of catastrophizing on residual limb pain intensity are conveyed through indirect effects of systemic inflammatory mediators.

Examination of plasma inflammatory markers reveals that patients with residual limb pain after suffering post-traumatic amputation while on active military duty have a significant increase in pro-inflammatory markers that correlates positively with pain intensity and pain catastrophizing. In addition, the association between pain catastrophizing and pain intensity was mediated by TNF-α, TNF-beta, SAA, and ICAM-1 levels in plasma.

Figure 1

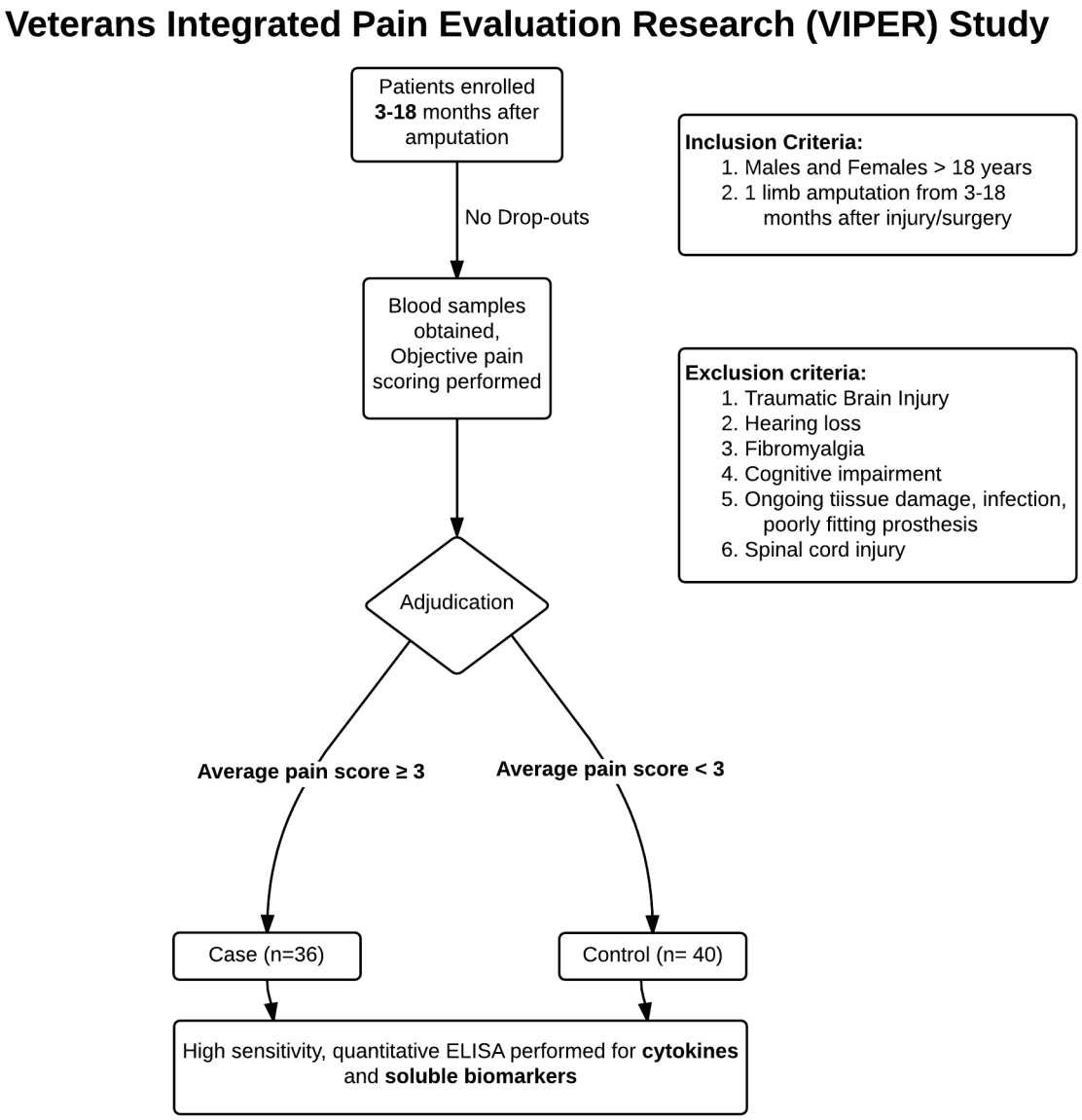
Veterans Integrated Pain Evaluation Research (VIPER) Study
Patients enrolled 3-18 months after amputation
No Drop-outs
Blood samples obtained, Objective pain scoring performed
Adjudication
Average pain score ≥ 3
Average pain score < 3
Case (n=36)
Control (n= 40)
High sensitivity, quantitative ELISA performed for cytokines and soluble biomarkers
Inclusion Criteria:
1. Males and Females > 18 years
2. 1 limb amputation from 3-18 months after injury/surgery
Exclusion criteria:
1. Traumatic Brain Injury
2. Hearing loss
3. Fibromyalgia
4. Cognitive impairment
5. Ongoing tiissue damage, infection, poorly fitting prosthesis
6. Spinal cord injury

