Award Number: W81XWH-12-2-0129

TITLE: Regional Anesthesia and Valproate Sodium for the Prevention of Chronic Post-Amputation Pain

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REPORT DATE: June 2018

TYPE OF REPORT: Final

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 *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
June 2018	Final	30 Sep 2012 - 29 Mar 2018

4. TITLE AND SUBTITLE
Regional Anesthesia and Valproate Sodium for the Prevention of Chronic Post-Amputation Pain

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-12-2-0129

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)

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8. PERFORMING ORGANIZATION REPORT NUMBER

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

U.S. Army Medical Research and Materiel Command
Fort Detrick, MD 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

This research was designed to test the efficacy of valproic in reducing the incidence of chronic pain after amputation, and to elucidate the underlying epigenetic mechanisms.
We have completed the randomized controlled trial at Duke University, WRNMMC, and the Durham VAMC. All patients received multimodal perioperative care including regional anesthesia and were randomized to valproic acid or placebo. Our data reveal an overall rate of chronic pain of 68.22% without a significant effect of valproic acid. The incidence of chronic pain was 65.45% in the treatment group and 71.15% in the placebo group.
We are pleased to note that the overall median pain scores decreased from baseline to follow up (median reduction of 2) with 74 patients (69.16%) noting a reduction. Patients additionally experienced functional improvement after surgery. BPI interference scores improved by a median of 12 and DVPRS supplemental scores improved by 7. **This research demonstrates that both pain and function significantly improve in the three months following surgical amputation when performed in the context of comprehensive perioperative care and regional anesthesia.**
We have completed the epigenetic studies to define the impact of methylation status and alterations on the susceptibility to chronic pain following surgery and are currently analyzing data for publication.

15. SUBJECT TERMS
Amputation, Postamputation pain, Post-surgical pain, Neuralgia, Epigenetics, Valproic Acid, DNA Methylation, Neuropathic pain, Pain Trajectory

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
U	U	U	UU	33	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

Page

INTRODUCTION

Chronic pain is a significant problem in patients undergoing surgery following military trauma and chronic vascular disease. Symptoms are typically treated with medications such as narcotics, anti-inflammatory drugs, and local anesthetics. Despite these therapies, more than 60% of patients who have an amputation or significant limb injury experience long-term chronic pain.(Reiber et al. 2010; Nikolajsen & Jensen 2001; Ephraim et al. 2005) Post-amputation pain, especially in young military personnel, may additionally impair a soldier's ability to ambulate or wear a prosthetic device, and may ultimately lead to the use of narcotic medications with associated risks. Identifying preventive mechanisms that can be employed at the time of surgery is of utmost importance for military and veteran health systems.

Amputation surgery itself is commonly viewed as a result of a failed re-vascularization procedure, a chronic infection that will not respond to antibiotic treatment, or an undesirable alternative when limb salvage is not an option. (Dardik 1997; Barshes et al. 2016; Uccioli et al. 2015) However, the trajectory of pain and recovery after amputation surgery is not well described and little is known about the effect of amputation surgery on pre-surgical pain.

Catheter-based regional blockade provides significant analgesia during surgical recovery.(Richman et al. 2006) and there are data to support the association between prolonged catheter use and reduced burden of chronic pain.(Borghi et al. 2010; Buchheit et al. 2015) Medications such as gabapentin, pregabalin, and ketamine have demonstrated analgesic and opioid sparing activities when used in the perioperative period.(Yan et al. 2014; Schmidt et al. 2013; Buvanendran et al. 2010; Jouguelet-Lacoste et al. 2015) However, these medications do not appear to reduce the burden of chronic pain when used at the time of amputation.(Nikolajsen et al. 2006; Hayes et al. 2004; Maier et al. 2003)

Valproates (valproate sodium, valproic acid, divalproex sodium) have been less frequently tested in the perioperative period despite their widespread use world-wide.(Perucca 2002) Their use is further supported by evidence-based guideline that recommended valproates as second-line therapy for painful diabetic neuropathy (Level B: evidence probably effective and should be considered).(Bril et al. 2011) It is also increasingly known that epigenetic are important factors in the development of persistent neuropathic pain(Zhang et al. 2011) and drugs that inhibit these changes have been shown to produce analgesia in animal models of both inflammatory and neuropathic pain.(Lu et al. 2010; Chiechio et al. 2009; Jones et al. 2005; Bai et al. 2010) Valproates such as valproic acid have the ability to alter histone acetylation and DNA methylation,(Dong et al. 2007; Yamanegi et al. 2010) modulating N-methyl-D-aspartate (NMDA) receptor function and pain processing.(Chen et al. 2005; Mitchell et al. 2005; Villeda et al. 2006; Dong et al. 2007) These combination of attributes make valproic acid an attractive possibility in the treatment and prevention of chronic post-amputation pain.

Our research investigates the effectiveness of valproic acid vs placebo when added to regional anesthesia in the reduction chronic pain after amputation or revision surgery. Additionally, we investigated the impact of epigenetic changes (methylation) for the suspectibility to, and development of chronic pain.

BODY:

HYPOTHESIS 1: THE USE OF COMBINED PERINEURAL LOCAL ANESTHETIC INFUSIONS AND VALPROATE SODIUM IN SURGICAL AMPUTATION PATIENTS WILL DECREASE THE INCIDENCE OF CHRONIC POSTAMPUTATION PAIN.

Specific Aim 1: In a blinded randomized controlled clinical trial at two centers, we will determine whether the combination of perineural catheter infusion and oral valproate sodium reduces the incidence of postamputation pain when compared with local anesthetic infusion alone.

Completion Timeframe	**Projected**	**Actual**
Task 1 (pre-study) – Human subjects approval (including HRPO): Months 1-24		
Milestone Pre-Study Task 1a – IRB and HRPO approval in Durham VAMC	*Month 9*	*Month 14*
Milestone Pre-Study Task 1b – IRB approval at WRNMMC	*Month 12*	*Month 17*
Milestone Pre-Study Task 1c – Duke IRB and HRPO approval (added site)	*Month 24*	*Month 26*

Task 1 was accomplished with the above noted timelines, with the third clinical site (Duke University) added in year 2 to improve enrollment.

Task 2 – Clinical Trial: Months 9-57

Aim 1*: Determine the efficacy of regional anesthesia and valproate in reducing the incidence of chronic post-amputation pain.*

Milestone Task 2a – First patient enrolled in Durham VAMC	*Month 9-10*	*Month 14*
Milestone Task 2b – First patient enrolled at WRNMMC	*Month 12-13*	*Month 20*
Milestone Task 2c – First patient enrolled at Duke	*Month 24*	*Month 26*
Milestone Task 2d – Endpoint adjudication meeting	*Month 18*	*Month 30*
Milestone Task 2e – Enrollment of 50% of projected subjects	*Month 45*	*Month 57*
Milestone Task 2f – Endpoint adjudication meeting	*Months 30*	*Month 35*
Milestone Task 2g – Endpoint adjudication meeting	*Month 36*	*Month 44*
Milestone Task – Endpoint adjudication meeting	*Month 42*	*Month 45*
Milestone Task – Endpoint adjudication meeting	*Month 48*	*Month 47*
Milestone Task – Endpoint adjudication meeting	*Month 52*	*Month 53*
Milestone Task 2h – Close of enrollment for clinical trial	*Month 57*	*Month 57*
Additional Milestone: Final adjudication analyses		*Month 66 & 71*

Task 2: Clinical Trial methodology, results, and analysis.

Methods:

This randomized controlled trial was conducted as part of an ongoing collaborative research initiative (Veterans Integrated Pain Evaluation Research (VIPER)) between Duke University Medical Center, Walter Reed National Military Medical Center (WRNMMC) and the Durham Veterans Affairs Medical Center (VAMC). The trial was designed to determine the extent to which the addition of oral valproic acid to regional anesthetic blockade (either peripheral nerve or epidural) and multimodal perioperative care decreases the incidence of chronic pain 3 months after amputation or amputation revision surgery. We also analyzed the trajectory of pain and functional recovery after surgery as well as epigenetic mechanisms associated with the development of chronic pain after nerve injury. Findings from the epigenetic analysis will be reported separately.

Patient Recruitment:

Prior to enrollment, the clinical trial was approved by the respective institutional review boards at WRNMMC, Durham VAMC, and Duke University Medical Center and the trial was registered at ClinicalTrials.gov (NCT01928849). Patients scheduled for surgical amputation or revision were screened for trial participation at the three Medical Centers from 2013 to 2017.

From the 1,365 patients screened, a total of 132 patients were consented and enrolled. Among those who were screened, a significant number had been added to the surgical schedules in a non-elective fashion at the Durham VAMC and Duke University Medical Center, and were not able to be approached for consent in a non-pressured environment given the short interim period between scheduling and surgery. Similarly, patients scheduled for amputations following trauma were often excluded because of the urgency of surgery.

Inclusion/Exclusion:

Criteria for inclusion were individuals, age 18 years and older presenting for amputation, stump revision, or surgery for mangled limb (defined as limb injury with sensory or motor deficits consistent with injury to a major nerve).

Criteria for exclusion were severe traumatic brain injury, significant cognitive deficits or dementia of any cause, substantial hearing loss without alternative means of communication, spinal cord injury with permanent deficits, current pregnancy or lactation, end-stage liver disease or hepatic encephalopathy, current therapy with valproic acid or other valproates, coumadin, chlorpromazine, olanzapine, zidovudine, or monoamine oxide inhibitors (medications affected by valproic acid metabolism), diagnosis of seizure disorder requiring anti-epileptic medication, current therapy with tricyclic antidepressants doses greater than 50mg/day, current diagnosis of malaria requiring anti-malaria medication, or allergy to valproates or valproic acid.

Randomization:
After enrollment, randomization was performed within the respective investigational drug pharmacy according to the schedule provided by our statistician. Randomization was stratified by site and surgical intervention, ie, amputation, amputation revision, or surgery for mangled limb with equal probability of assignment to the valproic acid group or the controlled placebo group. Patients, investigators, treating medical/surgical team, and study personnel were blinded to the assignment.

Intervention / Treatment:
The study medication (valproic acid oral solution 250 mg per 5 mL or placebo (similar tasting flavored syrup) was then administered in single-dispense units. The initial dose was given by the perioperative anesthesia team. Subsequent doses were stored on the hospital ward or in the ICU with the patient's other medications and administered by the nurse every 8 hours, up to 7 days or until time of patient discharge from the hospital, whichever came first. Blinding was kept intact throughout the study for all patients.

Patients in both the placebo and intervention study arms received regional anesthesia (either peripheral nerve or epidural), with multimodal perioperative management according to the standard of care at each of the three institutions. Research blood samples were collected preoperatively, postoperatively (at the end of treatment with study drug or placebo), and at clinic follow-up (3 months or at time of adjudication). Valproic acid levels were measured at the completion of drug administration to confirm study compliance.

