



Differential expression of systemic inflammatory mediators in amputees with chronic residual limb pain

Alexander Chamesian^a, Thomas Van de Ven^{b,c,*}, Thomas Buchheit^{b,c}, Hung-Lun Hsia^{b,c}, Mary McDuffie^d, Eric R. Gamazon^e, Colin Walsh^{f,g}, Stephen Bruehl^h, Chester 'Trip' Buckenmaier III^{d,i}, Andrew Shaw^h

Abstract

Chronic postsurgical pain impacts most amputees, with more than half experiencing neuralgic residual limb pain. The transition from normal acute postamputation pain to chronic residual limb pain likely involves both peripheral and central inflammatory mechanisms. As part of the Veterans Integrated Pain Evaluation Research 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 tumor necrosis factor (TNF)- α , TNF- β , interleukin (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 proinflammatory 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 postamputation residual limb pain is associated with excessive inflammatory response to injury or to inadequate resolution of the postinjury inflammatory state. Impact of pain catastrophizing on residual limb pain may be because of part to common underlying inflammatory mechanisms.

Keywords: Residual limb pain, Chronic post surgical pain, Neuropathic pain, Inflammatory markers, Inflammation

1. 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 because of 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% to 80%.^{12,13,34}

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.^{7,10,23} There is

now abundant evidence from preclinical animal models that the immune system plays a critical role in driving chronic pain.^{1,6,11,17} 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, pro-inflammatory mediators such as tumor necrosis factor (TNF) and interleukin-(IL)-2 were found to be significantly elevated compared with controls, whereas anti-inflammatory mediators such as IL-4 and IL-10 exhibited the opposite trend.²⁸ Similarly, a study comparing patients with painful vs nonpainful peripheral neuropathies demonstrated that patients with painful neuropathy had elevated systemic TNF and IL-2 (both protein and mRNA) compared with their nonpainful counterparts.²⁹

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 posttraumatic amputees in the Veterans Integrated Pain Evaluation Research (VIPER) study.⁴ This study used 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 comorbidities in the otherwise young and healthy study cohort, as well as the fact that both case and control groups experienced the same traumatic injury,

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

Departments of ^a Pharmacology and Cancer Biology and, ^b Anesthesiology, Duke University Medical Center, Durham, NC, ^c Division of Anesthesiology, Durham Veterans Affairs Medical Center, Durham, NC, ^d Defense and Veterans Center for Integrative Pain Management, Rockville, MD, ^e Department of Medicine, Division of Genetic Medicine, Vanderbilt University Medical Center, Nashville, TN, Departments of ^f Biomedical Informatics, ^g Medicine, and, ^h Anesthesiology, Vanderbilt University Medical Center, Nashville, TN, ⁱ Department of Military Emergency Medicine, Uniformed Services University, Bethesda, MD

*Corresponding author. Address: Department of Anesthesiology, Duke University Medical Center, Durham, NC 27710, USA. Tel.: 919-286-6938. E-mail address: thomas.vandeven@duke.edu (T. Van De Ven).

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minimizing the likelihood that injury status would confound results. The first aim of this 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 severity across various chronic pain conditions, including phantom limb pain postamputation.^{27,31} However, little is known about links between catastrophizing and chronic postamputation residual limb pain. Although 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.²⁵ A second aim of this study was therefore to examine associations between catastrophizing, chronic postamputation pain and systemic inflammatory profiles.

2. Methods

2.1. 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 residual limb 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 before enrollment (Fig. 1). Blood plasma samples were then sent to the Duke Biomarker Core facility for inflammatory marker detection using the Meso Scale Discovery System.

2.2. 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 posttraumatic 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 postinjury amputation of all or part of one limb. Amputation injury must also have occurred between 3 and 18 months before 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, and ongoing tissue damage that might cause pain, infection, heterotrophic ossification, poorly fitting prosthesis, or hip disarticulation.