Figure 2

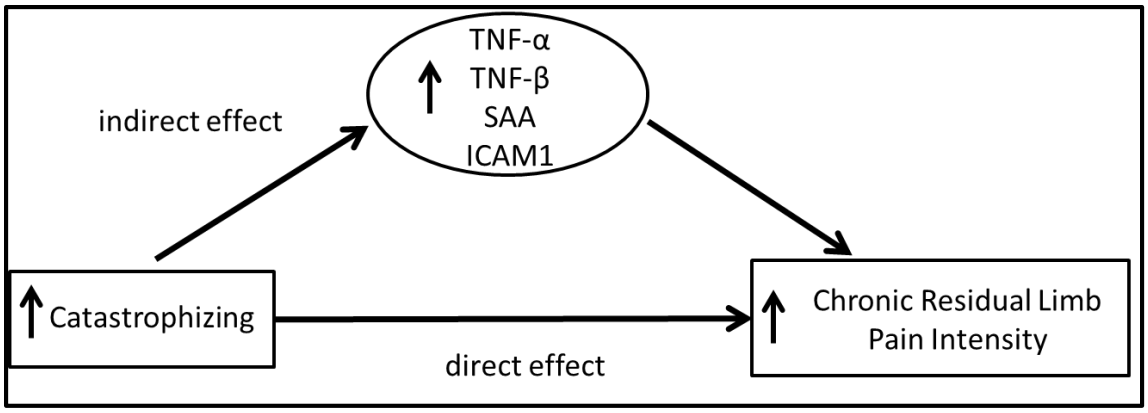
TNF-α
TNF-β
SAA
ICAM1
indirect effect
Catastrophizing
Chronic Residual Limb Pain Intensity
direct effect

Table 1. Characteristics of sample participants from the Veterans Integrated Pain Evaluation Research (VIPER) study.

	Control (N=40)	**Case (N=36)**	**p-value**
	Mean (SD)	*Mean (SD)*	
Age	25.2 (4.8)	27.7 (9.1)	0.1526
Body Mass Index	25.7 (2.8)	26.8 (3.6)	0.1427
Time since amputation (months)	7.6 (3.5)	8.9 (5.9)	0.2243
Smoking (ppd)	0.6 (0.5)	0.6 (0.54)	0.8168
Average Pain Intensity (0-10)	1.2 (0.73)	5.6 (1.38)	0.000
Pain Catastrophizing Scale	2.6 (4.53)	11.8 (11.09)	0.000
	N (%)	*N(%)*	
Male	40 (100)	35(97)	0.9577
Smokers	25 (63)	21 (58)	0.8918
Ethnicity	*N (%)*	*N(%)*	
American Indian/Alaska Native	0 (0)	0 (0)	1.0000
Asian	2 (5)	1 (3)	1.0000
Native Hawaiian or Other Pacific Islander	0 (0)	0 (0)	1.0000
Black or African American	3 (7)	3 (8)	1.0000
White	37 (93)	32 (89)	0.8836

Note: Continuous variables were analyzed using t-tests whereas categorical values were examined using a chi-squared test.

Table 2

Table 2 –Systemic Inflammatory Mediator Concentrations in Cases vs. Controls.

Mediator	**Case (n=36)** Median (Range)	**Control (n=40)** Median (Range)	Mann-Whitney U Test (p value)
IFN-γ	4.1 (1.5-34.4)	3.5(1.5-19.1)	0.252
IL-10	0.3 (0.1-0.9)	0.3 (0.0-3.0)	0.113
IL-13	0.8 (0.8-0.8)	0.8 (0.8-4.0)	0.099
IL-1β	0.0 (0.0-0.2)	0.0 (0.0-0.1)	0.859
IL-2	0.1 (0.1-0.9)	0.1 (0.1-0.8)	0.713
IL-4	0.0 (0.0-0.1)	0.0 (0.0 -0.1)	0.970
IL-6	0.8 (0.1-10.3)	0.5 (0.1 - 2.6)	0.138
IL-8	4.7 (2.3-25.2)	4.0 (1.9-9.9)	**0.041**
TNF-α	2.2 (1.2-5.0)	1.9 (1.1-3.5)	**0.031**
IL-12	140.1 (23.9-289.5)	129.8 (48.5-240.5)	0.131
IL-17	1.1 (1.1-72.5)	1.1 (1.1-6.5)	0.546
IL-5	0.3 (0.1-3.1)	0.3 (0.1-7.8)	0.983
IL-7	3.4 (0.8-18.7)	3.5 (0.5 -25.5)	0.768
TNF-β	0.2 (0.1-1.1)	0.1 (0.1-0.3)	**0.037**
VEGF	43.4 (16.0-309.3)	35.1 (16.5-82.3)	0.065
IL-15	1.9 (1.2-5.0)	1.8 (1.2-2.6)	0.914
IL-16	183.4 (114.2 - 292.9)	189.8 (93.8-444.2)	0.349
IL-1α	0.9 (0.1-111.5)	1.2 (0.2-12.4)	0.971
Eotaxin	74.7 (24.1-218.9)	86.4 (37.6-211.3)	0.938
Eotaxin-3	18.8 (11.4-1563.3)	22.3 (4.0-182.6)	0.399
IP-10	252.6 (99.7-11413.7)	209.8 (50.5-561.5)	0.293
MCP-4	45 (14.4-122.4)	52.4 (20.7-107.6)	0.182
MDC	660.4 (296.4-1647.4)	657 (427.7-1362.6)	0.979
MIP-1α	5.5 (5.5-300.4)	5.5 (5.5-625.0)	0.494
MIP-1β	53.8 (28.5-119.1)	45.0 (22.0-118.0)	0.302
TARC	58.5 (7.6-247.9)	71.9 (19.3-297.3)	0.163
Flt1	47.5 (22.8-145.0)	47.5 (23.9-171.2)	0.881