Clinical Assessment Tools:
Clinical assessments were performed at enrollment, daily during hospitalization, and at clinic follow-up (3 months). If a patient was not available at 3 months to collect adjudication data, the study outcome was determined at the 6-month research visit. Assessment tools for pain and function included the Brief Pain inventory, short form(BPI) (Keller et al. 2004) and the Defense and Veterans Pain Rating Scale (DVPRS) (Buckenmaier et al. 2013). Phantom and/or residual limb pain was assessed using the Groningen Questionnaire Problems Leg Amputation (GQPLA) questions (van der Schans et al. 2002). Neuropathic pain was measured using the Self-Reported Leeds Assessment of Neuropathic Symptoms and Signs (S-LANSS) tool (Bennett et al. 2005; Clarke et al. 2013). The presence of complex regional pain syndrome was determined using the Budapest Clinical Criteria (Harden et al. 2010). Opioid and medication use was evaluated preoperatively, during hospitalization, and at the final outcome determination.

Physical Exam and Clinical Adjudiction:
Physical exam was performed on the surgical limb if no wound dressing was present. Visual inspection for asymmetry of sweating, color, skin changes, and hair growth. Allodynia was tested with cotton wool brushing. The presence of a Tinel's sign was elicited by tapping on the most painful area and asking the patient if (s)he experienced any "sensations of pins and needles". Sensory and motor deficits were additionally noted.

An adjudication committee consisting of Drs Buchheit, Van de Ven and Hsia held 8 sessions between March 2014 and November 2017. All 3 members had to be present for quorum and a majority vote determined the phenotypic endpoint. At these meetings, the post-amputation pain subtypes were defined through the following process:

Step 1: Determine if surgery was a primary amputation, a revision amputation surgery, or surgery for mangled limb

Step 2: Determine preoperative case/control status. Cases were defined by either residual limb/phantom pain $\geq$ 3/10 on numeric rating scale

Step 3: If patient had a previous amputation, determine presence of preoperative phantom/residual limb pain

Step 4: Determine if pain was neuropathic vs somatic pain using S-LANSS. If the patient had an S-LANSS $\geq$ 12, it was defined as neuropathic.

Step 5: If patient had significant residual limb pain, then categorize subtype:

a. Neuroma: defined as a focal area of pain or tingling when the residual limb (stump) is palpated or tapped.

b. Complex Regional Pain Syndrome was detected with Budapest clinical criteria.
c. Other neuropathic pain was defined as Mosaic neuralgia.

Adjudication of Clinical Endpoint:
An adjudication committee consisting of Drs Buchheit, Van de Ven and Hsia met for 8 sessions between March 2014 and November 2017. All 3 members were required to be present for a quorum and a majority vote determined the phenotypic endpoint. At these meetings, the post-amputation pain subtypes were adjudicated using the Duke Post-Amputation Pain Algorithm (Buchheit et al. 2015).

Statistical Analysis:
Patient data were collected from patients at all study sites and entered into Research Electronic Data Capture (REDCap)TM. Data were analyzed using SAS version 9.4 (SAS Inc., Cary, NC).

Patient characteristics were summarized by treatment group using mean (SD) or median [Q1, Q3] for numeric variables and count (%) for categorical variables to assess randomization and ensure balance between treatment groups. We also compared the patient characteristics between those who did and did not return for follow-up to evaluate the possibility of response bias. Finally, plasma levels of valproic acid (VPA) were monitored to verify treatment adherence, and to determine the concentrations of VPA among treated patients.

The incidence of chronic pain, the primary endpoint, was reported by treatment arm and compared using a two-sample chi-square test and multivariable logistic regression adjusting for baseline pain severity, type of surgery (amputation, revision, other), and surgical indication (medical vs. trauma). We also investigated possible differences in treatment effect within patient sub-groups based on study site, surgery type, and reason for surgery.

Secondary endpoints and analysis of functional trajectory were planned including the change in BPI (pain and interference scores), DVPRS (pain and supplemental scores), opioid use, and neuropathic pain subtypes from baseline to time of adjudication endpoint. Median [Q1, Q3] of the changes of mean scales from baseline was computed by arm and by assessed time between the two treatment groups via Wilcoxon rank sum test, and overall by the Wilcoxon signed rank test. Frequency and percentage of the categorical variables in above endpoints was reported overall, by treatment arm, and by assessed time. Total opioid consumption during the first and second postoperative day was summarized via median [Q1, Q3], and compared between groups via Wilcoxon rank sum test. Rates of mortalities, liver disorders, and infections was summarized by group and compared by Fisher exact or chi-square tests as appropriate to evaluate safety of valproic acid administration.

Sample size was calculated for the primary endpoint based on the assumption that 65% of the non-treatment arm patients will experience significant chronic pain (NRS ≥3/10, averaged over previous week) based on this group's prior research cohort (Buchheit et al. 2015). With a 12% drop-out rate at 3 months secondary to death and loss to follow up, and a type 1 error rate of 0.05, the planned study of 224 patients would have 83% power to detect a difference in the incidence of chronic pain of 20% (45% vs 65%) between two arms using a two-sided chi-square test. Due to difficulties in enrollment the study was stopped early after 107 patients completed the primary endpoint evaluation.

Results:
There were 107 patients who completed the endpoint assessment and were available for adjudication analysis. In 11 out of 107 patients (10%), the 6-month visit data was used in primary endpoint analysis. Median age for patients enrolled was 51. Four participants dropped out prior to treatment (2 withdrawals and 2 for medication exclusion) and 21 patients were lost to follow-up. There was no difference in enrollment site, surgery type or baseline pain in those individuals lost to follow-up. There were 62 patients randomized to Valproic Acid and 66 randomized to Placebo and no significant difference in dropout between treatment groups (p=0.130) or between sites (p=0.136).

Figure 1: Flow Diagram of Patients Assessed and Eligible for Study

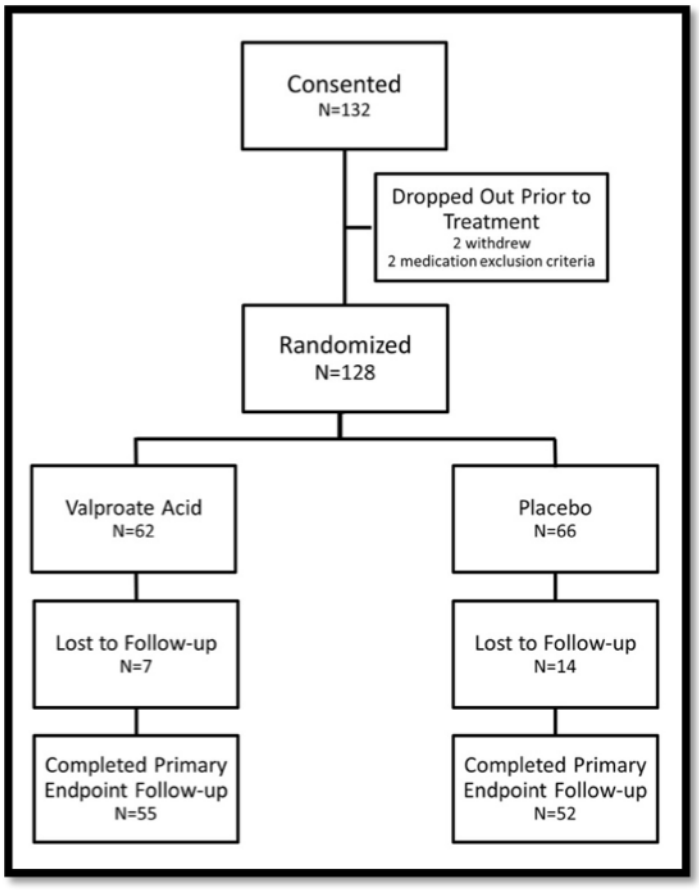


Table 1: Baseline Patient Characteristics

	Placebo (N=52)	Valproate (N=55)	Total (N=107)	p value
Site				0.9502[1]
Durham VA	11 (21.2%)	13 (23.6%)	24 (22.4%)	
Duke	16 (30.8%)	16 (29.1%)	32 (29.9%)	
WRNMMC	25 (48.1%)	26 (47.3%)	51 (47.7%)	
Surgery type				0.1805[1]
Primary amputation	23 (44.2%)	33 (60.0%)	58 (54.2%)	
Amputation revision	27 (53.8%)	22 (40.0%)	48 (44.9%)	
Surgery for mangled limb	1 (1.9%)	0 (0.0%)	1 (0.9%)	
Indication: Trauma vs Medical	25 (48.1%)	22 (40.0%)	47 (43.9%)	0.4001[1]
Sex (Male)	41 (78.8%)	45 (81.8%)	86 (80.4%)	0.6988[1]
Hispanic or Latino Ethnicity	2 (3.8%)	3 (5.5%)	5 (4.7%)	0.6936[1]
Race*				0.4589[1]
Asian	1 (1.9%)	0 (0.0%)	1 (0.9%)	
Black/African-American	11 (21.2%)	9 (16.7%)	20 (18.9%)	
Multi-racial	1 (1.9%)	0 (0.0%)	1 (0.9%)	
White	39 (75.0%)	45 (83.3%)	84 (79.2%)	

	Placebo (N=52)	Valproate (N=55)	Total (N=107)	p value
Age (years)	49.5 (32.0, 63.0)	57.0 (34.0, 67.0)	51.0 (33.0, 66.0)	0.4122[2]
Height (cm)	175.3 (167.6, 182.9)	177.8 (170.2, 182.9)	177.8 (167.6, 182.9)	0.3212[2]
Weight (kg)	88.5 (69.0, 109.0)	94.0 (75.8, 106.0)	91.0 (72.5, 107.0)	0.6626[2]
BMI	28.0 (23.7, 33.6)	29.0 (24.6, 32.7)	28.7 (24.1, 33.3)	0.8614[2]
Current smoker	38 (73.1%)	37 (67.3%)	75 (70.1%)	0.5122[1]
Opioid Use (morphine equiv)*	3.0 (0, 40.0)	5.0 (0, 40.0)	5.0 (0, 40.0)	0.9760[2]
Anticonvulsants*	17 (35.4%)	19 (37.3%)	36 (36.4%)	0.8493[1]
Tricyclic Antidepressants*	4 (8.3%)	6 (11.8%)	10 (10.1%)	0.5712[1]
Beta-blockers*	12 (25.0%)	12 (23.5%)	24 (24.2%)	0.8645[1]
Corticosteroids*	4 (8.3%)	4 (7.8%)	8 (8.1%)	0.9287[1]
Fish oil supplement*	1 (2.1%)	0 (0.0%)	1 (1.0%)	0.4848[3]
Comorbidities				
Charleson Comorbidity Index	1.0 (0.0, 3.0)	1.0 (0.0, 3.0)	1.0 (0.0, 3.0)	0.38122
Myocardial Infarct	4 (7.7%)	3 (5.5%)	7 (6.5%)	0.7109[3]
Congestive Heart Failure	1 (1.9%)	3 (5.5%)	4 (3.7%)	0.6184[3]
Peripheral vascular disease	14 (26.9%)	19 (34.5%)	33 (30.8%)	0.3935[1]
Cerebrovascular disease	4 (7.7%)	3 (5.5%)	7 (6.5%)	0.7109[3]
Significant Dementia	0	0	0	NT
Chronic pulmonary disease	4 (7.7%)	8 (14.5%)	12 (11.2%)	0.2615[1]
Connective Tissue disease	1 (1.9%)	1 (1.8%)	2 (1.9%)	>0.999[3]
Peptic ulcer disease	0 (0.0%)	1 (1.8%)	1 (0.9%)	>0.999[3]
Mild liver disease	2 (3.8%)	1 (1.8%)	3 (2.8%)	0.6110[3]
Diabetes without complications	8 (15.4%)	8 (14.5%)	16 (15.0%)	0.9032[1]
Diabetes with end organ damage	12 (23.1%)	10 (18.2%)	22 (20.6%)	0.5312[1]
Hemiplegia	0	0	0	NT
Moderate or severe renal disease	9 (17.3%)	8 (14.5%)	17 (15.9%)	0.6961[1]
Solid tumor (non-metastatic)	3 (5.8%)	6 (10.9%)	9 (8.4%)	0.3384[1]
Leukemia	0 (0.0%)	1 (1.8%)	1 (0.9%)	>0.999[3]
Lymphoma, Multiple myeloma	0 (0.0%)	1 (1.8%)	1 (0.9%)	>0.999[3]
Moderate or severe liver disease	1 (1.9%)	1 (1.8%)	2 (1.9%)	>0.999[3]
Metastatic solid tumor	0 (0.0%)	2 (3.6%)	2 (1.9%)	0.4957[3]
AIDS	0 (0.0%)	1 (1.8%)	1 (0.9%)	>0.999[3]
Depression	21 (40.4%)	14 (25.5%)	35 (32.7%)	0.0999[1]