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

Veterans Integrated Pain Evaluation Research (VIPER) Study

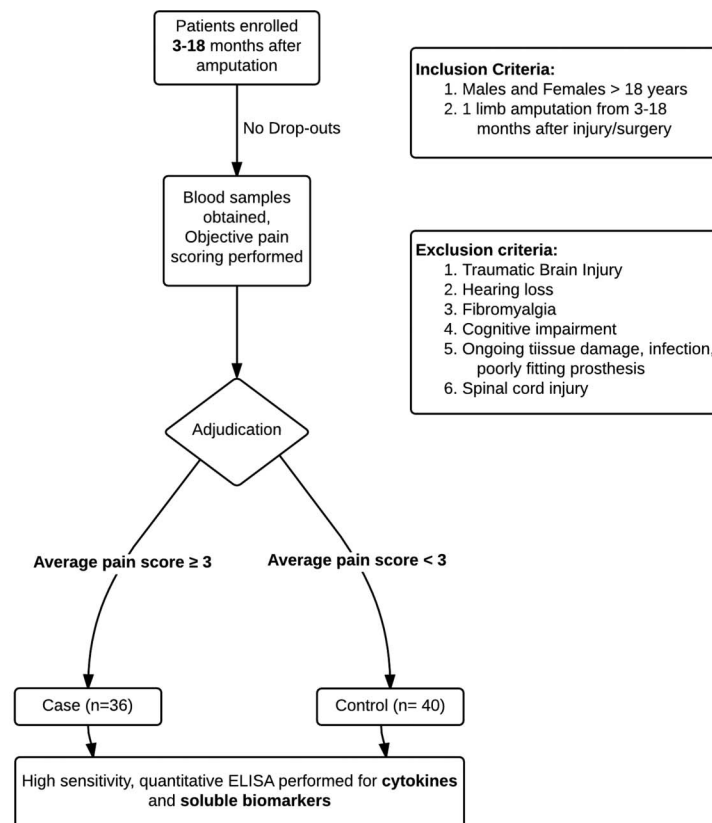


Figure 1. VIPER study flow diagram defining inclusion/exclusion criteria and patient adjudication results.

Subject characteristics are summarized in **Table 1**. There were no significant differences between groups in subject age, BMI, ethnicity, smoking status, amputation site, amputation level, 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. Cases also were more likely to use opioid medications of any kind. Ninety-four percent of cases reported at least some degree of phantom limb pain.

2.3. Procedures

After written informed consent was obtained, blood samples were obtained from each patient at one time point for subsequent analysis. For preparation of plasma, 6 mL of blood was collected in EDTA-containing K2 tubes and inverted to mix. Tubes were then spun at 3000 g for 20 minutes at 4°C. Plasma fraction was collected with a pipette and aliquoted into 1.5 mL cryovials and stored at –20°C for 24 hours and subsequently at –80°C.

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

2.4. Measures

Ratings of average pain severity 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 severity and neuropathic pain characteristics. Pain severity 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^{18,26} Focus in the present study was on overall level of catastrophizing as reflected in total PCS scores.

2.5. Inflammatory mediator assays

The Neuroinflammation Panel 1 by Meso Scale Discovery (MSD #K15210D) was used to quantify 37 acute inflammatory and injury markers in human serum. These sandwich immunoassays consist of 5 microplates, each precoated 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 (IFN γ , IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-13, and TNF- α), Cytokine Panel 1 (IL-1 α , IL-5, IL-7, IL-12/IL-23p40, IL-15, IL-16, IL-17a, TNF- β , and vascular endothelial growth factor [VEGF]), Chemokine Panel 1 (Eotaxin, MIP-1 β , Eotaxin-3, Thymus and Activation Regulated Chemokine (TARC), IP-10, Macrophage Inflammatory Protein 1-alpha (MIP-1 α), Monocyte Chemoattractant Protein 1 (MCP-1), Macrophage-Derived Chemokine (MDC), and Monocyte Chemoattractant Protein 4 (MCP-4)), Angiogenesis Panel 1 (VEGF-c, VEGF-D, Tyrosine-Protein Kinase Receptor TIE-2 (Tie2), Flt-1, Phosphatidylinositol Glycan Anchor Biosynthesis Class F (PGIF), and basic Fibroblast Growth Factor (bFGF)), and Vascular Injury Panel 2 (Serum amyloid A (SAA), CRP, VCAM-1, and Intercellular Adhesion Molecule 1 (ICAM-1)). Each of these panels is a V-plex assay indicating it is fully validated according to fit-for-purpose principles and the Food and Drug Administration’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 Lower limit of detection (LLOD) were defined as 1/2 LLOD when determining significant differences in inflammatory mediator concentration between cases and controls but not for correlational analysis.

