



# Of mice, microglia, and (wo)men: a case series and mechanistic investigation of hydroxychloroquine for complex regional pain syndrome

Elena S. Haight, Emily M. Johnson, Ian R. Carroll, Vivianne L. Tawfik\*

## Abstract

**Introduction:** Complex regional pain syndrome (CRPS) is a condition that occurs after minor trauma characterized by sensory, trophic, and motor changes. Although preclinical studies have demonstrated that CRPS may be driven in part by autoinflammation, clinical use of immune-modulating drugs in CRPS is limited. Hydroxychloroquine (HCQ) is a disease-modifying antirheumatic drug used to treat malaria and autoimmune disorders that may provide benefit in CRPS.

**Objectives:** To describe the use of HCQ in patients with refractory CRPS and investigate possible mechanisms of benefit in a mouse model of CRPS.

**Methods:** We initiated HCQ therapy in 7 female patients with refractory CRPS undergoing treatment at the Stanford Pain Management Center. We subsequently undertook studies in the mouse tibial fracture–casting model of CRPS to identify mechanisms underlying symptom reduction. We evaluated behavior using mechanical allodynia and spinal cord autoinflammation by immunohistochemistry and enzyme-linked immunosorbent assay.

**Results:** We treated 7 female patients with chronic, refractory CRPS with HCQ 200 mg twice daily for 2 months, followed by 200 mg daily thereafter. Two patients stopped HCQ secondary to lack of response or side effects. Overall, HCQ significantly improved average numerical rating scale pain from  $6.8 \pm 1.1$  before HCQ to  $3.8 \pm 1.9$  after HCQ treatment. In the tibial fracture–casting mouse model of CRPS, we observed reductions in allodynia, paw edema, and warmth following daily HCQ treatment starting at 3 weeks after injury. Spinal cord dorsal horn microglial activation and cytokine levels were also reduced by HCQ treatment.

**Conclusion:** Together, these preclinical and clinical results suggest that HCQ may benefit patients with CRPS at least in part by modulating autoinflammation and support further investigation into the use of HCQ for CRPS.

**Keywords:** Complex regional pain syndrome, Microglia, Neuroinflammation, Hydroxychloroquine, Translational research

## 1. Introduction

Complex regional pain syndrome (CRPS) is a chronic pain condition typically occurring after minor surgery or fracture, with an incidence of 5.5 to 26.2 per 100,000 per year.<sup>20,41</sup> Two subtypes have been identified, based on the absence (type I) or presence (type II) of nerve injury, although subtype-specific

mechanism research and treatments have not been pursued. Rather, it is accepted that irrespective of the subtype, CRPS has myriad mechanisms, including ischemic reperfusion injury,<sup>16</sup> peripheral and central sensitization,<sup>14</sup> autoinflammation/autoimmunity,<sup>1,6</sup> changes in “pain matrix” brain regions,<sup>4</sup> and genetic polymorphisms,<sup>48</sup> with combinations of these at play in a given patient. This multitude of mechanisms and their nonuniformity has made developing broadly effective therapies challenging, and physicians have used medications targeting neural circuitry, including tricyclic antidepressants, cation channel blockers, and *N*-methyl-D-aspartate antagonists.<sup>38</sup>

Recently, research has focused on immune mechanisms.<sup>15</sup> Limited clinical data identify autoantibodies against neuronal proteins in patients with CRPS,<sup>23</sup> and a benefit of cytokine antagonists<sup>22,35</sup> and steroids in CRPS,<sup>3,32</sup> suggesting an autoimmune component of CRPS. Further clinical studies of immune modulators have been limited by the serious side-effect profile of these drugs, especially with prolonged dosing. Interestingly, neuroinflammation characterized by central nervous system microglial activation and cytokine release has been observed in animal models<sup>17</sup> and in the postmortem spinal cord from a human subject with CRPS.<sup>21</sup> In addition, in rodent models

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Department of Anesthesiology, Perioperative and Pain Medicine, Stanford University, Stanford, CA, USA

\*Corresponding author. Address: Department of Anesthesiology, Perioperative & Pain Medicine, Stanford University School of Medicine, E.D. Stone Grant Building, S007, Stanford, CA 94305. Tel.: 650-724-7935. E-mail address: vivianne@stanford.edu (V.L. Tawfik).