	Placebo (N=52)	Valproate (N=55)	Total (N=107)	p value
PTSD	13 (25.0%)	9 (16.4%)	22 (20.6%)	0.2692[1]
Heterotopic ossification	13 (25.0%)	10 (18.2%)	23 (21.5%)	0.3908[1]
Baseline Pain Questionnaires				
DVPRS Pain Score*	5.0 (3.5, 8.0)	5.0 (3.0, 7.0)	5.0 (3.0, 7.5)	0.1462[2]
BPI average pain	5.1 (2.5)	4.2 (2.7)	4.7 (2.6)	0.0696[4]
NRS Pain Severity (average over past week)	8.0 (6.0, 10.0)	7.0 (4.0, 10.0)	8.0 (5.0, 10.0)	0.2725[2]

*contains small amount of missing data when surgery occurred on the same say as screening

P-Value Key: [1]Chi-Square [2]Wilcoxon [3]Fisher Exact [4]Equal Variance T-Test

Effect of Perioperative Valproic Acid Administration:

Overall rate of chronic pain (defined as pain ≥ 3/10 on the numeric rating scale (NRS) over the previous week) was 68.22% in the study cohort. There was no significant effect of perioperative valproic acid administration with a rate of 65.45% (n=36) in the treatment group and a rate of 71.15% (n=37) in the placebo group (chi-square p=0.53). The odds ratio for development of chronic pain with treatment was 0.77 (95% CI 0.34-1.74) which was not statistically significant. Also, no significant effect of valproic acid was found in any of the subgroups studied, including patients having surgical amputation following traumatic injury (OR 1.04, P-value 0.956). In the multivariable model analysis the only factor significantly associated with odds of chronic pain was baseline pain score. The odds of chronic pain were 1.27 times higher for each point increase in baseline pain (OR 1.27 95% CI (1.10, 1.47), p=0.001).

Table 2: Effect of VPA Administration by Site and Surgical Subgroups

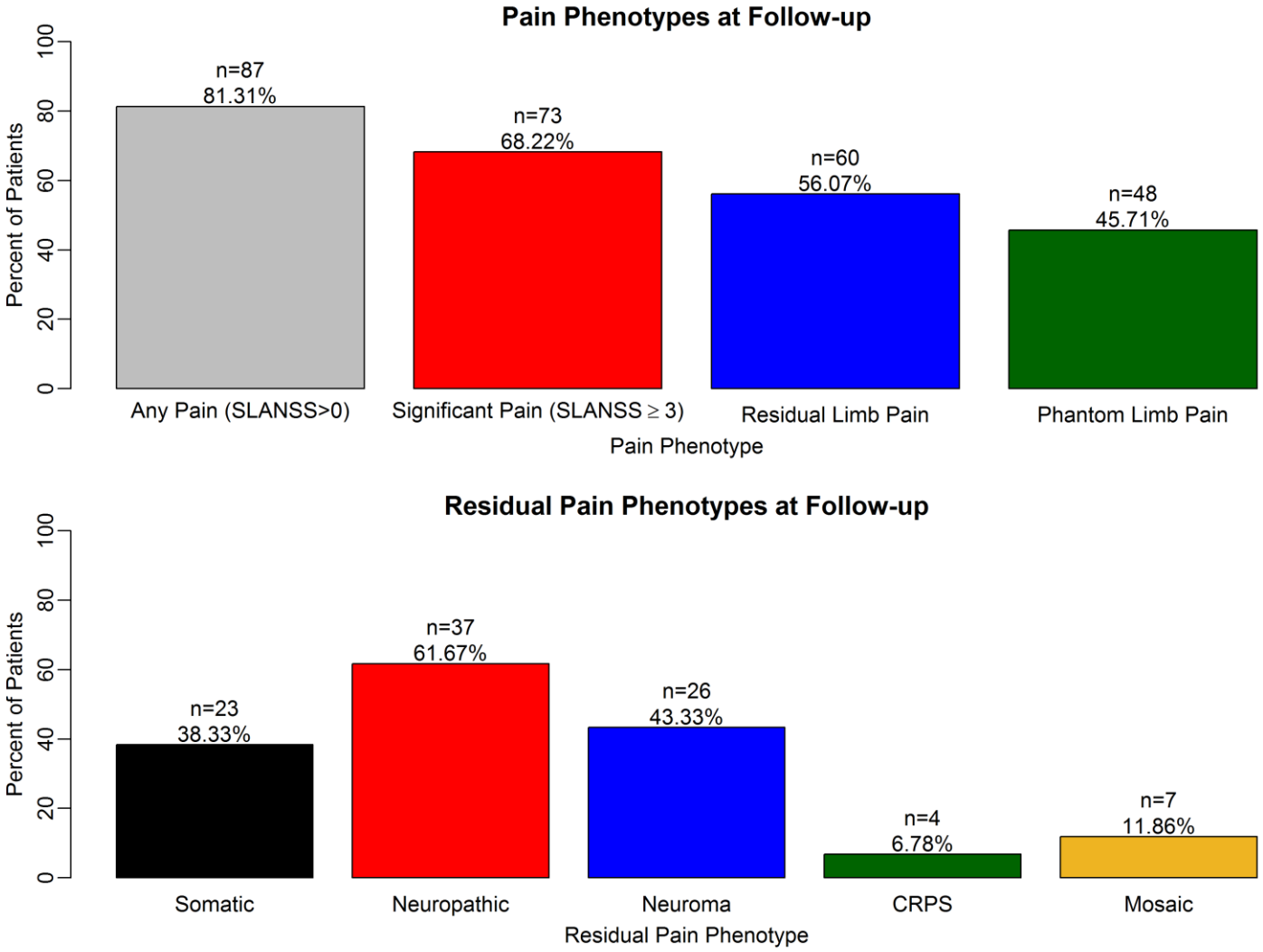
Group	**Chronic Pain Placebo**	**Chronic Pain Valproic Acid**	**OR (95% CI)**	**P-Value**
Overall	71.15	65.45	0.77 (0.34, 1.74)	0.5273
Site				
Durham VA	63.64	53.85	0.67 (0.12, 3.42)	0.6285
Duke	75.00	62.50	0.56 (0.11, 2.50)	0.4480
WRNMMC	70.83	73.08	1.06 (0.30, 3.67)	0.9313
Surgery Type				
Primary Amputation	73.91	63.64	0.62 (0.18, 1.95)	0.4197
Revision	67.86	68.18	1.02 (0.31, 3.45)	0.9805
Reason for Surgery				
Medical	70.37	60.61	0.65 (0.21, 1.89)	0.4314
Trauma	70.83	72.73	1.04 (0.29, 3.84)	0.9557

Pain Phenotypes at Follow-Up:

Analysis of pain phenotypes was performed both at study enrollment and at 3-month follow-up. At end-point adjudication, we observed that 81.3% of patients experienced pain of any severity, 68.22% experienced significant pain (≥3/10 NRS), 56.07% noted residual limb pain (RLP), and 45.71% described phantom limb

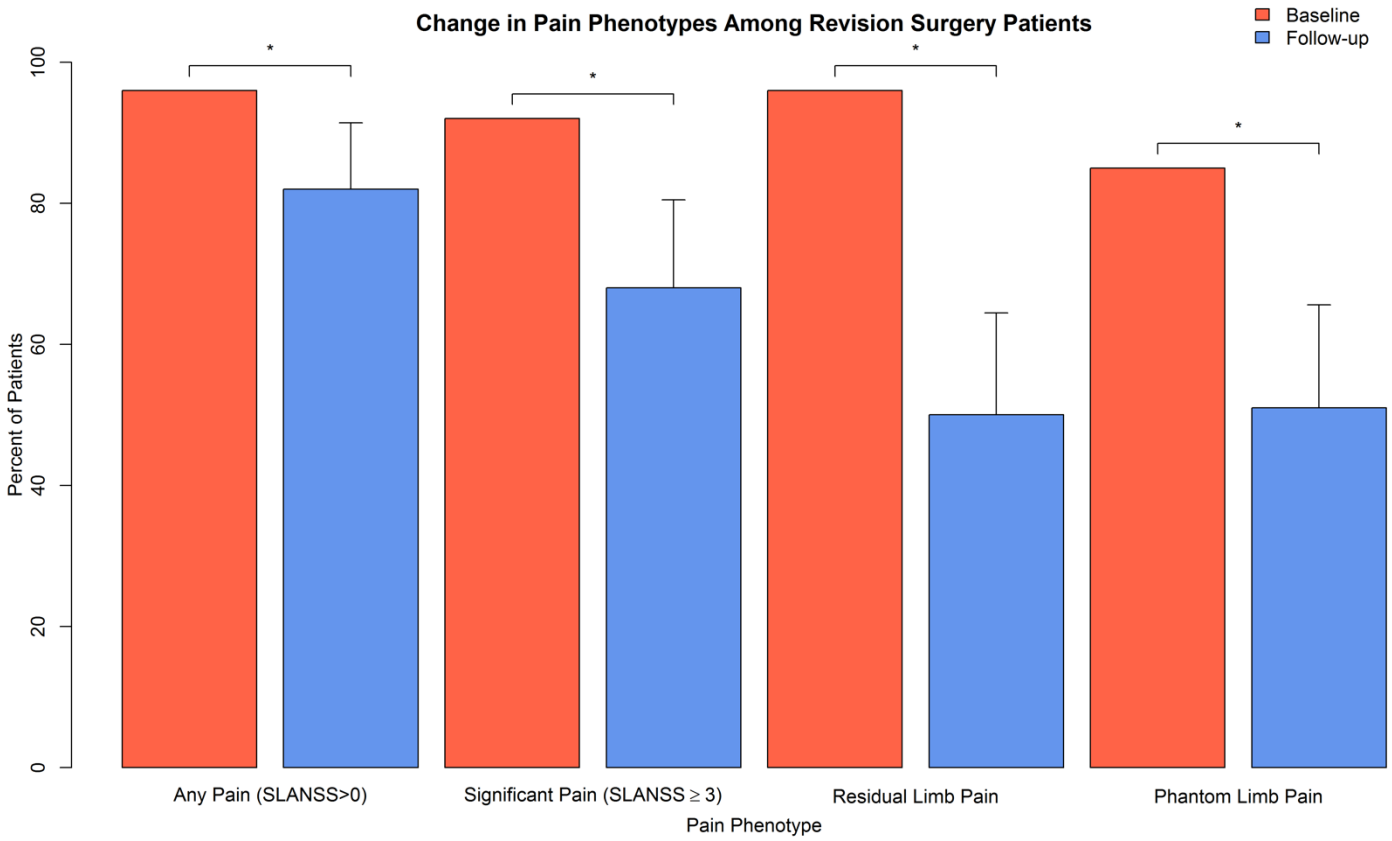
pain. In patients with RLP, we found the majority (61.67%) described neuropathic symptoms with one or more sensitized neuromas (43.33%).

