



# DNA methylation profiles are associated with complex regional pain syndrome after traumatic injury

Stephen Bruehl<sup>a,\*</sup>, Eric R. Gamazon<sup>b,c</sup>, Thomas Van de Ven<sup>d</sup>, Thomas Buchheit<sup>d</sup>, Colin G. Walsh<sup>e</sup>, Puneet Mishra<sup>a</sup>, Krishnan Ramanujan<sup>a</sup>, Andrew Shaw<sup>a</sup>

## Abstract

Factors contributing to development of complex regional pain syndrome (CRPS) are not fully understood. This study examined possible epigenetic mechanisms that may contribute to CRPS after traumatic injury. DNA methylation profiles were compared between individuals developing CRPS ( $n = 9$ ) and those developing non-CRPS neuropathic pain ( $n = 38$ ) after undergoing amputation following military trauma. Linear Models for Microarray (LIMMA) analyses revealed 48 differentially methylated cytosine-phosphate-guanine dinucleotide (CpG) sites between groups (unadjusted  $P$ 's  $< 0.005$ ), with the top gene *COL11A1* meeting Bonferroni-adjusted  $P < 0.05$ . The second largest differential methylation was observed for the *HLA-DRB6* gene, an immune-related gene linked previously to CRPS in a small gene expression study. For all but 7 of the significant CpG sites, the CRPS group was hypomethylated. Numerous functional Gene Ontology-Biological Process categories were significantly enriched (false discovery rate-adjusted  $q$  value  $< 0.15$ ), including multiple immune-related categories (eg, activation of immune response, immune system development, regulation of immune system processes, and antigen processing and presentation). Differentially methylated genes were more highly connected in human protein-protein networks than expected by chance ( $P < 0.05$ ), supporting the biological relevance of the findings. Results were validated in an independent sample linking a DNA biobank with electronic health records ( $n = 126$  CRPS phenotype,  $n = 19,768$  non-CRPS chronic pain phenotype). Analyses using PrediXcan methodology indicated differences in the genetically determined component of gene expression in 7 of 48 genes identified in methylation analyses ( $P$ 's  $< 0.02$ ). Results suggest that immune- and inflammatory-related factors might confer risk of developing CRPS after traumatic injury. Validation findings demonstrate the potential of using electronic health records linked to DNA for genomic studies of CRPS.

**Keywords:** Complex regional pain syndrome, CRPS, Methylation, Genetic, Immune, Inflammatory, Chronic pain, Risk

## 1. Introduction

Complex regional pain syndrome (CRPS) is a chronic pain condition characterized by allodynia and hyperalgesia, skin temperature and color changes, edema, and trophic changes.<sup>7,8</sup> Its pathophysiological mechanisms are only incompletely

understood, but available data suggest that neuropathic, central and autonomic nervous system, inflammatory, and immune mechanisms all are involved.<sup>7,8</sup> Complex regional pain syndrome is a somewhat heterogeneous diagnosis, with several distinct phenotypes<sup>9,10,15</sup>; multiple mechanisms may contribute to different degrees across patients and over time.<sup>8,10</sup>

Familial aggregation of CRPS cases have led to suspicions of a heritable component of CRPS risk.<sup>20,21,35</sup> Supporting this, prior work has suggested possible genetic risk factors for CRPS. Most frequently reported are genetic differences in the human leukocyte antigen (HLA) system, the system underlying the adaptive immune response.<sup>42</sup> Differences have been noted in a number of studies in the frequency of several HLA alleles between patients with CRPS and controls,<sup>40,67–69</sup> with replication observed for *HLA-DQ8* effects.<sup>22,70</sup>

Beyond putative genetic drivers of susceptibility, epigenetic modifications provide an additional path for transmitting CRPS risk. There have been multiple epigenetic processes identified, with one of the most widely studied being DNA methylation.<sup>14,65</sup> Methylation of the DNA sequence, particularly at cytosine-phosphate-guanine dinucleotide (CpG) sites, is known to be affected by both genetic and environmental factors (eg, diet, smoking, and trauma), and suppresses transcriptional activity.<sup>38,65</sup> To the best of our knowledge, no prior studies have evaluated associations between CRPS and DNA methylation profiles. However, limited animal and human work support the potential importance of altered gene expression in CRPS.

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

S. Bruehl and E.R. Gamazon contributed equally to this manuscript.

<sup>a</sup> Department of Anesthesiology, Vanderbilt University Medical Center, Nashville, TN, United States. Mr. Shaw is now with Department of Anesthesiology and Pain Medicine, University of Alberta, Edmonton, AB, Canada, <sup>b</sup> Division of Genetic Medicine, Department of Medicine, Vanderbilt Genetics Institute, Vanderbilt University Medical Center, Nashville, TN, United States, <sup>c</sup> Department of Anesthesiology, Clare Hall, University of Cambridge, Cambridge, United Kingdom, <sup>d</sup> Department of Anesthesiology, Duke University Medical Center, Durham, NC, United States, <sup>e</sup> Departments of Medicine and Biomedical Informatics, Vanderbilt University Medical Center, Nashville, TN, United States

\*Corresponding author. Address: Vanderbilt University Medical Center, 701 Medical Arts Building, 1211 Twenty-First Ave South, Nashville, TN 37212, United States. Tel.: (615) 936-1821. E-mail address: Stephen.Bruehl@vumc.org (S. Bruehl).

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site ([www.painjournalonline.com](http://www.painjournalonline.com)).

PAIN 160 (2019) 2328–2337

© 2019 International Association for the Study of Pain

<http://dx.doi.org/10.1097/j.pain.0000000000001624>

Reviews of animal research conclude that altered gene expression in dorsal root ganglion neurons could enhance inflammatory responses, contributing to CRPS risk.<sup>71,72</sup> Human work regarding gene regulatory effects on CRPS risk is quite limited. One study described altered expression of 18 micro-RNAs, which are involved in posttranscriptional regulation of gene expression, in patients with CRPS compared to controls.<sup>52</sup> A more recent study conducting genome-wide gene expression profiling of blood samples from 4 patients with CRPS and 5 pain-free controls revealed 80 genes that were differentially expressed.<sup>37</sup> A reanalysis of these data using Linear Models for Microarray analysis revealed 257 differentially expressed genes in patients with CRPS.<sup>62</sup> In both studies, 3 of the largest gene expression differences were for genes in the HLA system.<sup>37,62</sup>

No published studies have reported associations between CRPS and DNA methylation status, and no gene expression studies have compared patients with CRPS to other pain patients. The latter may be important because any gene expression differences between patients with CRPS and controls might reflect chronic pain status rather than CRPS per se. The current study therefore sought to evaluate whether presence of CRPS (vs non-CRPS neuropathic pain) after traumatic injury is associated with differential DNA methylation profiles. We further examined whether genes showing significant methylation differences in the primary sample could be independently validated (in terms of the genetically determined component of gene expression)<sup>26</sup> in a large deidentified clinical sample.<sup>25,57</sup>

## 2. Method

### 2.1. Primary methylation study

#### 2.1.1. Design

Patients were all enrolled in the Veterans Integrated Pain Evaluation Research (VIPER) study, a case-control study that included recent traumatic amputees from Operation Enduring Freedom/Operation Iraqi Freedom (OEF/OIF) who were enrolled 3 to 18 months after amputation. All subjects were enrolled between November 2011 and July 2013.

#### 2.1.2. Subjects

All study procedures were approved by the institutional review board of Walter Reed National Military Medical Center (WRNMMC). Subjects included 9 CRPS and 38 non-CRPS neuropathic pain patients, all of whom had experienced military trauma and had subsequently undergone posttraumatic amputation surgery. 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 performed 3 to 18 months previously. Patients were excluded if they were afflicted with severe traumatic brain injury, significant cognitive deficits, substantial hearing loss, spinal cord injury with permanent or persistent deficits, ongoing tissue damage that might cause pain, infection, heterotrophic ossification, poorly fitting prosthesis, or hip disarticulation.