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of CRPS, minocycline<sup>27</sup> and cannabinoid receptor-2 agonists<sup>49</sup>—both of which function in part as microglial modulators—provide analgesia. Currently, just 1 clinical trial exists to evaluate the effects of low-dose naltrexone, an opioid antagonist that may also act in part on the microglial receptor toll-like receptor 4 to limit microglial activation (ClinicalTrials.gov, NCT02502162).<sup>28</sup> Therefore, there remains an unmet need to study central nervous system autoinflammation in CRPS and to translate basic science observations into treatments for evaluation in clinical trials. In this study, we aimed to address this in a translational manner using the safe, well-tolerated disease-modifying antirheumatic drug, hydroxychloroquine (HCQ), in 7 patients with chronic refractory CRPS at the Stanford Pain Management Center and in a rodent model of CRPS to elucidate a mechanism of action. Ultimately, our reverse translational study aims to broaden the armamentarium of treatments for CRPS and identify the mechanism of HCQ in CRPS.

## 2. Materials and methods

### 2.1. Patients

Beginning in 2016, patients with severe, chronic CRPS for whom immune modulators (eg, thalidomide and intravenous immunoglobulin) were considered, but deemed high risk, were offered off-label HCQ at the Stanford Pain Management Center (I.R.C. and V.L.T.). Data extraction from patient charts was approved by the Stanford University Institutional Review Board as a chart review with the requirement for written informed consent waived. To understand HCQ's effects on CRPS, we identified patients with a CRPS type I/II diagnosis who met the Budapest criteria for CRPS<sup>29</sup> and were prescribed HCQ by I.R.C. and V.L.T. between March 1, 2016, and April 1, 2019. We included all patients who received HCQ during this time frame. Extracted information was deidentified and collected in an IRB-compliant database. The following data were collected: demographics, age at CRPS onset, duration of CRPS symptoms, and duration of HCQ therapy. We reviewed patient charts for descriptors of clinical change after HCQ initiation, the numerical rating scale (NRS) score at the visit before HCQ initiation, and at the first visit after HCQ initiation. The NRS score was unavailable for patient 2 and was not collected for patient 1 because she discontinued HCQ after <3 weeks.

As a safety measure, we followed dosing guidelines for systemic lupus erythematosus (SLE), given HCQ's extensive, FDA-approved use in this disease.<sup>33</sup> In most cases, we prescribed a loading dose of 400 mg per day (200 mg b.i.d.) for 8 weeks followed by 200 mg daily. Two patients used a loading dose of 600 to 800 mg daily (300–400 mg b.i.d.) for 8 weeks and decreased to 200 mg daily per ophthalmologist recommendation. Patients began HCQ after plateauing on their existing medication regimen, which continued unchanged during HCQ treatment. All patients established care with an ophthalmologist before beginning HCQ and were asked to follow-up annually to monitor for retinopathy.

### 2.2. Animals

All procedures were approved by the Stanford University Administrative Panel on Laboratory Animal Care in accordance with the American Veterinary Medical Association guidelines and the International Association for the Study of Pain. Mice (C57BL/6J, Jax #00664, 10–11 weeks) were housed 2 to 5 per cage and maintained on a 12-hour light/dark cycle in a temperature-controlled environment with ad libitum access to

food and water. Female mice were used because of the predominance of CRPS in female patients<sup>20</sup> and reported sex differences in pain mechanisms in animal models.<sup>43</sup> Mice were randomized to the following groups: (1) CRPS-vehicle (n = 5), (2) 10 mg/kg HCQ (n = 10), or (3) 50 mg/kg HCQ (n = 10). The same mice undergoing behavioral testing were used for immunohistochemistry (IHC) and enzyme immunoassay (CRPS-veh [n = 4–6], CRPS-HCQ-10 [n = 4–6], and CRPS-HCQ-50 [n = 4–6]). An additional n = 5 age-matched uninjured C57BL/6J mice were used as controls for IHC.