Figure 2: Neuropathic Pain Subtypes at Follow-up Visit



We also adjudicated patients undergoing revision amputation to determine the impact of surgery on the severity of different pain phenotypes. Subsequent to surgery, we noted improvements in the percentage of patients experiencing pain of any severity, significant pain (≥3/10 NRS), and subtypes of residual limb pain, and phantom pain.

Figure 3: Change in Pain Phenotypes with Revision Amputation Surgery



Pain and Functional Trajectories:

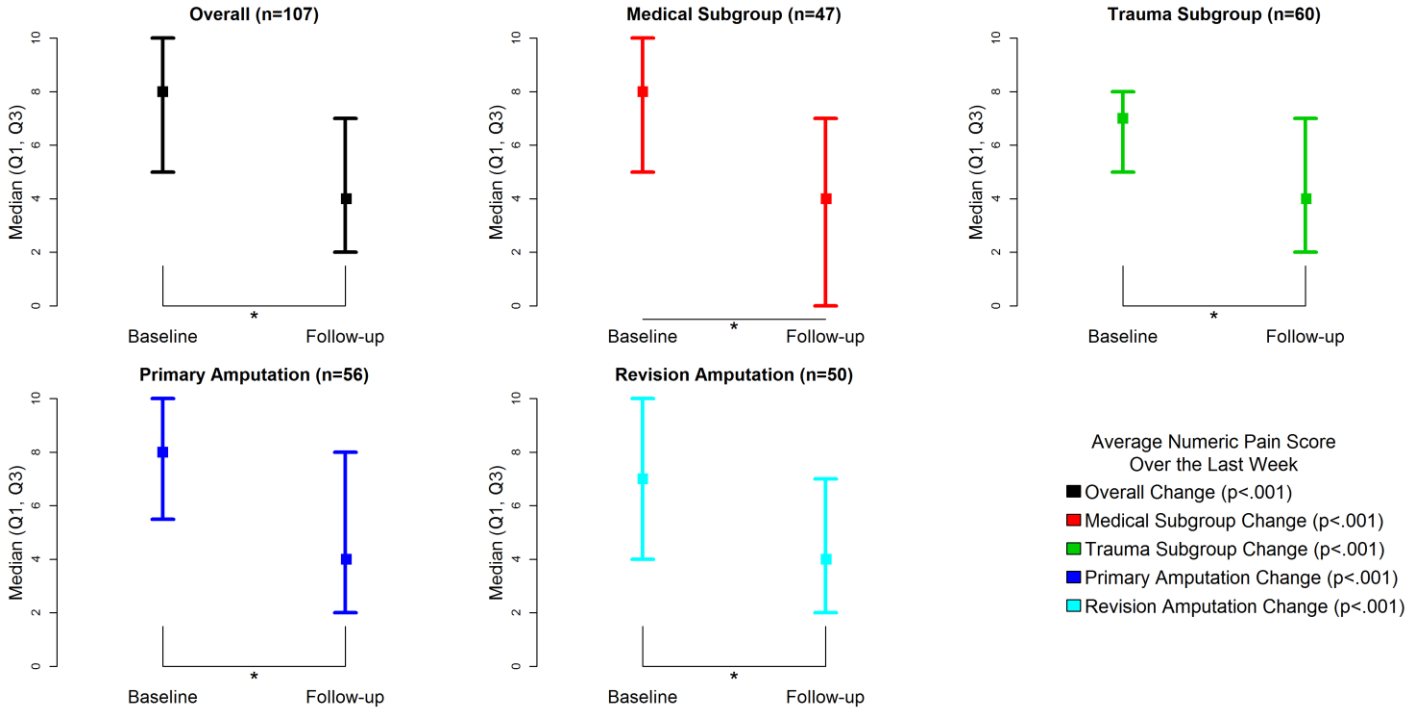
Overall the median NRS pain score at baseline was 8/10, and at follow-up was 4/10, with a median [Q1, Q3] reduction of -2 [-5, 0] points within patient (p<0.001). Following surgery, 77 patients (69.16%) reported a reduction in pain scores, while 18 patients (16.82%) experienced no change, and 15 (14.02%) reported an increase in pain score. There was no significant difference in the change in NRS pain score from baseline between placebo (median [Q1, Q3] -2 [-5, -1]) and treatment (median [Q1, Q3] -2 [-5, 0]) groups (p=0.349).

Changes in BPI pain scores (median [Q1, Q3] -1 [-3, 0], p<0.001), and DVPRS scores (median [Q1, Q3] -1 [-3, 0], p<0.001) also reflected overall improvement in pain after surgery. There was no evidence of a difference in the change in functional scores from baseline between placebo and treatment groups (See Table #3).

Table 3: Change in Pain and Function from Baseline

Questionnaire Change Score	**Placebo (N=52)**		**Valproate (N=55)**		**Overall (N=107)**		Group Diff WRS p-value	Overall WSR p-value
	n	med (Q1, Q3)	n	med (Q1, Q3)	**n**	**med (Q1, Q3)**		
NRS Pain	52	-2 (-5, -1)	55	-2 (-5, 0)	**107**	**-2 (-5, 0)**	0.3494	<0.001
DVPRS Score	43	-2 (-3, 0)	44	0 (-3.5, 0)	**87**	**-1 (-3, 0)**	0.4164	<0.001
BPI Score	49	-2 (-3, 0)	52	-1 (-3, 0)	**101**	**-1 (-3, 0)**	0.5901	<0.001

Figure 4: Average Numeric Pain Score Trajectory



Study patients also experienced self-perceived functional improvement after surgery by all measures assessed. BPI interference scores improved from a baseline median of 34.5 to a follow-up median of 10. BPI activity subscales improved from baseline median of 5 to follow-up score of 1. Likewise, DVPRS supplemental questions reflected significant improvements in function, improving from a baseline (18.5) to follow-up (7), with activity subscale improving from a median baseline of 5 to a follow-up median of 2.

Table 4: Trajectory of BPI and DVPRS Functional Scores

		Primary Amputation (N=56)		Revision (N=50)	
Questionnaire	**Time Point**	**n**	**med (Q1, Q3)**	**n**	**med (Q1, Q3)**
DVPRS Activity Question	Baseline	50	**7** (1, 10)	41	**4** (1, 8)
	Follow-up	55	**2** (0, 5)	46	**2** (0, 5)
DVPRS Supplemental Questions Sum	Baseline	50	**20** (10, 37)	41	**17** (10, 32)
	Follow-up	56	**7** (0, 19.5)	46	**6.5** (0, 14)
BPI Activity Question	Baseline	55	**6** (2, 10)	50	**3** (0, 8)
	Follow-up	55	**1** (0, 5)	46	**1** (0, 4)
BPI Interference Questions Sum	Baseline	55	**39** (20, 58)	50	**23.5** (10, 45)
	Follow-up	56	**10** (1.5, 37.5)	46	**9** (0, 24)