Patients were eligible for this study if they reported clinically significant residual limb pain at the time of evaluation, operationally defined as an average pain score over the past week of  $\geq 3/10$  on a numeric rating scale (NRS). Those patients with clinically significant pain were further adjudicated into pain subtypes (see Ref. 11 for full details). For use in the adjudication process, all patients underwent a history and physical

examination to assess signs and symptoms used in CRPS diagnosis. Per the diagnostic algorithm in the larger study, patients were initially classified into phantom or residual limb pain. Subsequently, the residual limb pain group was further classified as somatic vs neuropathic through use of the Self-Report Leeds Assessment of Neuropathic Symptoms and Signs (S-LANSS), based on the standard cutoff of  $\geq 12$  on the S-LANSS.<sup>4</sup> Finally, patients identified with neuropathic pain qualities were further characterized into diagnostic groups, including CRPS (based on the Budapest clinical criteria).<sup>33</sup> The non-CRPS neuropathic pain subgroup consisted of those patients meeting criteria for neuropathic pain who did not meet the Budapest criteria for CRPS. The sample reported in the current work included all CRPS and non-CRPS neuropathic pain patients as defined above in the VIPER sample who had DNA methylation assay data available. The final sample was 98% male and 89% white, with a mean age of 26.9 years.

#### 2.1.3. Procedures

After providing written informed consent, patients completed 0 to 10 NRS ratings of their pain intensity, with NRS anchors being 0 = "no pain" and 100 = "worst possible pain." Blood samples were also obtained from each patient at the time of enrollment for subsequent analysis. For plasma preparation, 6 mL of blood was collected in EDTA-containing K2 tubes and inverted to mix. Tubes were then spun at 3,000g for 20 minutes at 4°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 until assays were conducted.

### 2.2. DNA methylation assays

DNA was extracted from whole blood and then sent to the Molecular Genomics Shared Resource of the Duke Molecular Physiology Institute for methylation analysis. As recommended by Illumina, we used the Zymo EZ DNA Methylation kit (Zymo Research, Irvine, CA) for bisulfite conversion of DNA before running the Illumina Methylation 450 k protocol. Before beginning the main portion of the protocol, the CT Conversion Reagent was prepared. Then all steps were completed until step 2 where reagent was instead vortexed constantly for 10 minutes instead of frequently. The protocol was then continued starting with 500 ng of good-quality DNA and proceeding with the manufacturer's protocol using the alternative thermocycler conditions for Infinium Methylation assay in downstream applications for steps 4 and 5 of the Zymo EZ DNA Methylation protocol. These alternative conditions dictate instead of an incubation of 50°C for 16 hours, the thermocycler program is as follows: (95°C for 30 seconds, 50°C for 60 minutes)  $\times$  16 cycles, then 4°C hold.

To use the Illumina Methylation 450 k kit (Illumina, San Diego, CA), 4  $\mu$ L of bisulfite-converted DNA was used and the manufacturer's protocol for Infinium HD Methylation was followed. Briefly, the samples were denatured and amplified overnight for 20 to 24 hours. Fragmentation, precipitation, and resuspension of the samples followed overnight incubation. After resuspension, samples were then hybridized to the Illumina Infinium Methylation 450 k BeadChip for 16 to 24 hours. Finally, the BeadChips were washed to remove any unhybridized DNA and then labeled with nucleotides to extend the primers to the DNA sample. Following the Infinium HD Methylation protocol, the BeadChips were imaged using the Illumina iScan system (Illumina).

### 2.3. Data analysis

#### 2.3.1. Differential methylation analysis

We conducted Linear Models for Microarray (LIMMA)<sup>56</sup> analyses to identify differentially methylated CpG sites. LIMMA estimates the log-fold change (logFC) for each CpG site.

Let  $j$  be a given CpG site. LIMMA's Bayesian model assumes an inverse  $\chi^2$  prior for the unknown variance  $\sigma_j^2$  with mean  $s_0^2$  and degrees of freedom  $d_0$ :

$$\frac{1}{\sigma_j^2} \sim \left( \frac{1}{d_0 s_0^2} \right) \chi_{d_0}^2. \quad (1)$$

For statistical inference, the approach uses a modified  $t$ -statistic, in which the posterior variance is substituted for the regular variance in the classical  $t$ -statistic:

$$\tilde{s}_j^2 = \frac{d_0 s_0^2 + d_j s_j^2}{d_0 + d_j}. \quad (2)$$

Here,  $d_j$  is the residual degrees of freedom for the  $j$ th CpG site. Note that the variance estimates are “moderated” using a shared value from the Bayesian prior Equation 1, reducing the possibility of false positives that may arise from underestimation of the variance. The modified  $t$ -statistic has higher degrees of freedom,  $d_0 + d_j$ , in comparison with the ordinary  $t$ -statistic.

Most CpG sites were not variably methylated across the individuals. Hence, we filtered out those CpG sites that were not sufficiently variable. Only those with variance  $>0.002$  were analyzed for this report.

#### 2.3.2. Protein–protein interactions

We generated a protein–protein interaction (PPI) subnetwork from the genes annotated to the differentially methylated CpG sites using DAPPLE.<sup>58</sup> A direct interaction between 2 proteins, represented as nodes, from a database of high-confidence in vitro direct interactions (InWeb)<sup>46</sup> is represented by an “edge” (line) between the corresponding annotated genes. We tested the significance of the centrality index, defined as the average number of direct connections to a tested gene. The significance was evaluated as the proportion of within-degree node-label permutations ( $n = 1000$ ) with a permutation statistic greater than or equal to the centrality index value in the actual (nonpermuted) data.

#### 2.3.3. Enrichment for known pathways and functional annotations

Using DAVID,<sup>17</sup> we performed gene ontology analysis of the top 48 genes from the differential methylation analysis to determine which genes belong to known pathways, gene sets, and functional annotations. We used an enrichment analysis approach using the enrichGO function of the Cluster Profiler R package (<http://bioconductor.org/packages/release/bioc/html/clusterProfiler.html>). This analysis generated a list of pathways and functional annotations with significant enrichment, as indicated by a false discovery rate-adjusted (FDR)  $q$  value  $<0.15$  for the genes with differentially methylated sites. The number of annotated genes for significant categories is provided in Supplementary Figure 1 (available at <http://links.lww.com/PAIN/A818>) and Supplementary Figure 2 (available at <http://links.lww.com/PAIN/A819>).

### 2.4. Validation study

#### 2.4.1. Design

Results in the primary discovery sample were validated using a case-control design in a DNA biobank linked to an electronic health records (EHR) database.

#### 2.4.2. Sample

The validation sample was drawn from a large pool of clinical patients seen at Vanderbilt University Medical Center since 2002 who had DNA samples available in BioVU, the Vanderbilt biobank of deidentified DNA samples obtained for research purposes from discarded blood.<sup>55,57</sup> BioVU DNA samples were linked in a deidentified manner to pain-relevant phenotypes previously derived based on informatics algorithms applied to ICD-9 codes in the Synthetic Derivative, the Vanderbilt deidentified electronic medical records database.<sup>19</sup> The current study compared patients with a CRPS phenotype ( $n = 126$ ) to patients with a non-CRPS chronic pain phenotype ( $n = 19,768$ ). In the CRPS sample, 69% were female, vs only 50% being female in the non-CRPS sample. This predominance of female patients in the CRPS validation sample is consistent with the broader CRPS population.<sup>8</sup> Mean age in the 2 validation samples was 62.6 ( $\pm 14.18$ ) and 64.4 ( $\pm 20.7$ ), respectively.