### 2.3. Drug administration

Hydroxychloroquine sulfate (Sandoz Pharmaceuticals, Inc., Princeton, NJ) was dissolved in 0.1 M phosphate-buffered saline (PBS) and injected intraperitoneally in unanesthetized mice using a 25 G needle. Mice received 0.9% normal saline as a vehicle, 10 mg/kg HCQ, or 50 mg/kg HCQ. We used a human equivalent dose calculation<sup>37</sup> to convert the human dose of <5 mg/kg HCQ<sup>36</sup> to a mouse dose of 50 mg/kg. Ten mg/kg HCQ was selected as a low-dose alternative to assess dose-dependent effects of HCQ. Vehicle and HCQ treatments were administered daily for 7 days beginning 24 hours after cast removal to treat mice with active CRPS and mimic the typical clinical scenario (treatment, not prevention).

### 2.4. Tibial fracture complex regional pain syndrome model

Mice were anesthetized with isoflurane and underwent a closed right distal tibial fracture followed by casting.<sup>46</sup> The right hind limb was wrapped in gauze, and a hemostat was used to make a closed fracture of the distal tibia. The limb was wrapped in a casting tape (Scotchcast Plus) from the metatarsals to a spica around the abdomen. The cast was applied over the plantar paw surface with a window over the dorsum of the paw and ankle to prevent constriction with postfracture edema. After 3 weeks, mice were briefly anesthetized with isoflurane to remove casts.

### 2.5. Behavioral testing

In vivo behavioral testing was performed blinded between 7:00 AM and 1:00 PM in an isolated, temperature-, and light-controlled room. Mice acclimated for 30 to 60 minutes in the testing environment within clear plastic cylinders (4" D) on a metal mesh platform (24" H). Mice were randomized by cage before testing and placed randomly in a cylinder; after testing, identification was recorded. An uninjured control group was not included in behavior testing because each mouse was used as its own control. For allodynia and unweighting, preinjury behavior was collected as a control against postinjury/treatment behavior. For edema and temperature, the uninjured paw was a control against the injured paw at postinjury/treatment time points. In all behavioral studies, we followed established methods for evaluating mouse behavior in the tibial fracture model of CRPS.<sup>26</sup>

### 2.6. Mechanical nociception assays

To evaluate mechanical hypersensitivity, we applied von Frey filaments (Stoelting) ranging in gram force from 0.007 to 6.0 g. These were applied perpendicular to the plantar hind paw with sufficient force to cause slight bending of the filament. A positive response was characterized as rapid paw withdrawal within 4 seconds. We used the validated up-down method<sup>12</sup> to calculate

50% withdrawal mechanical thresholds. Longitudinal mechanical nociception testing was performed in the same cohort before fracture as a preinjury baseline, 1 day after cast removal ("pretreatment"), and 24 hours after the final dose of HCQ ("posttreatment").

### 2.7. Paw edema, unweighting, and temperature measurements

Hind paw edema was determined by measuring the hind paw dorsal-ventral thickness over the midpoint of the third metatarsal using calipers (VWR, Inc., Radnor, PA) in isoflurane-anesthetized mice. Data were analyzed as the difference between the fracture and contralateral thickness and averaged across groups.

Hind paw unweighting was measured using an incapacitance device (IITC, Inc., Woodland Hills, CA). Mice were held vertically over the apparatus with hind paws resting on separate scales, supporting the full body weight. Each measurement lasted 6 seconds, with 6 measurements obtained and averaged per mouse. A percent weight bearing was established using the formula:  $2 \times (\text{right paw weight bearing}) / (\text{right paw weight bearing} + \text{left paw weight bearing})$ .

Hind paw temperature was measured using a fine gauge thermocouple (Bioseb Lab Instruments). Temperature was measured over the hind paw dorsal skin between the first and second metatarsals (medial), the second and third metatarsals (central), and the fourth and fifth metatarsals (lateral). Measurements were averaged for the mean paw temperature. Data were expressed as the average difference between the ipsilateral and contralateral hind paw within an experimental group.