Figure 5: BPI Functional Trajectory

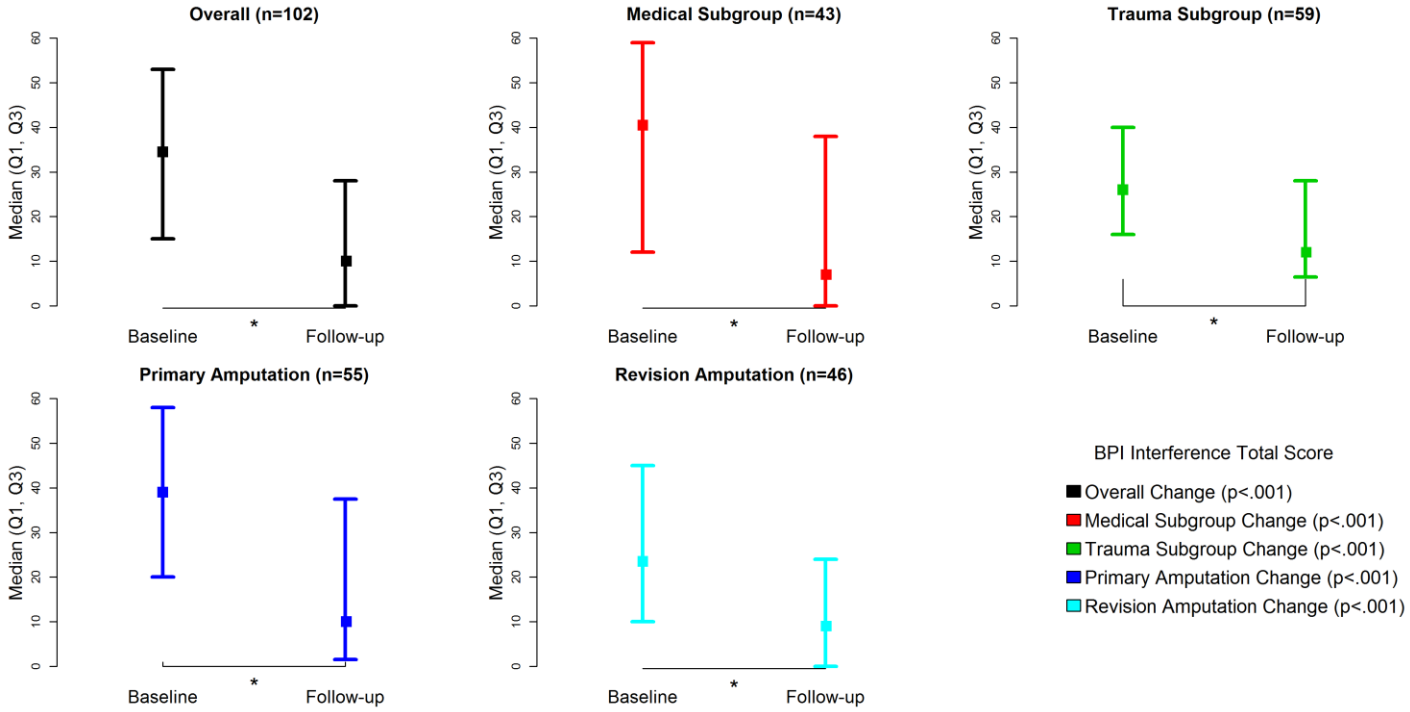
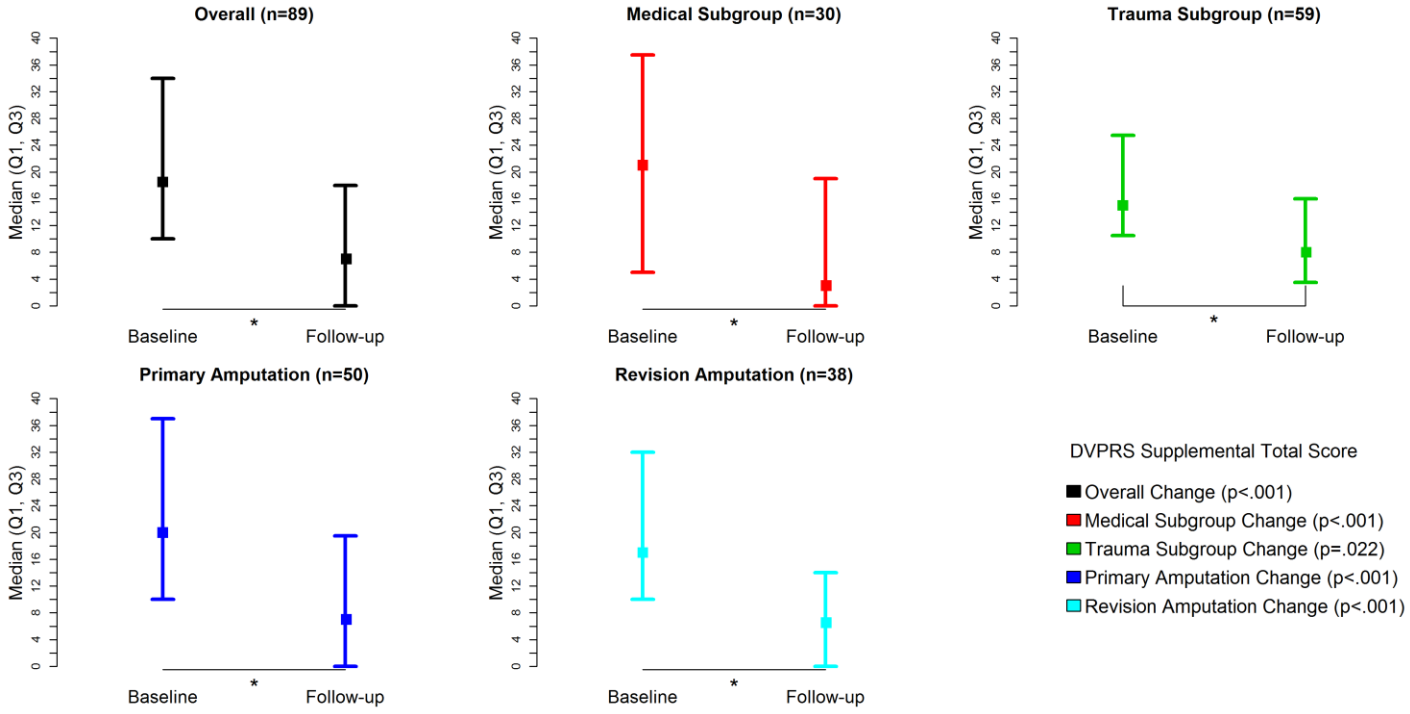


Figure 6: DVPRS Functional Trajectory



<u>Opioid consumption:</u>

We analyzed perioperative opioid consumption in morphine milligram equivalents (MME) at 2 time points (0-24 hours post-op and or the 24-48hours post-op) by Wilcoxon Rank Sum test. We did not find a significant reduction in opioid use in the VPA group at either time period. From 0-24 hours, the median consumption was 33 MME in the VPA group vs 59 MME in the placebo group (p=0.267), and from 24-48 hours, the median opioid consumption was 45 MME in the VPA group vs 49 MME in the placebo group (p=0.249).

Table 5: Opioid Consumption Postoperative Days 1 and 2

Postoperative Day	Placebo	Valproric Acid	Overall	Group Difference P-value
1	58.6 (20, 88.8)	32.6 (10, 108)	45 (18, 90)	0.2665
2	49 (24, 158.5)	45 (20, 114)	48 (20, 140)	0.2491

Valproic Acid Administration:
A study drug day was defined as a day during which the patient received 3 doses in a 24-hour period; if a dose was missed this was subtracted from the total number of treatment days for that patient. Among the 107 patients who completed the study, the average number of study drug days was 3.7 days.

Valproic acid serum levels were not detected in 2 patients in the treatment arm. These patients were included in their randomization group for the primary intention to treat analysis but excluded in the secondary per protocol analysis. Among the VPA patients with detectable blood levels, the measurement concentrations ranged from 4.2-59mg/L with a median [Q1, Q3] of 37.7mg/L [25.7, 45.5].

Adverse Events:
Among the 128 randomized and treated patients, 82 patients reported adverse events (46 placebo and 36 valproic acid, p=0.171), and 46 patients experienced severe adverse events (26 placebo and 20 valproic acid patients, p=0.400). There were a total of 6 mortalities (4 placebo and 2 valproic acid, p=0.681), 11 patients with liver function events (7 placebo, 4 valproic acid, p=0.402), and 28 patients with post-operative infections (16 placebo and 12 valproic acid, p=0.504). There were 46 patients (20 placebo and 26 valproic acid) who experienced no adverse events.

Table 6: Adverse Events

	Placebo (n=66)	Valproate (n=62)	p-value
Any Adverse Event	46 (69.7)	36 (58.1)	0.171[1]
Any Serious Adverse Event	26 (39.4)	20 (32.3)	0.400[1]
Mortality	4 (6.1)	2 (3.2)	0.681[2]
Hepatobiliary Disorder	7 (10.6)	4 (6.5)	0.402[1]
Infection or Infestation	16 (24.2)	12 (19.4)	0.504[1]
Serious Infection or Infestation	7 (10.6)	4 (6.5)	0.402[1]

Discussion:
In this randomized controlled trial we observed an incidence of chronic post-amputation pain similar to that noted in prior literature (Reiber et al. 2010; Ephraim et al. 2005; Buchheit et al. 2015), but we did not observe a significant reduction in chronic pain with the addition of valproic acid to a multimodal perioperative analgesic regimen that included the use of regional anesthetic techniques (either peripheral nerve or epidural).

Encouragingly, both BPI and DVPRS pain assessments improved significantly between the time of enrollment and final end-point adjudications. These improvements in pain were noted in all subgroups, regardless of the reason for amputation, ie, medical illness (diabetes or vascular disease) or traumatic injury. Even more impressive were the improvements in self-perceived function during this 3-month time period regardless of subgroup (medical disease vs trauma) or assessment tool (BPI interference score vs DVPRS supplemental questions).

Although patients experience functional gains as amputation sites heal, prostheses are fitted, and rehabilitation evolves, these prosthetic-facilitated functional gains are often not realized until up to a year or longer after surgery (Czerniecki et al. 2012). Our data support that the symptomatic and functional improvements in comprehensively managed patients are seen in a time course that appears to precede completion of rehabilitation.

HYPOTHESIS 2: THE TRANSITION FROM ACUTE TO CHRONIC PAIN IS MEDIATED VIA EPIGENETIC

MECHANISMS (SPECIFICALLY DIFFERENTIAL DNA METHYLATION) IN GENES INVOLVED IN INFLAMMATION AND NOCICEPTION. THESE EPIGENETIC CHANGES ARE MODIFIABLE WITH THE USE OF VALPROATE SODIUM, A KNOWN INHIBITOR OF ECTOPIC DNA METHYLATION.

Specific Aim 2: We will analyze differential DNA methylation region (DMR) patterns of patients with different types of post-amputation pain, and determine the way they are altered by valproate sodium. We will prioritize pathways of interest using plasma metabolomics and confirm the functional relevance of these epigenetic modifications using circulating leukocyte gene expression signatures.

Task 3 –Epigenetic Genomic and Gene Expression Analysis: Months 48-60

Aim 2: Determine the role of differential DNA methylation in post-amputation pain syndromes and their Treatment with valproate.

a. Determine the effect of pre-surgical methylation status on the incidence of chronic post surgical pain through methyl-seq (targeting 5.5 million cytosine sites) on 90 patients before and after surgery

Milestone Task 3a –Initial methylation sequence analysis of 60 samples collected	Month 50	Month 62
Milestone Task 3b – Methyl-seq initial analysis of 60 samples completed	Month 50	Month 62
Milestone Task 3c – Collect Methyl-seq sequence on 60 samples with initial analysis completed	Month 54	Month 62
Milestone Task 3d – Collect RNA seq data on 120 samples with initial analysis completed	Month 54	Month 59
Milestone Task 3e – Collect Methyl-seq sequence on 60 samples with initial analysis completed	Month 56	Month 62
Milestone Task 3f – Collect RNA sequencing data on 60 samples with initial analysis completed	Month 56	Month 59
Milestone Task 3g,3h – Targeted analysis of methylation status at promoter regions of genes of interest with confirmatory gene expression analysis using RT-PCR	Month 58	Month 69
Final Task 3 Milestone – Local investigator meeting for convergence analysis of epigenetic, genomic and RNA expression data	Month 60	Month 68

Methylation study deliverables:

September 2016:
RNA processing begun on pre-study drug samples with good yields noted

As of 12/31/2016:
The process of sample analysis (expression, sequencing, and methylation) was begun with good sample quality and yields. The plan for enriched methylation studies (approved 1/14/2016) is proceeding successfully with excellent early results.

As of 3/31/2017:
mRNA extractions was completed for 92% of the "day of surgery" samples and 90% of the "3 month" samples. The final extractions, including those for recently arrived samples for 6 more patients, should be completed by the end of the week. Extractions were unsuccessful for 1 "day of surgery" sample and one "3 month" samples. Extracted RNA samples were sent to the Sequencing Core.

As of 6/30/2017:
RNA sequencing on over 150 samples was completed and analysis has started. We are now starting extraction and processing of DNA samples in anticipation of Methylation sequencing.

As of 12/31/2017:
Methylation sequencing completed, analysis of sequence data commenced. Sample quality was excellent.
As of 3/20/18:
Initial analysis and QC performed

As of 5/15/2018:
Pathway analysis review with Core Epigenetic Lab

As of 6/1/2018:
Methylation summary review and pathway analysis completion

Epigenetic Studies:
Epigenetic and methylation studies were performed at the Duke Center for Genomic and Computational Biology with the collaboration of Dr David Corcoran. Because of recent advances in genomic and methylation analyses, these studies now include whole genome bisulfite methylation sequencing and targeted methyl-DNA immunoprecipitation sequencing as effective laboratory methods, allowing refinements in outcomes for this research grant. We chose this platform over microarrays. Arrays have problems with incorporating genetic information like SNP detection, discovery of new loci, and providing allele specific patterns. What's more, array probes are susceptible to batch effects and can potentially cross-hybridize with non-targeted DNA, confounding results and requiring secondary confirmation.