#### 2.4.3. Procedures

As a means of validating epigenetic effects observed in methylation analyses, we tested for the contribution of differential gene expression to CRPS using the PrediXcan methodology.<sup>25,26</sup> PrediXcan estimates the genetically determined component of gene expression and evaluates this component for association with the target phenotype (ie, CRPS). Using the weights  $\hat{\beta}_j$  derived from the gene expression imputation model<sup>31</sup> and the number of effect alleles  $X_{ij}$  at the variant predictor  $j$ , the genetically determined component of gene expression is calculated as the additive effect:

$$\hat{G}_i = \sum_j X_{ij} \hat{\beta}_j.$$

The imputation model was generated using Elastic Net. A significant association between the genetically determined component of gene expression and the CRPS phenotype (in this case, based on logistic regression) suggests a causal direction of effect (from gene expression to phenotype) because the germline genetic profile (on which the genetic component of gene expression is based) is not influenced by the CRPS phenotype.

#### 2.4.4. Data analysis

We performed logistic regression modeling CRPS vs non-CRPS chronic pain status using the genetically determined component of gene expression as the independent variable. We tested in the independent BioVU validation sample the 48 genes found to be significant in the differential methylation discovery analysis.

## 3. Results

### 3.1. Differential methylation between complex regional pain syndrome and non-complex regional pain syndrome neuropathic pain patients

Overall methylation levels by individual subject are provided in Supplementary Figure 3 (available at <http://links.lww.com/PAIN/A820>). We compared patients meeting vs not meeting diagnostic

criteria for CRPS to test whether presence of CRPS after traumatic injury is associated with differential methylation profiles. LIMMA analyses revealed 48 CpG sites differentially methylated ( $P < 0.005$ , uncorrected) between CRPS and non-CRPS groups (Table 1). For all but 7 CpG sites, the CRPS group was hypomethylated relative to the non-CRPS neuropathic pain group. A Manhattan plot summarizing differential methylation at all CpG sites highlights genomic regions in which this epigenetic mechanism may contribute to differences between the groups (Fig. 1). One CpG site (cg17820060, *COL11A1*,  $P = 9.98 \times 10^{-7}$ ) was differentially methylated at a Bonferroni-corrected  $P < 0.05$  level. Additional CpG sites at  $P < 5 \times 10^{-4}$  were noted for the following genes: *HLA-DRB6*, *TMEM173*, *GPR75*, *C2orf27A*, and *KCNH1*. Box and whisker plots for methylation differences across CRPS and non-CRPS groups for the top 2 genes identified (*COL11A1* and *HLA-DRB6*) are provided in Figures 2A and B, respectively. In both cases, hypomethylation was observed in the CRPS group, with wide variability in individual methylation levels noted, possibly reflecting the suspected heterogeneity in contributions of various CRPS mechanisms across individual patients.

To address potential pain confounders, we considered whether the 2 patient groups also differed in the primary clinical feature of chronic pain intensity based on responses on the NRS pain intensity rating scale used to determine study eligibility. Comparisons of mean limb pain intensity ratings across the 2 groups revealed that the CRPS group ( $4.7 \pm 1.41$ ) and the non-CRPS neuropathic pain group ( $4.5 \pm 2.00$ ) did not significantly differ [ $t(45) = -0.283$ ,  $P = 0.78$ ].

### 3.2. Functional analysis of differentially methylated cytosine-phosphate-guanine dinucleotide sites

Using the Gene Ontology (GO) classification system,<sup>27</sup> we tested whether CpG sites showing differential methylation in patients with CRPS exhibited patterns of functional enrichment. This functional enrichment analysis revealed numerous GO-Biological Process (GO-BP) categories that were significantly enriched for differentially methylated genes at FDR (q-value)  $< 0.15$ .<sup>60</sup> As indicated in Supplementary Figure 1 (available at <http://links.lww.com/PAIN/A818>), a notable subset of these enriched GO-BP categories involved immune function, including leukocyte-mediated cell toxicity, regulation of cell killing and negative regulation of cell killing, immune effector process, immune response and activation of immune response, production of molecular mediator of immune response, immune system development, regulation of immune system processes, negative and positive regulation of immune system process, antigen processing and presentation, and leukocyte activation.

Supplementary Figure 2 (available at <http://links.lww.com/PAIN/A819>) portrays the number of differentially methylated genes by GO-Molecular Function (GO-MF) categories. The 5 GO-MF categories with the largest number of differentially methylated genes were protein binding, organic cyclic compound binding, ion binding, heterocyclic compound binding, and antigen binding. This latter immune-related GO-MF category supports the pervasive immune-relevant findings in GO-BP enrichment analyses.

### 3.3. Protein-protein interactions for differentially methylated genes in patients with complex regional pain syndrome

We examined whether the 48 differentially methylated CpG sites reflected known PPIs. Among the genes annotated to the differentially methylated CpG sites, we found genes that assemble

in PPI subnetworks (Fig. 3). Using a within-degree node-label permutation, we found that the differentially methylated genes were more highly connected in human PPI networks than expected by chance ( $P = 0.03$ ), indicating that the genes are more likely to be essential genes.<sup>39</sup>

### 3.4. Independent validation

We sought to validate primary findings in a large deidentified clinical sample of patients who had provided DNA that was subjected to genome-wide level (GWAS) genetic assays.<sup>18</sup> Prior work by one of the authors (E.R.G.) had developed methods for using reference transcriptome data to impute the genetically determined component of gene expression from GWAS genetic information alone (PrediXcan).<sup>26</sup> Applying the PrediXcan approach, we expected that compared to patients with a non-CRPS chronic pain phenotype, patients displaying a CRPS phenotype would show evidence for differential genetically determined gene expression in similar genes as revealed in primary analyses.

Results (Table 2) indicated differential gene expression for the CRPS phenotype relative to the non-CRPS chronic pain phenotype for 7 of the 48 genes identified as loci for differential methylation in the primary sample. These 7 genes were *PTGR1*, *HS3ST3A1*, *FAM83B*, *CD81*, *GPR75*, *APBB2*, *AGT*, and *BTNL2*. Taken together, these results demonstrate that the genes identified as showing CRPS-related differential methylation in the primary analyses had some predictive power in an independent patient data set in which the genetic component of gene expression was estimated using the germline genetic profile.

## 4. Discussion

To evaluate epigenetic mechanisms that may be associated with CRPS after injury, we compared DNA methylation profiles in individuals with chronic pain who met Budapest diagnostic criteria for CRPS to those not meeting CRPS criteria. We identified 48 CpG sites that were (nominally) differentially methylated between the 2 groups. Of the 48, one met methylome-wide significance (*COL11A1*). Functional analyses revealed numerous GO-BP categories that were significantly enriched in the CRPS group at FDR (q-value)  $< 0.15$ .<sup>60</sup> A substantial portion of these functionally enriched categories were related to immune function. It is notable that differences in methylation profiles and functional enrichment patterns were observed despite both groups reporting similar pain intensity. Thus, differential methylation was not related simply to presence of chronic pain, but rather specifically to clinical features reflected in the diagnostic criteria defining the CRPS group (eg, edema, skin temperature changes). Validation sample findings regarding the genetically determined component of gene expression suggest that altered expression of implicated genes may confer risk of CRPS rather than simply being a consequence of CRPS.