2.6. 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 non-parametric 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 statistical mediation model in which the association of catastrophizing with chronic postamputation residual limb pain severity was conveyed in part through indirect effects of inflammatory mediators. To evaluate this statistical mediation model, the approach of Preacher and Hayes²¹ (2004) was used to test the significance of the indirect effects. 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.²¹ This bootstrap methodology tested each model in a series of 1000 random subsamples repeatedly drawn from the full sample, generating 95% confidence intervals (CIs) (bias corrected) around the indirect effect test statistic. If the 95% CI for the indirect effect generated by the model did not

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

	Control (N = 40), mean (SD)	Case (N = 36), mean (SD)	P
Age	25.2 (4.8)	27.7 (9.1)	0.153
Body mass index	25.7 (2.8)	26.8 (3.6)	0.143
Time since amputation, mo	7.6 (3.5)	8.9 (5.9)	0.224
Smoking (packs per day)	0.6 (0.5)	0.6 (0.54)	0.817
Average pain severity (0-10)	1.2 (0.73)	5.6 (1.38)	0.000
Pain catastrophizing scale	2.6 (4.53)	11.8 (11.1)	0.000
	Control (N = 40), N (%)	Case (N = 36), N (%)	P
Male	40 (100)	35 (97)	0.958
Smokers	25 (63)	21 (58)	0.892
Opioid use	3 (8)	19 (53)	0.000
Ethnicity			
American Indian/Alaska native	0 (0)	0 (0)	1.000
Asian	2 (5)	1 (3)	1.000
Native Hawaiian or other Pacific Islander	0 (0)	0 (0)	1.000
Black or African American	3 (7)	3 (8)	1.000
White	37 (93)	32 (89)	0.884
Amputation site			
Leg	37 (92)	32 (89)	0.587
Arm	3 (8)	4 (11)	0.587
Multiple	0 (0)	0 (0)	1.000
Proximal amputation	12 (30)	16 (44)	0.192

Continuous variables were analyzed using *t* tests whereas categorical values were examined using a χ^2 test.

include zero, this indicated that the hypothesized indirect (mediated) effect was significant at the $P < 0.05$ level. To minimize the risk of bias in estimation of indirect effects, inflammatory mediator values were normalized through log-transformations before 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 severity (Spearman's $\rho = -0.07$, $P = 0.572$) or catastrophizing scores on the PCS (Spearman's $\rho = -0.13$, $P = 0.274$), nor with most inflammatory mediator values. Exceptions to the latter were IL-12 (Spearman's $\rho = -0.24$, $P = 0.035$), IL-15 (Spearman's $\rho = 0.26$, $P = 0.026$), IL-16 (Spearman's $\rho = -0.27$, $P = 0.022$), IL-1 α (Spearman's $\rho = 0.27$, $P = 0.021$), and VCAM-1 (Spearman's $\rho = -0.23$, $P = 0.044$). Examination of interactions between time from injury and pain intensity on all inflammatory markers revealed only one significant interaction (for IL-2; $P < 0.04$). Because of the general absence of relevant pain duration effects for key outcomes of interest, it was not included as a control variable in the analyses reported below.

3. Results

3.1. 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 TNF- α , TNF- β , IL-8, ICAM-1, Tie2, CRP, and SAA (**Table 2**). Each of the elevated markers has mainly pro-inflammatory properties. Descriptive statistics for all the mediators tested are shown in **Table 2**.

3.2. 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, postamputation residual limb pain severity, and pain catastrophizing. Ratings of average residual limb pain severity and PCS scores were significantly correlated, $r(74) = 0.62$, $P < 0.001$. **Table 3** summarizes associations between systemic inflammatory mediators and both residual limb pain severity and PCS scores. Higher pain severity 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 (taking into account observed degree of intercorrelations among the mediators) were highly significant for both average pain severity and catastrophizing. These results indicate that the overall associations reported between these 2 variables and inflammatory status are unlikely to represent spurious findings.

Table 2

Systemic inflammatory mediator concentrations in cases vs controls.