Methylation sequencing of 181 samples was completed in January of 2018 using Roche Nimblegen methy-seq kit to prepare the libraries. This kit targets 5.5M CpG sites. The total size of the capture is ~90Mb. We were able to obtain high quality methylation and RNA sequencing data on 98% of samples including sufficient depth of sequencing for our analysis purposes. The methylation and RNA sequencing analysis is continuing but we have already obtained results comparing methylation changes before and after surgery in patients with significant chronic pain versus those without significant chronic pain. All genes with significant methylation changes (defined by unpaired t-test value of <0.05% (~800 genes) were entered into Ingenuity IPA pathway analysis software to determine pathways associated with the presence of chronic pain after amputation. The results are reported in Figure 7 below. Most interesting was the presence of a large number of inflammation and immune cell related pathways suggesting that changes in the inflammatory response to injury might predispose to chronic pain. This aligns well with the latest understanding that chronification of nociceptor sensitization is likely an inflammation based process. We will soon complete similar analysis comparing methylation changes before and after surgery and with accompanying RNA expression changes. The validation portion of the study using targeted RT-PCR is ongoing. RNA has been extracted from all additional patient samples and cDNA libraries have been created. Using RNA sequencing and RT-PCR, important changes in gene expression found in the main portion of the study described above will be validated in these remaining samples.

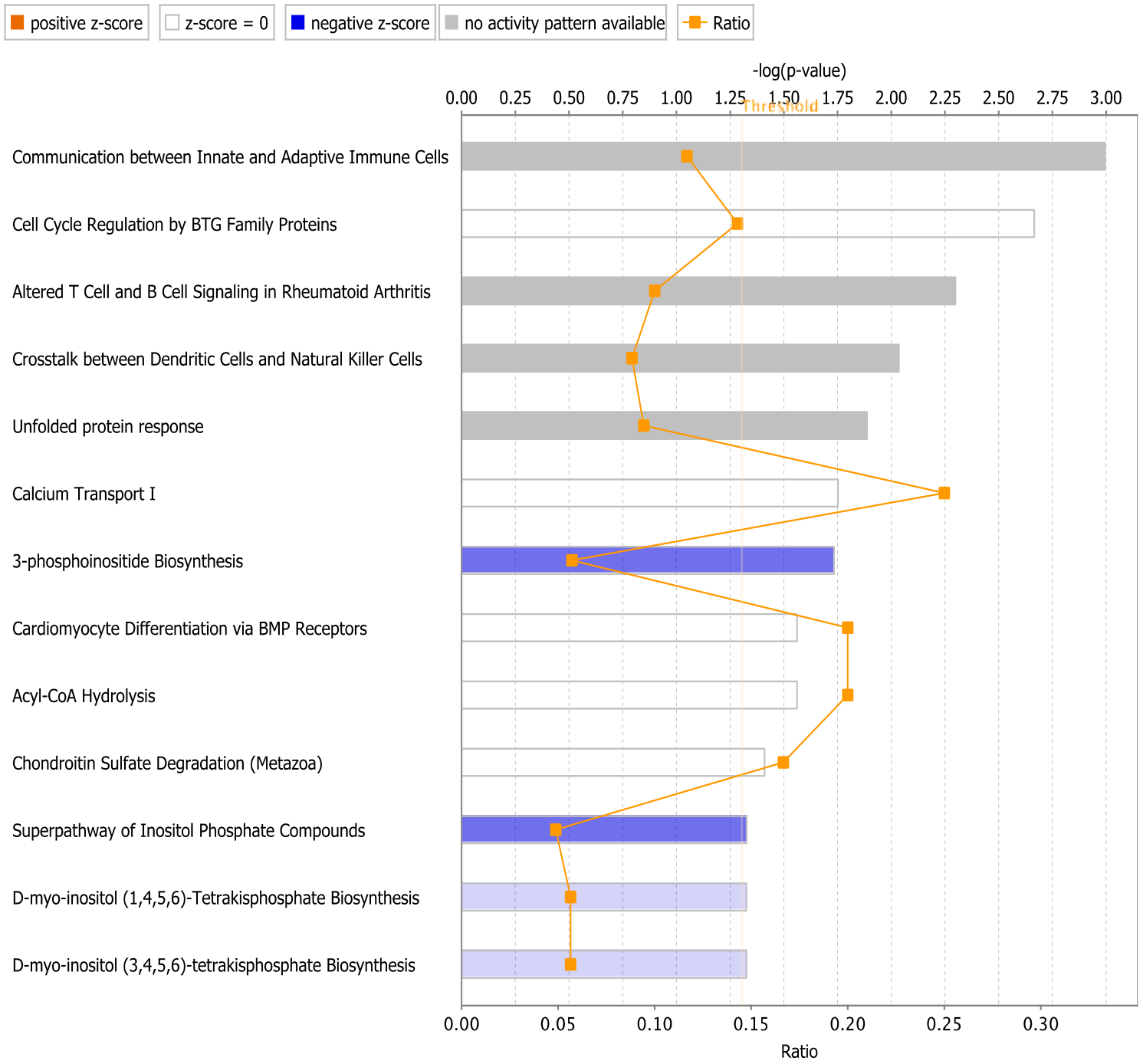


Figure 7: Pathways with the largest number of genes with significant methylation changes in patients who develop chronic pain after amputation or revision surgery compared to those who do not.

Proposed amendments to scientific justification

1) Methodology change for analysis of DNA methylation changes:

The initial study proposal utilized Illumina 450k methylation arrays to determine changes in DNA methylation patterns induced by surgery and treatment with the epigenetically active therapeutic, Valproic Acid. At the time of initial study design this array-based technology was the most robust and cost effective solution for genome-

wide DNA methylation analysis. The gold standard sequencing based method was cost prohibitive with per sample costs ranging from $10,000 to $20,000.

Array based technology for DNA methylation analysis has several weaknesses:

- Each cytosine assayed by the array has been chosen by a scientist with inherent bias toward analysis of his or her own gene or pathway of interest. We prefer our analysis to be genome wide and as unbiased as possible.
- Many array probes have been shown to bind to non-targeted genomic regions or regions with single nucleotide polymorphisms.
- All array analyses encounter batch effect errors during processing that are difficult to correct.
- The 450,000 cytosines analyzed by the array represent only a small fraction of all cytosines that can be potentially methylated genome-wide.

Over the past three years, next-generation sequencing technology has matured and per sample cost has lessened significantly. Direct methylation sequencing mitigates each of the problems described above. Sequencing an entire methylome eliminates the bias inherent in choosing probes to particular cytosines, batch effect and non-specific binding problems are eliminated, and genetic sequence information can be obtained simultaneously.

We proposed to use methyl-seq instead of methylation arrays for analysis of methylation status and methylation changes before and after amputation surgery and before and after Valproic Acid treatment. This will provide higher-quality, unbiased methylation analysis and also genetic sequence data. This is particularly exciting as the exome sequencing data obtained in our initial Veterans Integrated Pain Evaluation Research (VIPER) project has provided valuable insight into novel pain mechanisms.

2) Methodology change for analysis of RNA expression:

RNA expression array technology was also the most cost effective solution at the time we designed this study, but it has the same inherent flaws as DNA methylation arrays. The cost of next generation sequencing technology for analysis of RNA expression levels has now reached parity with RNA array analysis so we also propose to use RNA-seq for our RNA expression measurements. This will not require any alterations to the study budget.

3) Detailed DNA methylation and RNA expression study design to optimize completion of Specific Aim 2:

Below is an outline of our more detailed study design for molecular analysis.

Experiment 1: Effect of pre-surgical methylation status on susceptibility to chronic pain

We will compare methylation status (and corresponding RNA expression changes) in 90 patients before and after surgery and compare patients who go on to develop chronic pain to those that do not to determine methylation markers of susceptibility.

This will provide unprecedented genome wide, unbiased DNA methylation and DNA sequencing data to help determine genetic and epigenetic markers of susceptibility to chronic pain development.

Experiment 2: Effect of surgically induced methylation changes on incidence of chronic pain

We will compare methylation status (and corresponding RNA expression changes) before and after surgery to determine the effect of surgery itself on methylation status

Experiment 3: Effect of VPA on DNA methylation status and chronic post-amputation pain incidence

Although valproic acid treatment did not significantly change chronic pain incidence after amputation surgery we will determine the effect of this epigenetically active drug on methylation status in patients receiving drug compared to patients receiving placebo.

Problems or Challenges:

We received all approvals necessary to begin enrollment at the Durham VAMC on 22Nov13, 11 months after IRB submission. As our first year of enrollment (Grant Year 2) saw fewer numbers of eligible subjects because of reduced military conflict, we requested that Duke University Medical Center be added as a third enrollment site. We received approval for enrollment at DUMC on 19May14, from HRPO on 30Jul14, and from DOD on 02

Oct14. With this third study site, we were able to increase enrollment, although still experienced multiple potential study patients excluded by overly rigid inclusion criteria in regards to renal disease (the study drug is hepatically metabolized).

After meetings with the investigational pharmacist and a thorough review of the literature, we removed renal failure from the list of exclusion criteria at Duke University Medical Center on 24 Jun15. An amendment for the same was submitted to the Durham VAMC and approved on 10 Dec 15. This change of inclusion criteria is consistent with the pragmatic "real world" nature of this trial since one of the significant target audiences (chronically ill veterans with vascular disease and diabetes) experiences a high incidence of renal failure. Since the study medication is continued in the treatment of veterans and patients with neuropathic pain, chronic headaches, and bipolar disorder, we believed it appropriate to modify the inclusion/exclusion criteria to mirror standard clinical practice for the treatment of similar conditions.

During year 3 of this research project, we also analyzed our initial VIPER study data, revealing a 65% baseline incidence of chronic post-amputation pain, higher than anticipated at the start of this Valproate grant. (Buchheit et al. 2015) The principle investigator has also participated in a series of discussions with other investigators, including those in the IMMPACT Study Group regarding "meaningful" improvements needed to define significance in the setting of a clinical trial. The conclusions of these discussions are also supported by research literature with guidelines now recommending clinical significance to be defined as between 20-30% improvement. (Dworkin et al. 2005) With a baseline incidence of 65% chronic pain and a 20% threshold for clinically significant improvement, our statisticians report that 192 total enrolled patients would be required to maintain 80% power for clinical outcomes analysis. Study power expectations were adjusted accordingly during a rebudget process that improved methylation and expression analysis to the latest technology.

We extended our enrollment period into the no-cost extension year, which allowed for enrollment of 132 patients. We reviewed and confirmed the statistical analysis plan with the statistician in August 2017 and analysis began immediately afterwards. Of those enrolled, 128 patients were treated with standard anesthetic care, a perioperative regional catheter, and randomized to either valproic acid 250mg Q8hrs or placebo for the duration of hospitalization. The primary outcome was the proportion of patients with chronic pain at 3 months (>3/10 average over past week on numeric rating scale).