### 2.8. Immunohistochemistry

Mice were transcardially perfused with 10% formalin in PBS. The spinal cord (L3–L5 segments because the injury was to the lower extremity) was dissected, cryoprotected in 30% sucrose in PBS, and frozen in O.C.T. (Sakura Finetek, Inc., Torrance, CA). Spinal cord sections (40  $\mu\text{m}$ ) were prepared by cryostat (Leica Biosystems, Buffalo Grove, IL), incubated in blocking solution (5% normal donkey serum and 0.3% Triton X-100 in PBS) for 1 hour at room temperature, and incubated with the primary antibody (rat anti-CD11b; AbD Serotec #MCA711G, 1:500) at 4°C overnight. After wash with 1% normal donkey serum and 0.3% Triton X-100 in PBS, sections were incubated with the secondary antibody conjugated to AlexaFluor for 2 hours at room temperature. Sections were mounted on slides using the Fluoromount aqueous mounting medium with DAPI (#00-4959-52, Thermo Fisher Scientific, Waltham, MA). The entire ipsilateral dorsal horn was imaged with a BZ-X810 fluorescent microscope (Keyence, Itasca, IL).

### 2.9. Quantitative histological analysis

We imaged 3 to 4 dorsal horns per mouse with 4 to 6 mice per group to quantify CD11b expression in the spinal cord 24 hours after the final HCQ dose. Each image was taken with identical exposure with 0.4- $\mu\text{m}$ -step z-stacks of 19 slices at 20x objective magnification on the Keyence BZ-X810. CD11b expression was quantified using ImageJ/FIJI<sup>42</sup> by outlining the dorsal horn and setting the threshold (identical for all images) to minimize background and expressed as percent CD11b+ staining over the dorsal horn. Representative images were selected for figure presentation.

### 2.10. Protein extraction and cytokine quantification

Mice were perfused with 0.1 M PBS, and the spinal cord and paw skin were dissected 24 hours after the final HCQ dose. Tissue was minced in PBS with the protease inhibitor (Roche Applied Science, Basel, Switzerland) and homogenized using a Polytron device (Brinkmann Instruments, Fisher Scientific, Waltham, MA). Homogenates were centrifuged at 14,000 rpm for 15 minutes at 4°C, and supernatants were frozen at  $-80^{\circ}\text{C}$  until processing by enzyme-linked immunosorbent assay. An aliquot was subjected to protein assay (Bio-Rad Laboratories Inc) to normalize protein input levels. IL-1 $\beta$ , TNF- $\alpha$ , and IL-6 levels were determined by enzyme-linked immunosorbent assay kits (Invitrogen, Carlsbad, CA) using spectrophotometric absorbance of standards and samples at 450 nm (Bio-Rad Laboratories Inc., Hercules, CA). Results were plotted against the standard curve linear range, and protein concentration of each sample was expressed as pg cytokine/mg total protein.

### 2.11. Study design and statistical analyses

Numerical rating scale scores were analyzed using the paired *t* test with each patient's pre-HCQ NRS paired to their post-HCQ NRS. Cohort sizes for preclinical studies were determined by a power analysis, requiring a minimum of 5 animals per group to provide >80% power to discover 25% differences with  $P < 0.05$  between groups for all behavioral outcomes. Groups were unblinded after each experiment for statistical analysis. Data are reported in the Results as mean  $\pm$  SD and in figures as the mean + SEM with individual data points. Data were analyzed using ordinary one-way analysis of variance followed by the Tukey multiple comparisons test, or two-way analysis of variance with the Tukey post hoc test, as indicated in the main text or figure captions. No data were excluded.

## 3. Results

### 3.1. Hydroxychloroquine decreases pain in patients with refractory complex regional pain syndrome

Given HCQ's off-label status for CRPS and lack of previous studies, we initiated HCQ only in patients with chronic CRPS refractory to conventional medications and procedural treatments. Through retrospective chart review, we identified 7 female patients in whom we initiated HCQ. Each patient had plateaued on a medication regimen that was continued unchanged during

**Table 1**

**Clinical characteristics of patients prescribed HCQ for refractory CRPS.**

Patient number	Current age	Gender	Age at symptom onset (y)	Symptom duration (y)	CRPS type	HCQ duration
1	21	F	14	7	2	1 mo
2	40	F	35	4	1	7 mo
3	42	F	39	2	1	8 mo
4	62	F	55	6	2	9 mo
5	47	F	21	25	1	1.5 y
6	37	F	31	5	2	3 y
7	25	F	17	8	2	3 y
Avg (SD)	39 (13)	N/A	29 (14)	8 (8)	N/A	17 (14) mo

Avg, average; CRPS, complex regional pain syndrome; F, female; HCQ, hydroxychloroquine.