Interpretation of these findings is best considered in context of clinical and experimental data regarding CRPS mechanisms. Research has increasingly highlighted a likely role for immune mechanisms in CRPS. Several studies suggest that autoimmune mechanisms are relevant to CRPS<sup>24,28</sup> with, for example, over one-third of CRPS patients across 3 studies exhibiting higher levels of antineuronal antibodies than population norms.<sup>6,23,43,44</sup> Moreover, a passive transfer model has demonstrated that serum IgG from patients with CRPS can induce CRPS in animals after tissue injury.<sup>30,63</sup> A key role for inflammatory mechanisms has also been suggested. Compared to both healthy controls and

Table 1

**Cytosine-phosphate-guanine dinucleotide sites (and associated genes) showing differential methylation at an uncorrected  $P < 0.005$  level between CRPS and non-CRPS chronic pain patients.**

CpG site	Gene	Log FC	t value	P
cg17820060_chr1:103343469	<i>COL11A1</i>	-0.166219474	-5.623121047	9.98E-07
cg15591384_chr6:32525960	<i>HLA-DRB6</i>	-0.176122632	-3.955685059	0.000256198
cg00070715_chr5:138862796	<i>TMEM173</i>	-0.08300769	-3.939373128	0.000269618
cg01876338_chr2:54087008	<i>GPR75</i>	-0.065696053	-3.922495243	0.000284217
cg17854650_chr2:132481613	<i>C2orf27A</i>	-0.062572719	-3.882752019	0.000321678
cg09518270_chr1:211308115	<i>KCNH1</i>	-0.237310731	-3.785369214	0.000434713
cg23803868_chr19:2340235	<i>SPPL2B</i>	-0.412810322	-3.730180616	0.000514858
cg14832904_chr2:54087028	<i>GPR75</i>	-0.068758392	-3.671198847	0.000616167
cg20571420_chr22:25160308	<i>TOP1P2; PIWIL3</i>	-0.056746842	-3.54875351	0.000890954
cg10560079_chr2:191398806	<i>TMEM194B</i>	0.057021579	3.531565975	0.000937857
cg04375683_chr19:52692431	<i>PPP2R1A</i>	-0.058455731	-3.506453217	0.001010652
cg10226967_chr19:16606648	<i>C19orf44; CALR3</i>	-0.055366199	-3.496832101	0.001039946
cg15975960_chr19:1035450	<i>CNN2</i>	-0.183742719	-3.482898424	0.001083811
cg04610742_chr1:230513809	<i>PGBD5</i>	-0.205357602	-3.434243521	0.001251244
cg25115829_chr17:29057586	<i>SUZ12P</i>	-0.053363596	-3.380452852	0.00146496
cg13627197_chr22:25160378	<i>TOP1P2; PIWIL3</i>	-0.059896462	-3.354150242	0.001581692
cg23565757_chr7:157646605	<i>PTPRN2</i>	-0.080209854	-3.344032445	0.001628906
cg02675527_chr2:114030824	<i>PAX8</i>	0.119200439	3.341899694	0.001639028
cg03287111_chr2:121625634	<i>GLI2</i>	-0.051736842	-3.314028541	0.001776912
cg23206520_chr2:189158094	<i>GULP1</i>	-0.062407164	-3.30687498	0.001814038
cg26858423_chr1:6149477	<i>KCNAB2</i>	0.084953041	3.277223241	0.001975912
cg04573316_chr19:54216680	<i>MIR519D</i>	-0.063664591	-3.230689232	0.002257805
cg22451923_chr6:32370879	<i>BTNL2</i>	-0.085751053	-3.230266241	0.002260534
cg23964386_chr9:114362249	<i>PTGR1</i>	-0.067227339	-3.229947345	0.002262593
cg01122627_chr11:1474386	<i>BRSK2</i>	-0.079350234	-3.210120217	0.002394169
cg03848831_chr11:2397685	<i>CD81</i>	-0.091655	-3.206372603	0.002419837
cg16929496_chr19:58952249	<i>ZNF132</i>	-0.054303684	-3.200189318	0.002462757
cg26805579_chr6:32372962	<i>BTNL2</i>	-0.083768012	-3.195461202	0.00249606
cg03870448_chr4:41214687	<i>APBB2</i>	-0.055146374	-3.183598257	0.0025815
cg02027878_chr7:150711138	<i>ATG9B; NOS3</i>	-0.049219181	-3.182685168	0.002588189
cg10330187_chr16:88666383	<i>ZC3H18</i>	-0.063355117	-3.169273341	0.002688349
cg24474852_chr1:230850050	<i>AGT</i>	-0.07147886	-3.158041045	0.002775033
cg09701145_chr11:2019436	<i>MIR675; H19</i>	0.049746433	3.157926055	0.002775934
cg19846314_chr2:171680113	<i>GAD1</i>	0.052606901	3.153575381	0.002810223
cg18323236_chr1:24743029	<i>NIPAL3</i>	-0.066710292	-3.131413078	0.002991171
cg15975890_chr5:11903145	<i>CTNND2</i>	-0.05833652	-3.084777665	0.003408353
cg18584440_chr6:32370815	<i>BTNL2</i>	-0.084480673	-3.079664007	0.003457285
cg24212267_chr7:137801780	<i>AKR1D1</i>	-0.051366959	-3.054308822	0.003709806
cg15214448_chr17:13506230	<i>HS3ST3A1</i>	0.058285497	3.052583402	0.003727604
cg14989959_chr6:54711471	<i>FAM83B</i>	0.056408596	3.041369712	0.003845243
cg18683523_chr6:91297516	<i>MAP3K7</i>	-0.057792047	-3.01326307	0.00415557
cg22835630_chr15:25434030	<i>SNORD115</i>	-0.071314181	-3.007297156	0.004224389
cg07065756_chr7:2119340	<i>MAD1L1</i>	-0.045774766	-2.97828233	0.004574559
cg16320788_chr8:73794096	<i>KCNB2</i>	-0.05781769	-2.963333609	0.004765386

(continued on next page)

Table 1 (continued)

CpG site	Gene	Log FC	t value	P
cg17275074_chr5:135701422	<i>TRPC7</i>	-0.058669094	-2.958925012	0.004823067
cg02844892_chr6:31370412	<i>MICA</i>	-0.046332544	-2.957764382	0.004838361
cg13713922_chr2:121625577	<i>GLI2</i>	-0.050111784	-2.954838104	0.004877122
cg12580156_chr10:112588051	<i>RBM20</i>	-0.04473193	-2.948632141	0.004960286

Negative logFC and t values indicate lower methylation in the CRPS group.  
CpG, cytosine-phosphate-guanine dinucleotide; CRPS, complex regional pain syndrome.

non-CRPS pain patients, CRPS patients display significantly elevated levels of proinflammatory cytokines (eg, TNF-alpha, IL-1 beta).<sup>2,49,66,74,75</sup> A study using informatics-based network analysis<sup>34</sup> suggests that such proinflammatory changes may derive in part from upstream alterations in nfKappaB, an inducible transcription factor involved in immune regulation and inflammation,<sup>64</sup> a finding supported in a preclinical model of CRPS.<sup>16</sup>

In light of evidence for immune and inflammatory mechanism in CRPS, several aspects of the current findings are notable. One of the top 2 CpG sites most strongly associated with CRPS was in *HLA-DRB6*, an HLA class II pseudogene. This gene has previously exhibited differential gene expression between patients with CRPS and pain-free controls.<sup>37</sup> Class II HLA genes are involved in immune regulation, and are expressed by B cells, activated T cells, and dendritic cells.<sup>42</sup> *HLA-DRB6* is hypomethylated in rheumatoid arthritis, a painful autoimmune condition,<sup>32</sup> a pattern similar to patients with CRPS in the current study. Also, among the 48 CpG sites exhibiting differential methylation were genes including *MICA*, *TMEM173*, and *BTNL2*, all of which were hypomethylated in patients with CRPS. *MICA* is an HLA class I gene, expressed by somatic cells rather than specifically in immune cells.<sup>42</sup> Variations in the *MICA* gene have been linked with other painful (Sjogren syndrome)<sup>12</sup> and nonpainful autoimmune conditions (cutaneous lupus erythematosus).<sup>45</sup> *TMEM173* produces the stimulator of interferon genes (STING)

protein, a regulator of innate immune responses that also is involved in autoimmune diseases<sup>48,50</sup> and promotes inflammation.<sup>78</sup> *BTNL2*, a gene identified both in the primary methylation sample and in the validation sample, is an HLA class II-related gene that has been associated with painful autoimmune conditions due to its being in strong linkage disequilibrium with *HLA-DQB1* and *HLA-DRB1* haplotypes.<sup>53</sup> Interestingly, work in CRPS suggests that both the *HLA-DQB1* and *HLA-DRB1* genes are differentially expressed (downregulated) in patients with CRPS compared to pain-free controls.<sup>37,62</sup>