Mediator	Case (n = 36), median (IQR)*	Control (n = 40), median (IQR)*	Mann-Whitney U test (P)
IFN- γ	4.1 (3.3-6.4)	3.5 (3.0-6.2)	0.252
IL-10	0.3 (0.2-0.4)	0.3 (0.2-0.4)	0.113
IL-13	0.8 (0.8-0.8)	0.8 (0.8-0.8)	0.099
IL-1 β	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.859
IL-2	0.1 (0.1-0.3)	0.1 (0.1-0.2)	0.713
IL-4	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.970
IL-6	0.8 (0.4-1.2)	0.5 (0.3-0.9)	0.138
IL-8	4.7 (3.8-6.1)	4.0 (3.1-5.0)	0.041
TNF- α	2.2 (1.8-2.6)	1.9 (1.5-2.3)	0.031
IL-12	140 (103-191)	130 (83-154)	0.131
IL-17	1.1 (1.1-2.3)	1.1 (1.1-1.1)	0.546
IL-5	0.3 (0.1-0.6)	0.3 (0.2-0.6)	0.983
IL-7	3.4 (2.1-5.6)	3.5 (2.2-5.9)	0.768
TNF- β	0.2 (0.1-0.2)	0.1 (0.1-0.2)	0.037
VEGF	43.4 (32.0-56.5)	35.1 (28.3-44.1)	0.065
IL-15	1.9 (1.6-2.0)	1.8 (1.5-2.0)	0.914
IL-16	183 (152-230)	190 (165-272)	0.349
IL-1 α	0.9 (0.5-2.1)	1.2 (0.5-1.9)	0.971
Eotaxin	74.7 (62.7-104)	86.4 (62.0-108)	0.938
Eotaxin-3	18.8 (14.1-26.4)	22.3 (17.1-25.3)	0.399
IP-10	253 (170-361)	210 (155-329)	0.293
MCP-4	45 (32.2-60.6)	52.4 (38.6-69.7)	0.182
MDC	660 (481-824)	657 (532-746)	0.979
MIP-1 α	5.5 (5.5-12.7)	5.5 (5.5-12.0)	0.494
MIP-1 β	53.8 (38.9-68.0)	45.0 (37.8-62.0)	0.302
TARC	58.5 (30.8-101.0)	71.9 (38.9-124.0)	0.163
Flt1	47.5 (37.5-53.8)	47.5 (38.1-57.8)	0.881
PIGF	28.2 (22.5-34.2)	25.0 (18.8-28.1)	0.056
Tie2	6380 (5680-7530)	5550 (4830-6280)	0.008
VEGF-C	43.5 (33.9-68.2)	61.0 (42.9-93.7)	0.112
VEGF-D	540 (382-1060)	750 (453-2240)	0.172
bFGF	7.7 (2.0-22.8)	7.2 (4.4-32.3)	0.298
CRP	4010 (2010-10,600)	2150 (775-5300)	0.034
SAA	3880 (2420-8640)	1980 (840-4160)	0.002
ICAM-1	421 (351-586)	379 (310-421)	0.007
VCAM-1	415 (340-462)	415 (344-484)	0.873

* All concentrations are expressed in pg/mL with the exception of CRP, SAA, ICAM-1, and VCAM-1, which are reported in ng/mL. Mediators differing significantly in cases versus controls ($p < 0.05$) are highlighted in bold. IL, interleukin; IQR, interquartile range (Q1-Q3); TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

3.3. Do inflammatory mediators contribute to associations between pain catastrophizing and residual limb pain severity?

We considered the possibility that the positive association between pain catastrophizing levels (PCS) and residual limb pain severity might be accounted for in part by indirect effects

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	PCS	P
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-1 β	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-1 α	0.06	0.632	-0.03	0.831
MIP-1 β	0.14	0.238	0.11	0.358
TARC	-0.11	0.366	0.01	0.947
Fit-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

IL, interleukin; PCS, Pain Catastrophizing Scale; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor. Mediators differing significantly in cases versus controls ($p < 0.05$) are highlighted in bold.

conveyed through systemic inflammatory mediators. Although the only requirement for conducting analyses to test this model was that catastrophizing needed to be associated with pain severity, we restricted our analyses to only those inflammatory mediators showing significant associations with the outcome

of interest (residual limb pain severity) to limit the number of analyses conducted.

Results using bootstrapped mediation tests indicated significant indirect effects between PCS scores and residual limb pain severity through 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 severity independent of systemic inflammatory mediators (P 's < 0.001). Because both significant direct and indirect effects were observed for these mediators, results can be interpreted as indicating that the positive association between pain catastrophizing and residual limb pain severity was statistically mediated in part (rather than fully mediated) by TNF- α , TNF-beta, SAA, and ICAM-1 levels in plasma (model summarized in **Fig. 2**). Tests of indirect effects for other systemic inflammatory mediators showing associations with residual limb pain severity in correlational analyses were all nonsignificant (P 's > 0.05).

4. Discussion

In this study, we found elevated systemic levels of several pro-inflammatory mediators in amputees with residual limb pain (Cases) compared with those without clinically significant residual limb pain (Controls). Specifically, the pro-inflammatory mediators TNF- α , TNF-b, IL-8, CRP, SAA, Tie2, and ICAM-1 were significantly elevated in cases compared with controls.

Across both patient groups, severity 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.¹⁹ 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 after amputation.