HCQ treatment. Based on SLE dosing guidelines, we started most patients on HCQ 200 mg b.i.d. for 8 weeks and then decreased to a maintenance dose of 200 mg daily. In 2 cases (patients 6 and 7), based on symptom severity, patients started at 400 mg b.i.d. for 8 weeks and then decreased to 200 mg daily.<sup>24</sup> Patients had an average CRPS duration of  $8 \pm 8$  years, an average age at CRPS onset of  $29 \pm 14$  years, and an average HCQ duration of  $17 \pm 14$  months at the time of chart review (Table 1). Two patients discontinued HCQ therapy: Patient 1 cited nausea after 3 weeks and patient 4 secondary to lack of benefit after 9 months. Five patients (71%) noted improvement in CRPS symptoms with HCQ. We extracted average NRS scores, and NRS during flares/exacerbations, for patients before and after HCQ initiation. The average NRS score before HCQ was  $6.8 \pm 1.1$  and decreased to  $3.8 \pm 1.9$  after HCQ treatment (Table 2 and Fig. 1A, \* $P < 0.05$  using the paired  $t$  test). The NRS score during exacerbations was  $8.6 \pm 1.1$  and decreased to  $6.0 \pm 2.3$  after HCQ treatment (Table 2 and Fig. 1B, \* $P < 0.05$  using the paired  $t$  test). We detail 1 case below to highlight the clinical course after initiating HCQ.

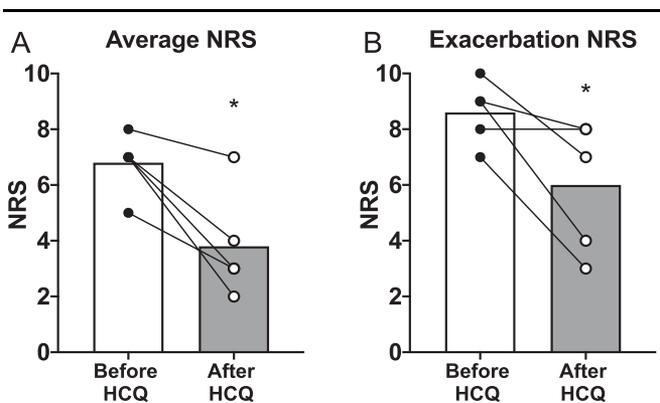
**3.2. Patient 7**

Patient 7 is a 25-year-old woman with an 8-year history of bilateral CRPS-II after lower extremity cyst excision. She presented with allodynia, edema, erythema, and warmth along the medial, dorsal, and lateral right foot extending midway up her right lower extremity. She was diagnosed with CRPS, which spread to the left lower extremity within 6 months. In addition to physical therapy, she trialed anticonvulsants (pregabalin 300 mg daily; gabapentin 3600 mg daily in divided doses; levetiracetam 1000 mg daily in divided doses; and lamotrigine 100 mg daily), serotonin-norepinephrine reuptake inhibitors (duloxetine 120 mg daily), tricyclic antidepressants (desipramine 150 mg daily and nortriptyline 125 mg daily), steroids, low-dose naltrexone (9 mg nightly), ketamine (5-day infusion of 40 mg/hour; intranasal spray up to 45 mg), adrenergic agonists (clonidine transdermal patch 0.2 mg per day; oral 0.1 mg daily), and opioids. Most were discontinued because of poor efficacy or side effects. She pursued unconventional approaches including pentoxifylline, tadalafil, and beta blockers without benefit. She was prescribed HCQ (400 mg b.i.d. for 8 weeks, then 200 mg daily) during a prolonged pain flare (4 years after CRPS onset). Decreases in swelling and trophic changes were documented within 2 months of HCQ initiation (Fig. 2); however, after 8 months, she questioned lasting benefit and discontinued HCQ. Swelling and trophic changes returned to their pre-HCQ severity. She restarted HCQ (200 mg b.i.d. for 8 weeks followed by 200 mg daily)

**Table 2**  
Average numerical rating scale (NRS) and exacerbation NRS reported by patients before and after initiation of HCQ therapy.