The current findings are also relevant in terms of inflammatory CRPS mechanisms. Among the 48 genes displaying differential methylation in CRPS were the *COL11A1* and *MAP3K7* genes. These genes are part of an nfKappaB-related gene network.<sup>64,73,76</sup> Activation of the nfKappaB pathway triggers cytokine release, leading to inflammation.<sup>34</sup> Inflammatory clinical features (eg, edema and erythema) and elevated proinflammatory cytokine levels are both characteristic of CRPS, particularly in its early stages.<sup>5,10,47</sup> Differential methylation was also noted in the *GPR75* gene, another gene validated in terms of the genetic component of gene expression in our large informatics sample. Impaired *GPR75* function has been linked to neuroinflammation.<sup>36</sup> Oxidative stress is also linked to inflammation,<sup>3</sup> and current findings of differential methylation of *PTGR1* in patients with CRPS may be relevant in this regard. *PTGR1* codes for

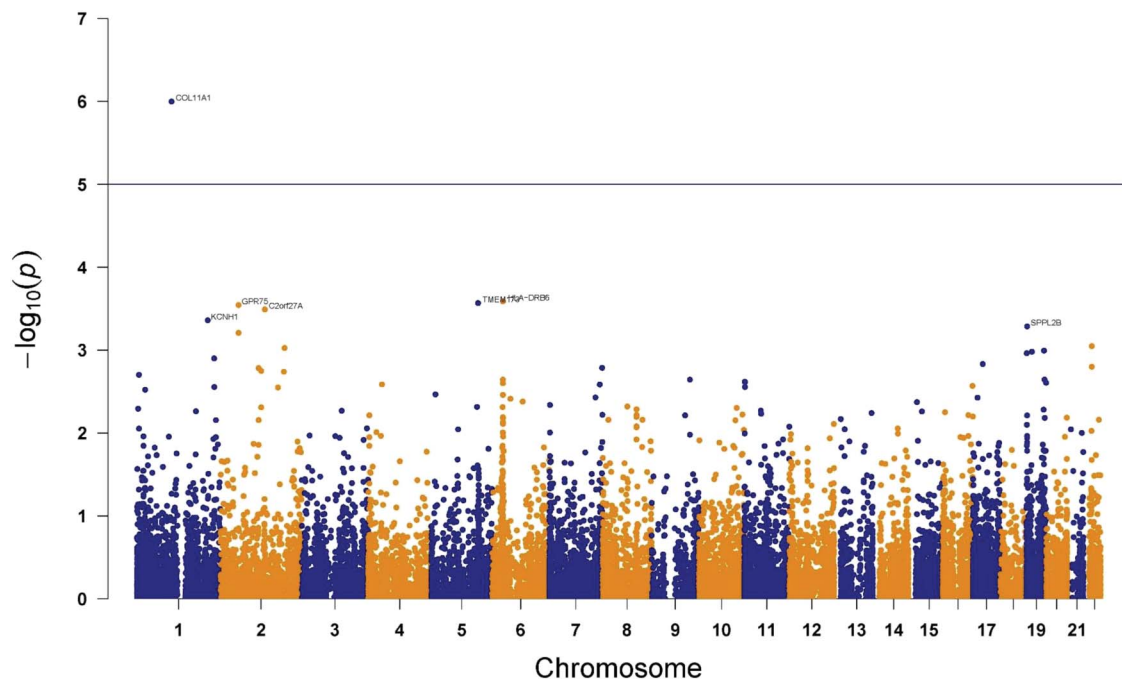


Figure 1. Manhattan plot for differential methylation across all CpG sites evaluated. Any gene above the horizontal line indicates Bonferroni-corrected  $P < 0.05$ . One gene *COL11A1* (nominal  $P = 9.98E-07$ ) passed this threshold. CpG, cytosine-phosphate-guanine dinucleotide.



**Table 2**  
**Genes displaying CRPS-related differential methylation profiles in the primary sample that showed additional support (defined as  $P < 0.05$ ) in the BioVU validation sample in terms of the genetic component of gene expression.**

Gene	Beta	Validation $P$
PTGR1	-0.728786	1.28E-03
HS3ST3A1	1.53113	1.64E-02
FAM83B	0.620845	2.41E-02
CD81	-2.49833	2.43E-02
GPR75	3.61601	2.86E-02
APBB2	0.550918	3.87E-02
AGT	-0.441226	4.70E-02
BTNL2	2.24399	1.39E-02

Beta is the beta-weight observed in the logistic regression analysis for each gene indicated. CRPS, complex regional pain syndrome.

Preclinical CRPS models<sup>13,41</sup> and meta-analysis of human studies<sup>1</sup> suggest that CRPS risk after injury may be mitigated by administration of antioxidants, consistent with a possible role for oxidative stress in CRPS.

The largest differential methylation effect in the current work was for the *COL11A1* gene. *COL11A1* is involved in collagen formation, and notably, the only prior gene expression work in CRPS found that one of the top differentially expressed genes was also a collagen-related gene, *MMP9*.<sup>37</sup> There is no prior evidence of a specific role for collagen-related factors in CRPS, although we might speculate that given the role of collagen in skin formation,<sup>54</sup> altered expression of genes such as *COL11A1* and *MMP9* might contribute to altered skin growth often characteristic of CRPS.<sup>7,8</sup> In addition, it may be relevant that both the *COL11A1* gene and the *KRT16* gene are part of the *TFAP2A* gene regulatory network.<sup>77</sup> The protein expressed by the *KRT16* gene has been shown to be a target for autoantibody responses in a preclinical CRPS model.<sup>61</sup>

Our results identified numerous PPIs related to the differentially methylated genes observed between groups. These findings of known functional interactions between proteins coded for by differentially methylated genes linked to CRPS strengthen arguments for the biological importance of these genes in CRPS. The 2 most prominent nodes reflected hormonal regulation processes and immune function.

The current findings further support the potential of immune-focused therapies for CRPS.<sup>29</sup> To the extent that immune differences may play a mechanistic role in only a subset of patients with CRPS, profiles of differential methylation in CRPS-related immune genes could potentially help target immune-focused treatments to patients most likely to respond. The epigenetic changes indicated by CRPS-related methylation profiles might also help identify novel CRPS risk mechanisms that could point towards new treatments. Finally, from the methodological perspective, findings in the validation sample demonstrate the promise of using electronic health records linked to DNA for genomic studies of CRPS.

Several study limitations are acknowledged. First, although our sample of patients with CRPS was more than twice as large as any prior epigenetic work in patients with CRPS, the sample was nonetheless small and there were a large number of statistical tests. Risk of type I error was mitigated in part through the LIMMA analysis used, which accounts for intercorrelations among measures and thereby helps minimize type I error. We note that one CpG site in the *COL11A1* gene was significant even after Bonferroni correction. In addition, the fact that 7 of the genes

displaying differential methylation in the primary study were validated in the informatics-based clinical sample also supports at least some of the observed methylation differences as being real effects. Nonetheless, the *ICD-9*-based CRPS phenotype in this validation sample might limit interpretation of these validation results. Another potential limitation is that we cannot address the possibility that pain management medications, which may differ in patients with CRPS, or other clinical factors may have impacted on methylation patterns after development of CRPS. Finally, generalizability of study results must be considered in light of the relatively low mean pain intensity observed (4.7/10), the unusual initiating event (amputation), and atypical sample demographics, although all patients with CRPS did meet the Budapest criteria. Patients with CRPS seeking clinical care often report severe pain and are more likely to be female and older,<sup>8</sup> whereas the current sample was primarily younger and male, due to the military nature of the sample. Replication of these findings in more traditional clinical CRPS samples would be desirable. The fact that 1 of the top 2 CpG sites exhibiting significant differential methylation in this study also displayed differential gene expression between patients with CRPS and nonpain controls in a prior nonmilitary sample<sup>37</sup> suggests that current results might generalize to more clinically representative samples.