This study also appears to be the first to examine associations between postamputation residual limb pain and levels of pain catastrophizing, a cognitive factor previously shown to exacerbate chronic pain severity across a variety of other pain conditions.^{22,31} Results, not surprisingly, indicated that elevated catastrophizing was correlated with greater residual limb pain severity. Although the causal nature of these effects cannot be ascertained because of the design of this study, the results nonetheless add to the existing literature by extending the apparent negative effects of pain catastrophizing into the postamputation residual limb pain population.

Finally, the current findings, to our knowledge, are among the first to systematically examine possible links among catastrophizing, chronic pain severity and a comprehensive array of inflammatory mediators. Although 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 postamputation residual limb pain, elevated catastrophizing levels were associated with higher levels of IL-8, IL-12, TNF- β , PIGF, and

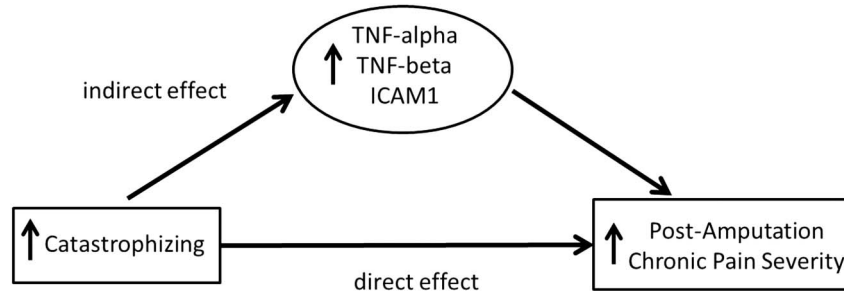


Figure 2. Model in which effects of catastrophizing on residual limb pain severity are conveyed through indirect effects of systemic inflammatory mediators.

ICAM-1, and lower levels of IL-13. These findings are consistent with generally pro-inflammatory influences of catastrophizing. Interestingly, the positive association between catastrophizing and residual limb pain severity was statistically mediated by TNF- α , TNF- β , SAA, and ICAM-1 levels. Although causation cannot be inferred from this study, results are consistent with the possibility that catastrophizing might be linked with an elevated pro-inflammatory profile, which in turn produces elevated chronic pain severity. Definitive conclusions regarding this causal model must await replication using a design with evaluation of pain catastrophizing, inflammatory mediator levels, and chronic pain severity over time. Extending these results to other chronic pain conditions would also be worthwhile.

4.1. 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 with amputees without significant 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 after nerve injury.^{14,15,32,33} IL-8, although 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 temporomandibular disorders (TMD) sufferers and in patients with interstitial cystitis.^{16,24} IL-2 and IL-12 have both been shown to be pro-algesic in animal models and in clinical studies of complex regional pain syndrome and painful small fiber neuropathy; IL-2 was shown to be elevated at the protein and mRNA level from blood samples.^{28,29}

One finding of particular interest relates to 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. There is also evidence that patients with other pain states, such as lumbar radiculopathy and stenosis, have high normal CRP levels.³⁰ 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.²⁰ The median CRP value of \sim 4 mg/L in the case group, although still within the range of normal CRP

values, is high enough to suggest that these individuals could be at increased risk for future cardiovascular disease.

4.2. Limitations

As a cross-sectional, observational study, this trial was not designed to determine causation regarding 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. Time from injury and pain intensity did not interact to show any consistent influence on inflammatory markers. Despite this, the possibility that the differential mediator profiles in these patients may have been present before injury cannot be excluded. Another possible limitation is that the degree of wound healing at the time of enrollment could have affected inflammatory mediator levels. However, active infection, heterotopic ossification, or signs of poor wound healing on physical examination were all exclusion criteria in this study, which should have minimized any effects of these potential confounds. Not surprisingly, opioid use in patients with significant residual limb pain was higher than controls. Opioids have been shown to favor an anti-inflammatory environment and could have affected inflammatory mediator concentrations in this patient cohort. We would expect, however, the anti-inflammatory effects of opioids to favor the null hypothesis, potentially strengthening the results presented here. 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. Also, because a large proportion of the plasma concentration values for IL-13, IL-2, IL-4, and IL-1 β were below the lower level of detection of the inflammatory mediator assay, it is possible that the significant correlations for IL-13 and IL-2 may be spurious; however, this limitation is not present for the 7 other mediators found to correlate with pain score. Finally, 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 severity and catastrophizing levels argue against our reported findings being spurious.

5. 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 severity and higher pain catastrophizing levels. These results generate intriguing hypotheses regarding the links between causation and resolution of the inflammatory state and chronic pain after nerve trauma. The mediators measured here may have utility as potential biomarkers of nerve injury-induced pain.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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