Patient number*	NRS pre-HCQ	NRS post-HCQ	Flare NRS pre-HCQ	Flare NRS post-HCQ
3	5	3	7	3
4	7	2	9	4
5	8	7	9	8
6	7	3	10	7
7	7	4	8	8
Avg (SD)	6.8 (1.1)	3.8 (1.9)	8.6 (1.1)	6.0 (2.3)

\* Note that patient 1 and patient 2 did not have a relevant NRS score recorded in chart and therefore were not included.  
HCQ, hydroxychloroquine; pre-HCQ, score noted at the clinic visit before starting HCQ; post-HCQ, score noted at the next clinic visit once HCQ was initiated.

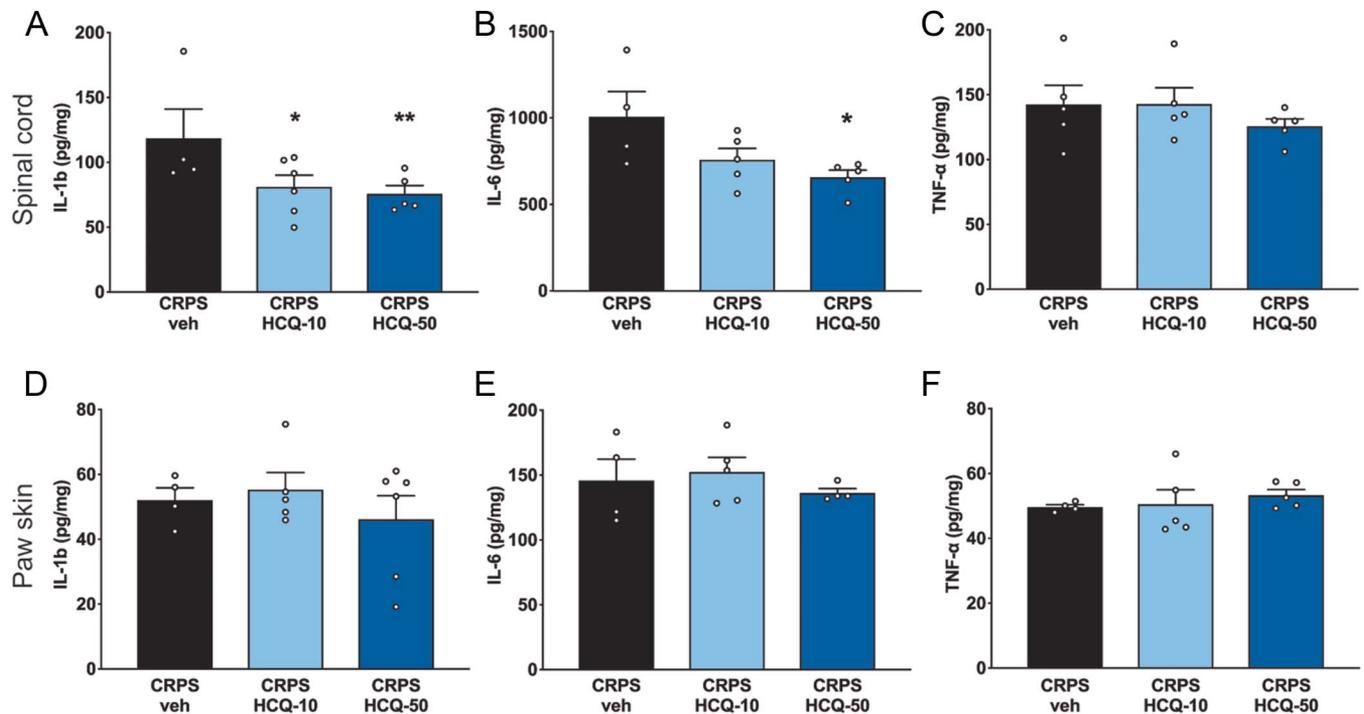


**Figure 1.** HCQ improves average and maximum pain scores in patients with chronic refractory CRPS. (A) There was a significant improvement in the average NRS score after HCQ treatment was initiated;  $n = 5$ , \* $P < 0.05$  using the paired  $t$  test. (B) The NRS score during CRPS pain flares/exacerbations was significantly improved after HCQ treatment;  $n = 5$ , \* $P < 0.05$  using the paired  $t$  test. All data points are shown, and lines connect each individual patients' "before" and "after" NRS scores. CRPS, complex regional pain syndrome; HCQ, hydroxychloroquine; NRS, numerical rating scale.