In summary, this study for the first time suggests a potential role for epigenetic mechanisms (differential DNA methylation) in risk of CRPS after injury. The pattern of results suggests that immune and inflammatory mechanisms in particular may be involved in influencing CRPS risk. Although our results require replication, these epigenetic findings build on prior studies increasingly pointing towards the potential value of interventions targeting immune and inflammatory pathways for enhancing care of patients with CRPS.

### Conflict of interest statement

The authors have no conflicts of interest to declare.

### Acknowledgments

The authors acknowledge the assistance of Melissa Chont in this project.

This research was supported by Congressionally Directed Medical Research Programs and Department of Defense awards MR130082, W81XWH-15-2-0046, W81XWH-12-2-0129, W81XWH-11-2-0003, and NIH grant T32GM008600. Support was also provided by a research grant from the Reflex Sympathetic Dystrophy Syndrome Association. The data set used for validation analyses was obtained from Vanderbilt University Medical Center's BioVU which is supported by institutional funding and CTSA award No. UL1 TR002243 from the NIH/ National Center for Advancing Translational Sciences. The contents of this manuscript are solely the responsibility of the authors and do not necessarily represent official views of the National Center for Advancing Translational Sciences, the Department of Defense, the Department of Veterans Affairs, or the U.S. Government.

### Appendix A. Supplemental digital content

Supplemental digital content associated with this article can be found online at <http://links.lww.com/PAIN/A818>, <http://links.lww.com/PAIN/A819>, and <http://links.lww.com/PAIN/A820>.



**Article history:**

Received 26 November 2018

Received in revised form 8 May 2019

Accepted 13 May 2019

Available online 22 May 2019

**References**

- [1] Aïm F, Klouche S, Frison A, Bauer T, Hardy P. Efficacy of vitamin C in preventing complex regional pain syndrome after wrist fracture: a systematic review and meta-analysis. *Orthop Traumatol Surg Res* 2017;103:465–70.
- [2] Alexander GM, van Rijn MA, van Hilten JJ, Perreault MJ, Schwartzman RJ. Changes in cerebrospinal fluid levels of pro-inflammatory cytokines in CRPS. *PAIN* 2005;116:213–219.
- [3] Basu S. Bioactive eicosanoids: role of prostaglandin F(2 $\alpha$ ) and F $_2$ -isoprostanes in inflammation and oxidative stress related pathology. *Mol Cells* 2010;30:383–91.
- [4] Bennett MI, Smith BH, Torrance N, Potter J. The S-LANSS score for identifying pain of predominantly neuropathic origin: validation for use in clinical and postal research. *J Pain* 2005;6:149–58.
- [5] Birklein F, Drummond PD, Li W, Schlereth T, Albrecht N, Finch PM, Dawson LF, Clark JD, Kingery WS. Activation of cutaneous immune response in complex regional pain syndrome. *J Pain* 2014;15:485–95.
- [6] Blaes F, Schmitz K, Tschernatsch M, Kaps M, Krasenbrink I, Hempelmann G, Bräu ME. Autoimmune etiology of complex regional pain syndrome (M. Sudeck). *Neurology* 2004;63:1734–6.
- [7] Bruehl S. An update on the pathophysiology of complex regional pain syndrome. *Anesthesiol* 2010;113:713–25.
- [8] Bruehl S. Complex regional pain syndrome. *BMJ* 2015;351:h2730.
- [9] Bruehl S, Harden RN, Galer BS, Saltz S, Backonja M, Stanton-Hicks M. Complex regional pain syndrome: are there distinct subtypes and sequential stages of the syndrome? *PAIN* 2002;95:119–24.
- [10] Bruehl S, Maihöfner C, Stanton-Hicks M, Perez RS, Vatine JJ, Brunner F, Birklein F, Schlereth T, Mackey S, Mailis-Gagnon A, Livshitz A, Harden RN. Complex regional pain syndrome: evidence for warm and cold subtypes in a large prospective clinical sample. *PAIN* 2016;157:1674–81.
- [11] Buchheit T, Van de Ven T, Hsia HL, McDuffie M, MacLeod DB, White W, Chamesian A, Keefe FJ, Buckenmaier CT, Shaw AD. Pain phenotypes and associated clinical risk factors following traumatic amputation: results from veterans integrated pain evaluation research (VIPER). *Pain Med* 2016;17:149–61.
- [12] Carapito R, Gottenberg JE, Kotova I, Untrau M, Michel S, Naegely L, Aouadi I, Kwemou M, Paul N, Pichot A, Locke J, Bowman SJ, Griffiths B, Sivits KL, Sibilia J, Inoko H, Micelli-Richard C, Nocturne G, Ota M, Ng WF, Mariette X, Bahram S. A new MHC-linked susceptibility locus for primary Sjögren's syndrome: MICA. *Hum Mol Genet* 2017;26:2565–76.
- [13] Coderre TJ, Xanthos DN, Francis L, Bennett GJ. Chronic post-ischemia pain (CPIP): a novel animal model of complex regional pain syndrome-type I (CRPS-I; reflex sympathetic dystrophy) produced by prolonged hindpaw ischemia and reperfusion in the rat. *PAIN* 2004;112:94–105.
- [14] Deichmann U. Epigenetics: the origins and evolution of a fashionable topic. *Dev Biol* 2016;416:249–54.
- [15] de Mos M, Huygen FJ, van der Hoeven-Borgman M, Dieleman JP, Ch Stricker BH, Sturkenboom MC. Outcome of the complex regional pain syndrome. *Clin J Pain* 2009;25:590–7.
- [16] de Mos M, Laferrère A, Millesamps M, Pilkington M, Sturkenboom MC, Huygen FJ, Coderre TJ. Role of NF $\kappa$ B in an animal model of complex regional pain syndrome-type I (CRPS-I). *J Pain* 2009;10:1161–9.
- [17] Dennis G Jr, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, Lempicki RA. DAVID: database for annotation, visualization, and integrated discovery. *Genome Biol* 2003;4:P3.