PIGF	28.2 (16.0-39.6)	25.0 (8.6-47.7)	0.056
Tie2	6384.7 (1602.6-9030.0)	5546.9 (1093.2-8823.6)	**0.008**
VEGF-C	43.5 (6.9-157.2)	61.0 (6.9- 232.7)	0.112
VEGF-D	540.4 (124.3-3474.5)	749.6 (120.7-6020.1)	0.172
bFGF	7.7 (0.4-158.1)	7.2 (1.0-68.6)	0.298
CRP	4011.5 (62.9-53937.9)	2147.0 (79.7-24416.1)	**0.034**
SAA	3878.7 (787.8-316972.6)	1981.9 (371.8-22428.1)	**0.002**
ICAM-1	421.2 (264-2-810.3)	379.3 (266.6-607.7)	**0.007**
VCAM-1	414.8 (282.0-660.0)	414.9 (257.0-683.8)	0.873

* All concentrations in pg/ml except with the exception of CRP, SAA, ICAM-1 and VCAM-1, which are in ng/ml

Table 3. Spearman correlations between inflammatory mediators, average residual limb pain intensity, and pain catastrophizing in the full sample (n=76).

Systemic Mediator	Average Pain	P value	PCS	P value
IFN-γ	0.11	0.339	0.18	0.119
IL-10	0.15	0.191	0.03	0.804
IL-13	**-0.45**	**0.000**	**-0.29**	**0.010**
IL-1β	0.06	0.604	0.13	0.280
IL-2	**-0.24**	**0.038**	-0.15	0.184
IL-4	-0.22	0.054	-0.06	0.612
IL-6	0.17	0.137	0.10	0.374
IL-8	**0.26**	**0.024**	**0.25**	**0.030**
TNF-α	**0.30**	**0.008**	0.18	0.129
IL-12	**0.31**	**0.006**	**0.24**	**0.037**
IL-17	0.21	0.065	0.10	0.369
IL-5	0.04	0.706	0.08	0.517
IL-7	-0.03	0.774	0.12	0.293
TNF-β	**0.43**	**0.000**	**0.39**	**0.001**
VEGF	0.19	0.104	0.17	0.139
IL-15	-0.01	0.906	0.02	0.855
IL-16	0.09	0.439	0.21	0.077
IL-1a	0.11	0.334	0.03	0.773
Eotaxin	0.02	0.856	-0.04	0.775
Eotaxin-3	**-0.27**	**0.017**	-0.10	0.385
IP-10	0.07	0.557	0.02	0.887
MCP-1	-0.03	0.813	0.01	0.917
MCP-4	-0.08	0.496	-0.06	0.628
MDC	0.04	0.728	-0.04	0.763
MIP-1α□□	0.06	0.632	-0.03	0.831
MIP-1β	0.14	0.238	0.11	0.358
TARC	-0.11	0.366	0.01	0.947
Flt-1	0.13	0.262	0.15	0.194
PIGF	**0.31**	**0.008**	**0.34**	**0.003**
Tie2	**0.35**	**0.002**	0.22	0.059
VEGF-C	-0.15	0.185	-0.04	0.756
VEGF-D	-0.05	0.664	0.20	0.087
bFGF	-0.13	0.270	0.06	0.595

CRP	0.20	0.092	0.05	0.671
SAA	**0.33**	**0.004**	0.21	0.072
ICAM-1	**0.43**	**0.000**	**0.44**	**0.000**
VCAM-1	0.11	0.346	0.21	0.074
Set-wise p value	**---**	**0.008**	**---**	**0.005**