The most significant challenge we have encountered in this research study has been study enrollment. At WRNMMC this has occurred for good reasons (reduction in military traumatic injuries). At Durham VAMC enrollment has mostly been challenged by patients with significant comorbid disease and the desire of veterans to not participate in a research study. At Duke, the surgical schedule logistics have frequently prevented discussion of the research with the patient in a non-pressured environment, and therefore reduced the number of potential study candidates.

Despite these hurdles, our research team and study coordinators have continued to work tirelessly (over 1,300 patients screened). However, with the results of the analysis (rate of chronic pain of 65.45% in the treatment group and a rate of 71.15% in the placebo grouop) it is important to point out that ***the primary outcome measure was not affected by low enrollment***. This differential between rates of chronic pain between treatment and placebo group would not have achieved significance even if the trial had enrolled 1,000 patients.

Important Dates at Durham VAMC

12/21/2012	Protocol submitted to VA IRB
11/22/2013	Approval to enroll at Durham VAMC
08/05/2014	50% of enrolled patients have completed the study.
09/02/2014	100% of enrolled patients have completed the month 3 visit (endpoint).
09/25/2014	Request for Human Studies Continuing Review (CR) was submitted to the DVAMC IRB. The IRB requested that the previously approved ICF be reformatted into the DVAMC IRB June 2014 format. The reformatted ICF without changes was submitted with this Continuing Review request.
11/24/2014	Continuing Review Approval received from DVAMC IRB
12/22/2014	S. Becky Perfect and Meghan Jones were added to staff listing at the DVAMC.

03/04/2015 Lori Walther, HRPO, requested a consent form revision because it lacked required DoD language. Documents submitted to DVAMC IRB on 04/16/2016 and approved on 16 June 2015.

03/16/2015 Veotria (Veda) Byrd was added to the staff listing at the DVAMC.

07/08/2015 Received Continuing Review Acceptance from Kimberly Odam, Human Subjects Protection Scientist, HRPO

09/17/2015 Received approval of the annual CR with approval through 9 September 2016.

10/2015 Submitted CR approvals to Lori Walther.

03/07/2016 The submitted continuing review report and supporting documentation have been reviewed by the HRPO and found to be in compliance.

09/28/2016 Continuing Review Approval received from DVAMC IRB

05/18/2017 Continuing Review documents sent to DVAMC IRB

7/27/2017 Continuing Review Approval received from DVAMC IRB

Important Dates at Duke University Medical Center

06/30/2014 Submission of requested Duke protocol documents, IRB approvals and re-budget information to Lori Walther, Dr. Patricia Henry, and Lisa Wells Roark, to request the addition of DUMC as an additional study site.

07/30/2014 Received approval for enrollment at DUMC; protocol was reviewed by USAMRMC, ORP, HRPO and found to comply with applicable DOD, US Army, and USAMRMC human subjects protection requirements. The total number of subjects approved is 420 across all sites; 155 subjects are approved at DUMC.

07/30/2014 An initial response to the SOW and Rebudget request was received from Dr. Henry. She asked for clarification regarding the subject fees and number of subjects, and supporting documents to demonstrate that the intermediate time point (7 days) and the mild pain phenotype would not be helpful for the metabolomics analyses as outlined in our Rebudget justification. Additional comments were made to the SOW.

08/06/2014 In-service for the Duke Acute Pain Service and PI training on the study protocol.

08/08/2014 The study team provided revised SOW and Rebudget request along with Volcano plots supporting changes to the metabolomics component of the study.

08/25/2014 The study at Duke was approved for CR by Duke IRB and given an expiration date of 08/28/2015.

08/28/2014 Dionne Apedjihoun (CRC) met with Nancy Payne (Clinical Nurse/Limb Loss Specialist) to trouble shoot potential patient recruitment for in and out-patients and to delineate a mechanism for screening and potential enrollment.

09/02/2014 Hung-Lun (John) Hsia MD., and Dionne Apedjihoun, met with Duke Personnel (Donna Hamel, Clinical Trials Project Leader at DCRU) to discuss storage of biological samples prior to shipping to the Genome Science Research Building I (GSRBI) on campus. Samples will be stored in the -20 ^{o}F at Hanes House and the -80^{o}F at Duke South.

10/02/2014 Mrs. Lisa L. Wells Roark notified the study team of approval of the SOW and Re-budget submitted on 06/30/2015

12/04/2014 The CR Submission Form along were submitted to HRPO for the study at Duke.

04/30/2015 Received CR Acceptance from Sharon Evans, Deputy Director, HRPO

06/24/2015 Amendment submitted and approved to open the study for enrollment in patients with renal disease to avoid unnecessary patient exclusions.

08/25/2015 The study at Duke was approved for CR by Duke IRB

08/28/2015 The CR Form was submitted to HRPO for the study at Duke.

10/29/2015 Submitted an updated scientific justification and budget for Year 4 to Jennifer Shankle, Contract Specialist.

11/04/2015 Received prior approval for purchase of equipment. Ms. Shankle requested a revised SOW to correspond with the updated scientific justification.

11/19/2015 A revised SOW was submitted to Jennifer Shankle

12/15/2015 A one-time 12 month EWOF was requested and sent to Jennifer Shankle.

01/14/2016 Fully executed modification of the award received

08/03/2017 The study was approved for CR by Duke IRB and given an expiration date of 08/28/2018.

11/15/2017 Extension Without Funds request sent to Jennifer Shankle

11/17/2017 Approval received for 6 month EWOF. New period of performace is through 3/29/2018

Important Dates of Multi-Site Study Coordination

03/24/2014 Clinical phenotype adjudication performed on initial patients reaching 3 month follow-up period

01/09/2015 Clinical phenotype adjudication meeting

03/20/2015 Meeting held with COL Buckenmaier, Peter Bedocs, Kelly Kiser, Dr. Thomas Buchheit, Dr. Van de Ven, Alex Chamessian, Dr. Hsia, Mary McDuffie, Dr. Kent, Nancy Kwon, Rachel Morales, and Veda Byrd in attendance. Dr. Kent was formally introduced to the whole team. Future goals, expected adverse events, deviations, and case report forms were discussed.

04/09/2015 WRNMMC shipped 5 complete blood sample kits to Duke. The samples were logged in our database and are being stored at GSRBI.

09/01/2015 Initial 4 blood samples were transferred from WRNMMC to Duke, logged in the study database and stored at our laboratory at Duke GSRB1.

9/02/2015 A teleconference held with Mary McDuffie, Veda Byrd, Drs Hsia, and Buchheit. New data collection points and protocol language discussed.

10/16/2015 A teleconference held with Drs. Buchheit, Van de Ven, Sandra Yee-Benedetto, Rachel Morales, Veda Byrd, Col Buckenmaier, Kelly Kiser, Nancy Kwon, Mary McDuffie. Budget plans, financials considerations, protocol changes, and enrollment updates were discussed.

10/27/2015 Adjudication meetings held on October 9th and 27th for patients meeting 3 month end point analysis. The number of patients with 3 month data was 36.

10/28/2015 Amendment approval received at WRNMMC to collect new data points: wound vac therapy?, and the PHQ-2 at the 3 and 6 month follow up visits.

10/30/2015 Annual report for the period of 30 Sep 2014 – 29 Sep 2015 was submitted.

11/05/2015 9 blood samples were successfully transferred from WRNMMC to Duke GSRB1.

11/09/2015 Annual Continuing Review approved at WRNMMC

11/30/2015 Adjudication meetings held on Novemeber 18 and 30 for patients meeting 3 month end point analysis. The number of patients with 3 month data was 43.

12/02/2015 The revised SOW for #W81XWH-12-2-0130 was submitted to Jennifer Shankle.

12/22/2015 Dr. Buckenmaier, partnering PI at WRNMMC and HJF, also requested a one-time EWOF

01/14/2016 Approval was received for HJF for the revised SOW and EWOF

01/15/2016 DSMB Report received and recommendations are listed below. Adverse events, protocol deviations and enrollment for all three sites were reviewed.

1.Continue to enroll patients into the trial.

2. We encourage the study team to closely monitor the incidence of liver enzyme elevation and mental status changes. Given the side effect profile of the medication and the patient population of interest, these are not unexpected events.

3. An interim analysis is planned at 50% enrollment, anticipated to be within the next calendar year. At this point, if continued occurrences of elevations in liver enzymes are noted, particular attention will be paid to this in the statistical review.

01/19/2016 Clinical phenotype adjudication performed by Adjudication Committee

05/17/2016 Year 4 Quarter 2 Report was reviewed and accepted.

05/17/2016	A teleconference was held with Drs. Buchheit, Van de Ven, Rachel Morales, Veda Byrd, Col Buckenmaier, Nancy Kwon, Mary McDuffie, Peter Bedocs. Protocol adjustments, enrollment update, sample shipping, and site visit plans were discussed.
07/2016	Adjudication meeting held for patients meeting 3 month end point analysis
09/2016	Adjudication meeting held for patients meeting 3 month end point analysis
09/13/2016	RNA extraction and sample processing begun in Nerve Injury and Pain Mechanisms Laboratory. RNA extraction completed on initial 92 samples.
10/03/2016	Dr. Buchheit, Dr. Van de Ven and Rachel Morales visited WRNMMC. They met with COL Chester Buckenmaier, Mary McDuffie, Kelly Kiser, Peter Bedocs, and Nancy Kwon. Protocol procedures, enrollment practices and future analyses were discussed. Dr. Buchheit and Dr. Van de Ven presented preliminary study results to a receptive crowd of 75 at the WRNMMC MATC at noon.
01/06/2017	Meeting held with Drs. Buchheit and Van de Ven, Rachel Morales, Mary Cooter and Lani Banez to begin preparations for statistical analysis of clinical outcomes
2/13/2017	WRNMMC shipped samples to our laboratory at Duke GSRB1.
2/14/2017	Adjudication meeting held for patients meeting 3 month end point analysis
2/27/2017	WRNMMC shipped samples to our laboratory at Duke GSRB1.
3/18/2017	Research Team Meeting with Duke/WRNMMC/DVAMC collaborators (Dr. Buchheit, Dr. Van de Ven, COL Chester Buckenmaier, Dr. Kent, Mary McDuffie, and Nancy Kwon) during the American Academy of Pain Medicine Annual Meeting. Protocol procedures, enrollment practices and current analysis was discussed.
3/29/2017	WRNMMC shipped samples to our laboratory at Duke GSRB1.
05/05/2017	Adjudication meeting held for patients meeting 3 month end point analysis
05/30/2017	Dr. Buchheit and Rachel Morales met with CRC Management Team to plan final enrollment, follow-up visits, completion of REDCap data fields, and to prepare for study closure and statistical analysis of clinical findings.
06/12/2017	Adjudication meeting held for patients meeting 3 month end point analysis
07/06/2017	Meeting held with Drs. Buchheit, Van de Ven, Hsia, DUMC and VAMC CRCs, Mary Cooter (biostatistician) to discuss end of clinical enrollment and preparation for statistical anyalsis plan
07/18/2017	Adjudication meeting held for patients meeting 3 month end point analysis
08/08/2017	Meeting held with Dr. Buchheit, Rachel Morales and Mary Cooter to discuss statistical anyalsis plan
08/21/2017	Meeting held with Dr. Buchheit, Rachel Morales and Jessica Hall to review REDCap data
11/06/2017	Adjudication meeting held for patients meeting 3 month end point analysis
11/06/2017	Meeting held with Mary Cooter and Rachel Morales to review VPA Blood levels
11/13/2017	A teleconference was held with Drs. Buchheit, Van de Ven and Kent, Rachel Morales, Col Buckenmaier, Mary McDuffie, and Peter Bedocs. End of enrollment, statistical anaylsis plan, and processing of samples discussed.
12/04/2017	Remaining WRNMMC samples brought to our laboratory at Duke GSRB1.
01/09/2018	Teleconference held with Drs. Buchheit, Van de Ven, Rachel Morales and Mary Cooter to discuss initial statistical analysis results
02/13/2018	Teleconference held with Drs. Buchheit, Van de Ven, Rachel Morales and Mary Cooter to discuss initial statistical analysis results
03/04/2018	Poster presentation at American Pain Society in Anaheim, CA
04/10/2018	Meeting held with Drs. Buchheit, Van de Ven and Rachel Morales to discuss VPA statistical analysis results, manuscript preparation, and AE table
05/09/2018	Meeting held with Drs. Buchheit, Van de Ven, Rachel Morales and Mary Cooter to discuss statistical analysis results
06/2018	Manuscript submitted for Publication to Pain Medicine