- [18] Denny JC, Bastarache L, Ritchie MD, Carroll RJ, Zink R, Mosley JD, Field JR, Pulley JM, Ramirez AH, Bowton E, Basford MA, Carrell DS, Peissig PL, Kho AN, Pacheco JA, Rasmussen LV, Crosslin DR, Crane PK, Pathak J, Bielinski SJ, Pendergrass SA, Xu H, Hindorf LA, Li R, Manolio TA, Chute CG, Chisholm RL, Larson EB, Jarvik GP, Brilliant MH, McCarty CA, Kullo IJ, Haines JL, Crawford DC, Masy DR, Roden DM. Systematic comparison of phenotype-wide association study of electronic medical record data and genome-wide association study data. *Nat Biotechnol* 2013;31:1102–10.
- [19] Denny JC, Ritchie MD, Basford MA, Pulley JM, Bastarache L, Brown-Gentry K, Wang D, Masy DR, Roden DM, Crawford DC. PheWAS: demonstrating the feasibility of a phenotype-wide scan to discover gene-disease associations. *Bioinformatics* 2010;26:1205–10.
- [20] de Rooij AM, de Mos M, Sturkenboom MC, Marinus J, van den Maagdenberg AM, van Hilten JJ. Familial occurrence of complex regional pain syndrome. *Eur J Pain* 2009;13:171–7.
- [21] de Rooij AM, de Mos M, van Hilten JJ, Sturkenboom MC, Gosso MF, van den Maagdenberg AM, Marinus J. Increased risk of complex regional pain syndrome in siblings of patients? *J Pain* 2009;10:1250–5.
- [22] de Rooij AM, Florencia Gosso M, Haasnoot GW, Marinus J, Verduijn W, Claas FH, van den Maagdenberg AM, van Hilten JJ. HLA-B62 and HLA-DQ8 are associated with Complex Regional Pain Syndrome with fixed dystonia. *PAIN* 2009;145:82–5.
- [23] Dirckx M, Schreurs MW, de Mos M, Stronks DL, Huygen FJ. The prevalence of autoantibodies in complex regional pain syndrome type I. *Mediators Inflamm* 2015;2015:718201.
- [24] Dubuis E, Thompson V, Leite MI, Blaes F, Maihöfner C, Greensmith D, Vincent A, Shenker N, Kuttikat A, Leuwer M, Goebel A. Longstanding complex regional pain syndrome is associated with activating autoantibodies against alpha-1a adrenoceptors. *PAIN* 2014;155:2408–17.
- [25] Gamazon ER, Segrè AV, van de Bunt M, Wen X, Xi HS, Hormozdiari F, Ongen H, Konkashbaev A, Derks EM, Aguet F, Quan J; GTEx Consortium, Nicolae DL, Eskin E, Kellis M, Getz G, McCarthy MI, Dermizakis ET, Cox NJ, Ardlie KG. Using an atlas of gene regulation across 44 human tissues to inform complex disease- and trait-associated variation. *Nat Genet* 2018;50:956–67.
- [26] Gamazon ER, Wheeler HE, Shah KP, Mozaffari SV, Aquino-Michaels K, Carroll RJ, Eyler AE, Denny JC; GTEx Consortium, Nicolae DL, Cox NJ, Im HK. A gene-based association method for mapping traits using reference transcriptome data. *Nat Genet* 2015;47:1091–8.
- [27] Gene Ontology Consortium. Creating the gene ontology resource: design and implementation. *Genome Res* 2001;11:1425–33.
- [28] Goebel A, Blaes F. Complex regional pain syndrome, prototype of a novel kind of autoimmune disease. *Autoimmun Rev* 2013;12:682–6.
- [29] Goebel A, Jacob A, Frank B, Sacco P, Alexander G, Phillips C, Bassett P, Moots R. Mycophenolate for persistent complex regional pain syndrome, a parallel, open, randomised, proof of concept trial. *Scand J Pain* 2018;18:29–37.
- [30] Goebel A, Leite MI, Yang L, Deacon R, Cendan CM, Fox-Lewis A, Vincent A. The passive transfer of immunoglobulin G serum antibodies from patients with longstanding complex regional pain syndrome. *Eur J Pain* 2011;15:504.e1–6.
- [31] GTEx Consortium; Laboratory, Data Analysis & Coordinating Center (LDACC)—Analysis Working Group; Statistical Methods groups—Analysis Working Group; Enhancing GTEx (eGTEx) groups; NIH Common Fund; NIH/NCI; NIH/NHGRI; NIH/NIMH; NIH/NIDA; Biospecimen Collection Source Site—NDRI; Biospecimen Collection Source Site—RPCI; Biospecimen Core Resource—VARI; Brain Bank Repository—University of Miami Brain Endowment Bank; Leidos Biomedical—Project Management; ELSI Study; Genome Browser Data Integration & Visualization—EBI; Genome Browser Data Integration & Visualization—UCSC Genomics Institute, University of California Santa Cruz; Lead analysts; Laboratory, Data Analysis & Coordinating Center (LDACC); NIH program management; Biospecimen collection; Pathology; eQTL manuscript working group, Battle A, Brown CD, Engelhardt BE, Montgomery SB. Genetic effects on gene expression across human tissues. *Nature* 2017;550:204–13.
- [32] Guo S, Zhu Q, Jiang T, Wang R, Shen Y, Zhu X, Wang Y, Bai F, Ding Q, Zhou X, Chen G, He DY. Genome-wide DNA methylation patterns in CD4+ T cells from Chinese Han patients with rheumatoid arthritis. *Mod Rheumatol* 2017;27:441–7.
- [33] Harden RN, Bruehl S, Perez RS, Birklein F, Marinus J, Maihöfner C, Lubenow T, Buvanendran A, Mackey S, Graciosa J, Mogilevski M, Ramsden C, Chont M, Vatine JJ. Validation of proposed diagnostic criteria (the “Budapest criteria”) for complex regional pain syndrome. *PAIN* 2010;150:268–74.
- [34] Hettne KM, de Mos M, de Bruijn AG, Weeber M, Boyer S, van Mulligen EM, Cases M, Mestres J, van der Lei J. Applied information retrieval and multidisciplinary research: new mechanistic hypotheses in complex regional pain syndrome. *J Biomed Discov Collab* 2007;2:2.
- [35] Higashimoto T, Baldwin EE, Gold JI, Boles RG. Reflex sympathetic dystrophy: complex regional pain syndrome type I in children with mitochondrial disease and maternal inheritance. *Arch Dis Child* 2008;93:390–7.
- [36] Ignatov A, Robert J, Gregory-Evans C, Schaller HC. RANTES stimulates Ca $^{2+}$  mobilization and inositol trisphosphate (IP $_3$ ) formation in cells transfected with G protein-coupled receptor 75. *Br J Pharmacol* 2006;149:490–7.
- [37] Jin EH, Zhang E, Ko Y, Sim WS, Moon DE, Yoon KJ, Hong JH, Lee WH. Genome-wide expression profiling of complex regional pain syndrome. *PLoS One* 2013;8:e79435.
- [38] Jones PA. Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nat Rev Genet* 2012;13:484–92.

- [39] Joyce AR, Palsson BØ. Predicting gene essentiality using genome-scale in silico models. *Methods Mol Biol* 2008;416:433–57.
- [40] Kemler MA, van de Vusse AC, van den Berg-Loonen EM, Barendse GA, van Kleef M, Weber WE. HLA-DQ1 associated with reflex sympathetic dystrophy. *Neurology* 1999;53:1350–1.
- [41] Kim JH, Kim YC, Nahm FS, Lee PB. The therapeutic effect of vitamin C in an animal model of complex regional pain syndrome produced by prolonged hindpaw ischemia-reperfusion in rats. *Int J Med Sci* 2017;14:97–101.
- [42] Klein J, Sato A. The HLA system. First of two parts. *N Engl J Med* 2000;343:702–9.
- [43] Kohr D, Singh P, Tschernatsch M, Kaps M, Pouokam E, Diener M, Kummer W, Birklein F, Vincent A, Goebel A, Wallukat G, Blaes F. Autoimmunity against the  $\beta_2$  adrenergic receptor and muscarinic-2 receptor in complex regional pain syndrome. *PAIN* 2011;152:2690–700.
- [44] Kohr D, Tschernatsch M, Schmitz K, Singh P, Kaps M, Schäfer KH, Diener M, Mathies J, Matz O, Kummer W, Maihöfner C, Fritz T, Birklein F, Blaes F. Autoantibodies in complex regional pain syndrome bind to a differentiation-dependent neuronal surface autoantigen. *PAIN* 2009;143:246–51.
- [45] Kunz M, König IR, Schillert A, Kruppa J, Ziegler A, Grallert H, Müller-Nurasyid M, Lieb W, Franke A, Ranki A, Panelius J, Koskenmies S, Hasan T, Kere J, Rönn AC, Simon JC, Schmidt E, Wenzel J, Tüting T, Landsberg J, Zeller T, Blankenberg S, Gläser R, Patsinakidis N, Kuhn A, Ibrahim SM. Genome-wide association study identifies new susceptibility loci for cutaneous lupus erythematosus. *Exp Dermatol* 2015;24:510–15.
- [46] Lage K, Karlberg EO, Störing ZM, Olason PI, Pedersen AG, Rigina O, Hinsby AM, Tümer Z, Pociot F, Tommerup N, Moreau Y, Brunak S. A human phenome-interactome network of protein complexes implicated in genetic disorders. *Nat Biotechnol* 2007;25:309–16.
- [47] Lenz M, Uçeyler N, Frettlöh J, Höffken O, Krumova EK, Lissek S, Reinersmann A, Sommer C, Stude P, Waaga-Gasser AM, Tegenthoff M, Maier C. Local cytokine changes in complex regional pain syndrome type I (CRPS I) resolve after 6 months. *PAIN* 2013;154:2142–9.
- [48] Li Y, Wilson HL, Kiss-Toth E. Regulating STING in health and disease. *J Inflamm (Lond)* 2017;14:11.
- [49] Maihöfner C, Handwerker HO, Neundörfer B, Birklein F. Mechanical hyperalgesia in complex regional pain syndrome: a role for TNF-alpha? *Neurology* 2005;65:311–13.
- [50] McCaffary D. STING signalling: an emerging common pathway in autoimmunity and cancer. *Immunopharmacol Immunotoxicol* 2017;39:253–8.
- [51] Nguyen T, Nioi P, Pickett CB. The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. *J Biol Chem* 2009;284:13291–5.
- [52] Orlova IA, Alexander GM, Qureshi RA, Sacan A, Graziano A, Barrett JE, Schwartzman RJ, Ajit SK. MicroRNA modulation in complex regional pain syndrome. *J Transl Med* 2011;9:195.
- [53] Orozco G, Eerligh P, Sánchez E, Zhenakova S, Roep BO, González-Gay MA, López-Nevo MA, Callejas JL, Hidalgo C, Pascual-Salcedo D, Balsa A, González-Escribano MF, Koeleman BP, Martín J. Analysis of a functional BTNL2 polymorphism in type 1 diabetes, rheumatoid arthritis, and systemic lupus erythematosus. *Hum Immunol* 2005;66:1235–41.
- [54] Philips N, Auler S, Hugo R, Gonzalez S. Beneficial regulation of matrix metalloproteinases for skin health. *Enzyme Res* 2011;2011:427285.
- [55] Ritchie MD, Denny JC, Crawford DC, Ramirez AH, Weiner JB, Pulley JM, Basford MA, Brown-Gentry K, Balsler JR, Masys DR, Haines JL, Roden DM. Robust replication of genotype-phenotype associations across multiple diseases in an electronic medical record. *Am J Hum Genet* 2010;86:560–72.
- [56] Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK. Limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* 2015;43:e47.
- [57] Roden DM, Pulley JM, Basford MA, Bernard GR, Clayton EW, Balsler JR, Masys DR. Development of a large-scale de-identified DNA biobank to enable personalized medicine. *Clin Pharmacol Ther* 2008;84:362–9.
- [58] Rossin EJ, Lage K, Raychaudhuri S, Xavier RJ, Tatar D, Benita Y. International Inflammatory Bowel Disease Genetics Consortium, Cotsapas C, Daly MJ. Proteins encoded in genomic regions associated with immune-mediated disease physically interact and suggest underlying biology. *PLoS Genet* 2011;7:e1001273.
- [59] Sánchez-Rodríguez R, Torres-Mena JE, Quintanar-Jurado V, Chagoya-Hazas V, Rojas Del Castillo E, Del Pozo Yauner L, Villa-Treviño S, Pérez-Carreón JL. Ptgr1 expression is regulated by NRF2 in rat hepatocarcinogenesis and promotes cell proliferation and resistance to oxidative stress. *Free Radic Biol Med* 2017;102:87–99.
- [60] Storey JD, Tibshirani R. Statistical significance for genomewide studies. *Proc Natl Acad Sci U S A* 2003;100:9440–5.
- [61] Tajerian M, Hung V, Khan H, Lahey LJ, Sun Y, Birklein F, Krämer HH, Robinson WH, Kingery WS, Clark JD. Identification of KRT16 as a target of an autoantibody response in complex regional pain syndrome. *Exp Neurol* 2017;287:14–20.
- [62] Tan W, Song Y, Mo C, Jiang S, Wang Z. Analysis of gene expression profile microarray data in complex regional pain syndrome. *Mol Med Rep* 2017;16:3371–8.
- [63] Tékus V, Hajna Z, Borbély É, Markovics A, Bagoly T, Szolcsányi J, Thompson V, Kemény Á, Helyes Z, Goebel A. A CRPS-IgG-transfer-trauma model reproducing inflammatory and positive sensory signs associated with complex regional pain syndrome. *PAIN* 2014;155:299–308.
- [64] Tian B, Brasier AR. Identification of a nuclear factor kappa B-dependent gene network. *Recent Prog Horm Res* 2003;58:95–130.
- [65] Tost J. DNA methylation: an introduction to the biology and the disease-associated changes of a promising biomarker. *Methods Mol Biol* 2009;507:3–20.
- [66] Uçeyler N, Eberle T, Rolke R, Birklein F, Sommer C. Differential expression patterns of cytokines in complex regional pain syndrome. *PAIN* 2007;132:14–15.
- [67] van de Beek WJ, Roep BO, van der Slik AR, Giphart MJ, van Hilten BJ. Susceptibility loci for complex regional pain syndrome. *PAIN* 2003;103:93–7.
- [68] Vaneker M, van der Laan L, Allebes WA, Goris J. Genetic factors associated with Complex Regional Pain Syndrome I: HLA DRB and TNF alpha promoter gene polymorphism. *Disabil Med* 2002;2:69–74.
- [69] van Hilten JJ, van de Beek WJ, Roep BO. Multifocal or generalized tonic dystonia of complex regional pain syndrome: a distinct clinical entity associated with HLA-DR13. *Ann Neurol* 2000;48:113–6.
- [70] van Rooijen DE, Roelen DL, Verduijn W, Haasnoot GW, Huygen FJ, Perez RS, Claas FH, Marinus J, van Hilten JJ, van den Maagdenberg AM. Genetic HLA associations in complex regional pain syndrome with and without dystonia. *J Pain* 2012;13:784–9.
- [71] Wang F, Stefano GB, Kream RM. Epigenetic modification of DRG neuronal gene expression subsequent to nerve injury: etiological contribution to complex regional pain syndromes (Part I). *Med Sci Monit* 2014;20:1067–77.
- [72] Wang F, Stefano GB, Kream RM. Epigenetic modification of DRG neuronal gene expression subsequent to nerve injury: etiological contribution to complex regional pain syndromes (Part II). *Med Sci Monit* 2014;20:1188–200.
- [73] Wen H, Lei Y, Eun SY, Ting JP. Plexin-A4-semaphorin 3A signaling is required for Toll-like receptor- and sepsis-induced cytokine storm. *J Exp Med* 2010;207:2943–57.
- [74] Wesseldijk F, Huygen FJ, Heijmans-Antonissen C, Niehof SP, Zijlstra FJ. Six years follow-up of the levels of TNF-alpha and IL-6 in patients with complex regional pain syndrome type 1. *Mediators Inflamm* 2008;2008:469439.
- [75] Wesseldijk F, Huygen FJ, Heijmans-Antonissen C, Niehof SP, Zijlstra FJ. Tumor necrosis factor-alpha and interleukin-6 are not correlated with the characteristics of Complex Regional Pain Syndrome type 1 in 66 patients. *Eur J Pain* 2008;12:716–21.
- [76] Yang T, Wang S, Yang X, Zheng Q, Wang L, Li Q, Wei M, Du Z, Fan Y. Upregulation of Bcl-2 and its promoter signals in CD4+ T cells during neuromyelitis optica remission. *Front Neurosci* 2017;11:11.
- [77] Zhang M. Transcriptional regulatory element database. Available at: <http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=home>. Accessed March 5, 2019.
- [78] Zhao Q, Wei Y, Pandol SJ, Li L, Habtezion A. STING signaling promotes inflammation in experimental acute pancreatitis. *Gastroenterology* 2018;154:1822–35.