KEY RESEARCH ACCOMPLISHMENTS

- **Operational success of a randomized clinical trial taking place at at VA Hospital (Durham VAMC), a Military Medical Centre (WRNMMC), and an Academic Medical Center (Duke University). This "Three-System" trial coordination paves the way for future research collaborations.**
- **Successful use of a clinical phenotyping tool that utilizes validated questionnaire instruments and a basic physical exam to define post-ampuation clinical pain subtypes.**
- **Demonstration that pain severity improves significantly after amputation or revision surgery when performed in conjunction with the use of regional anesthesia and multimodal analgesia, and with a time course that preceeds prosthetic rehabilitation.**
- **The methylation status of genes found within immune cell and inflammatory signaling pathways correlate with the presence of chronic pain after surgery.**
- **Targeting of these specific inflamatory pathways may be critical for prevention of chronic pain after amputation surgery. This provides opportunity to develop future non-opioid targeted therapies.**

Recommended changes or future work to better address the research topic may also be included. However, changes to the original SOW shall be approved by the USAMRAA Grants Officer through an award modification prior to initiating any changes.

Based on our research findings, we would recommend widespread deployment of multimodal perioperative techniques and the use of regional anesthesia catheter infusions for all amputation and revision surgeries. This comprehensive approach is noted to improve both pain and function in patients by 3 months, a time course that preceeds the completion of prosthetic rehabilitation.

REPORTABLE OUTCOMES:

PUBLICATIONS, ABSTRACTS AND PRESENTATIONS

Buchheit T, Hsia HL, Cooter M, Shortell C, Kent M, McDuffie M, Shaw A, Buckenmaier C, and Vandeven T. The impact of surgical amputation and valproic acid on pain and functional trajectory: results from the Veterans Integrated Pain Evaluation Research (VIPER) randomized, double-blinded placebo controlled trial. *Submitted for Publication. Pain Medicine*

The impact of surgical amputation and valproic acid on pain and functional trajectory: results from the Veterans Integrated Pain Evaluation Research (VIPER) randomized, double-blinded placebo controlled trial. Authors: Thomas Buchheit, Hung-Lun John Hsia, Mary Cooter, Cynthia Shortell, Michael Kent, Mary McDuffie, Andrew D Shaw, Chester "Trip" Buckenmaier, and Thomas Van de Ven. American Pain Society, Anaheim, CA, March 4-6, 2018.

Kent ML, Hsia H-LJ, Van de Ven TJ, Buchheit TE. Perioperative Pain Management Strategies for Amputation: A Topical Review. Pain medicine (Malden, Mass). The Oxford University Press; 2016 Jul 8; pnw110–6.

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.

CONCLUSIONS:

In this research, we found that perioperative use of valproic acid in the context of multimodal analgesic management does not further reduce chronic pain after surgical amputation in patients with either medical illness or traumatic injury. Nonetheless, patients noted significant improvements in pain and function during the early months after surgical amputation, even before the rehabilitation process is typically completed. These post-surgical improvements were observed in all patient subgroups analyzed, regardless of the need for amputation. Further, the therapeutic effect of this comprehensive approach appears to be beneficial in patients undergoing both primary amputation and revision surgery. In addition, although the epigenetic changes caused by valproic acid may not have been sufficient to prevent chronic pain, the results of the initial methylation

sequencing analysis of patient samples from this study suggests that therapies preventing methylation or activation of specific immune modulatory pathways around the time of amputation surgery may reduce the transition from acute to chronic pain.

APPENDICES

Attachment 1- IPA pathway analysis

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INGENUITY
PATHWAY ANALYSIS

Analysis Name: VDV_RNA-Seq - 2018-06-01 10:29 AM
Analysis Creation Date: 2018-06-01
Build version: 470319M
Content version: 43605602 (Release Date: 2018-03-28)

Analysis Settings

Reference set: User Dataset
Relationship to include: Direct and Indirect
Does not Include Endogenous Chemicals
Optional Analyses: My Pathways My List

Filter Summary:
Consider only relationships where
confidence = Experimentally Observed

Top Canonical Pathways

Name	p-value	Overlap
Communication between Innate and Adaptive Immune Cells	1.01E-03	11.7 % 7/60
Cell Cycle Regulation by BTG Family Proteins	2.16E-03	14.3 % 5/35
Altered T Cell and B Cell Signaling in Rheumatoid Arthritis	5.03E-03	10.0 % 6/60
Crosstalk between Dendritic Cells and Natural Killer Cells	9.22E-03	8.8 % 6/68
Unfolded protein response	1.30E-02	9.4 % 5/53

Top Upstream Regulators

Upstream Regulator	p-value of overlap	Predicted Activation
MEF2C	2.93E-05	
CD40	1.06E-04	
CD40LG	1.18E-04	
dalfampridine	1.24E-04	
A23187	1.41E-04	Inhibited

Top Diseases and Bio Functions

Diseases and Disorders

Name	p-value	#Molecules
Cardiovascular Disease	2.65E-02 - 1.84E-04	64
Hereditary Disorder	2.57E-02 - 1.84E-04	31
Organismal Injury and Abnormalities	2.65E-02 - 1.84E-04	230
Respiratory Disease	2.61E-02 - 1.84E-04	19
Cancer	2.61E-02 - 3.19E-04	197

Molecular and Cellular Functions

Name	p-value	#Molecules
Cellular Development	2.59E-02 - 2.02E-05	87
Cellular Growth and Proliferation	2.59E-02 - 2.02E-05	79
Cellular Assembly and Organization	2.24E-02 - 2.21E-04	32
Molecular Transport	2.65E-02 - 3.50E-04	24
Nucleic Acid Metabolism	6.66E-03 - 3.50E-04	6

Physiological System Development and Function

Name	p-value	#Molecules
Embryonic Development	2.65E-02 - 2.02E-05	63
Organ Development	2.65E-02 - 2.02E-05	56
Organismal Development	2.65E-02 - 2.02E-05	88
Skeletal and Muscular System Development and Function	2.38E-02 - 2.02E-05	44
Tissue Development	2.32E-02 - 2.02E-05	77

Top Tox Functions

Assays: Clinical Chemistry and Hematology

Name	p-value	#Molecules
Increased Levels of Potassium	3.30E-02 - 8.03E-03	3
Decreased Levels of Hematocrit	1.35E-02 - 1.35E-02	2
Increased Levels of Red Blood Cells	4.15E-01 - 5.24E-02	3
Increased Levels of Creatinine	2.56E-01 - 8.92E-02	3
Decreased Levels of Potassium	1.02E-01 - 1.02E-01	1

Cardiotoxicity

Name	p-value	#Molecules
Pulmonary Hypertension	2.15E-01 - 1.84E-04	8
Cardiac Arrythmia	2.45E-01 - 7.03E-04	7
Congenital Heart Anomaly	5.50E-01 - 7.03E-04	7
Heart Failure	5.03E-01 - 7.03E-04	8
Tachycardia	1.26E-01 - 2.07E-03	2

Hepatotoxicity

Name	p-value	#Molecules
Liver Hematopoiesis	1.77E-02 - 1.77E-02	2
Liver Hyperplasia/Hyperproliferation	1.00E00 - 2.65E-02	199
Hepatocellular Carcinoma	3.32E-01 - 3.03E-02	32
Liver Cirrhosis	1.96E-01 - 5.24E-02	8
Liver Damage	1.00E00 - 7.75E-02	4

Nephrotoxicity

Name	p-value	#Molecules
Renal Proliferation	1.84E-01 - 9.82E-03	7
Renal Inflammation	1.00E00 - 1.77E-02	10
Renal Nephritis	1.00E00 - 1.77E-02	10
Glomerular Injury	6.10E-01 - 2.65E-02	8
Kidney Failure	6.10E-01 - 2.65E-02	8

Top Regulator Effect Networks

ID	Regulators	Diseases & Functions	Consistency Score
1	RETNLB	Differentiation of epithelial tissue	1.155

Top Networks

ID	Associated Network Functions	Score
1	Cell Morphology, Skeletal and Muscular System Development and Function, Developmental Disorder	51
2	Decreased Levels of Hematocrit, Hematological System Development and Function, Cellular Function and Maintenance	43
3	Protein Synthesis, Behavior, Nervous System Development and Function	41
4	Embryonic Development, Organismal Development, Cell Death and Survival	37
5	Humoral Immune Response, Protein Synthesis, Hematological System Development and Function	32

Top Tox Lists

Name	p-value	Overlap
Decreases Permeability Transition of Mitochondria and Mitochondrial Membrane	1.35E-02	28.6 % 2/7
Increases Liver Steatosis	2.41E-02	7.1 % 6/84
Genes Downregulated in Response to Chronic Renal Failure (Rat)	2.65E-02	100.0 % 1/1
Increases Renal Nephritis	4.32E-02	8.0 % 4/50
Cytochrome P450 Panel - Substrate is an Eicosanoid (Mouse)	7.75E-02	33.3 % 1/3

Top Analysis-Ready Molecules

Expr Log Ratio up-regulated

Molecules	Expr. Value	Expr. Chart
CYP26B1	↑ 6.001	
ADAM29	↑ 2.701	
ARNT2	↑ 2.631	
KRT17P1	↑ 2.416	
DLX2	↑ 2.363	
KCNK1	↑ 2.220	
ANTXRL	↑ 2.199	

CRB1	2.175
C3orf67	1.791
FAM156A/FAM156B*	1.707

Expr Log Ratio down-regulated

Molecules	Expr. Value	Expr. Chart
IGKV1D-16	-2.426	
C6orf222	-2.120	
UCHL1	-1.780	
IGLV2-34	-1.729	
MICC	-1.674	
POU2F3	-1.568	
RN7SL124P	-1.532	
IGLV2-28	-1.531	
IGLV4-60	-1.521	
FN1	-1.490	