with subsequent documented improvements in pain, swelling, and trophic changes. She reports continued analgesia with HCQ and



**Figure 2.** HCQ attenuates CRPS signs in patients with chronic refractory CRPS. Patient 7 experienced significant edema and trophic changes for approximately 4 years before initiating HCQ. These cardinal features of CRPS diminished within 2 months of beginning HCQ. CRPS, complex regional pain syndrome; HCQ, hydroxychloroquine.



**Figure 3.** HCQ abrogates cardinal CRPS signs in a mouse model of CRPS. (A) At the time of cast removal, all mice exhibit a significantly decreased mechanical threshold compared with their baseline threshold ( $****P < 0.0001$  by two-way ANOVA, Tukey post hoc test). After 7 days of treatment, CRPS-HCQ-10 and CRPS-HCQ-50 mice have a significantly higher mechanical threshold than vehicle-treated mice ( $####P < 0.0001$  by two-way ANOVA, Tukey post hoc test), and CRPS-HCQ-50 mice have a significantly higher mechanical threshold than CRPS-HCQ-10 mice ( $††P = 0.004$  by two-way ANOVA, Tukey post hoc test). (B) At the posttreatment time point, CRPS-HCQ-10 and CRPS-HCQ-50 mice have a significantly higher % of baseline (uninjured) mechanical threshold than CRPS-veh mice, and this is dose dependent ( $****P < 0.0001$  vs CRPS-veh;  $##P = 0.003$  CRPS-HCQ-10 vs CRPS-HCQ-50, by one-way ANOVA, Tukey post hoc test). (C and D) CRPS-HCQ-50 mice have significantly less CRPS-like temperature (C;  $*P = 0.04$  by one-way ANOVA, Tukey post hoc test) and edema changes (D;  $*P = 0.015$  by one-way ANOVA, Tukey post hoc test) than do CRPS-veh mice, whereas HCQ has no effect on weight bearing (E). ANOVA, analysis of variance; CRPS, complex regional pain syndrome; HCQ, hydroxychloroquine; veh, vehicle.

near-complete resolution of skin changes, and is enrolled in the medical school.

### 3.3. Mouse tibial fracture and casting produces the signs of complex regional pain syndrome

To study the mechanism of HCQ's efficacy in CRPS, we used the tibial fracture model of CRPS.<sup>8</sup> We performed a tibial fracture model followed by 3 weeks of immobilization, at which time casts were removed and behavioral testing was performed. This was the "pretreatment" time point. As expected, we observed the signs of CRPS—mechanical sensitivity, increased paw temperature and thickness, and decreased weight bearing on the injured limb (Fig. 3).

### 3.4. Hydroxychloroquine treatment attenuates the signs of complex regional pain syndrome in a dose-dependent manner

We sought to determine whether treatment with HCQ mitigates signs of CRPS in our rodent model. After pretreatment behavioral testing, we initiated 7 days of vehicle/HCQ treatment. We repeated behavior testing 24 hours after the final vehicle/HCQ dose to assess CRPS-like changes. This was the "posttreatment" time point. Although CRPS-veh mice remained severely allodynic with a mechanical threshold of  $0.13 \pm 0.08$  g, HCQ-treated groups were less allodynic, with a mechanical threshold of  $0.79 \pm$

$0.23$  g ( $P < 0.0001$ ) in the CRPS-HCQ-10 group and  $1.16 \pm 0.23$  g ( $P < 0.0001$ ) in the CRPS-HCQ-50 group (Fig. 3A). In addition, the difference in the mechanical threshold between CRPS-HCQ-10 mice and CRPS-HCQ-50 mice was significant ( $P = 0.004$ ), suggesting dose-dependent antiallodynic effects of HCQ. To quantify the extent to which HCQ supports recovery from allodynia in CRPS, we calculated a % baseline mechanical threshold value for each mouse at the posttreatment time point.



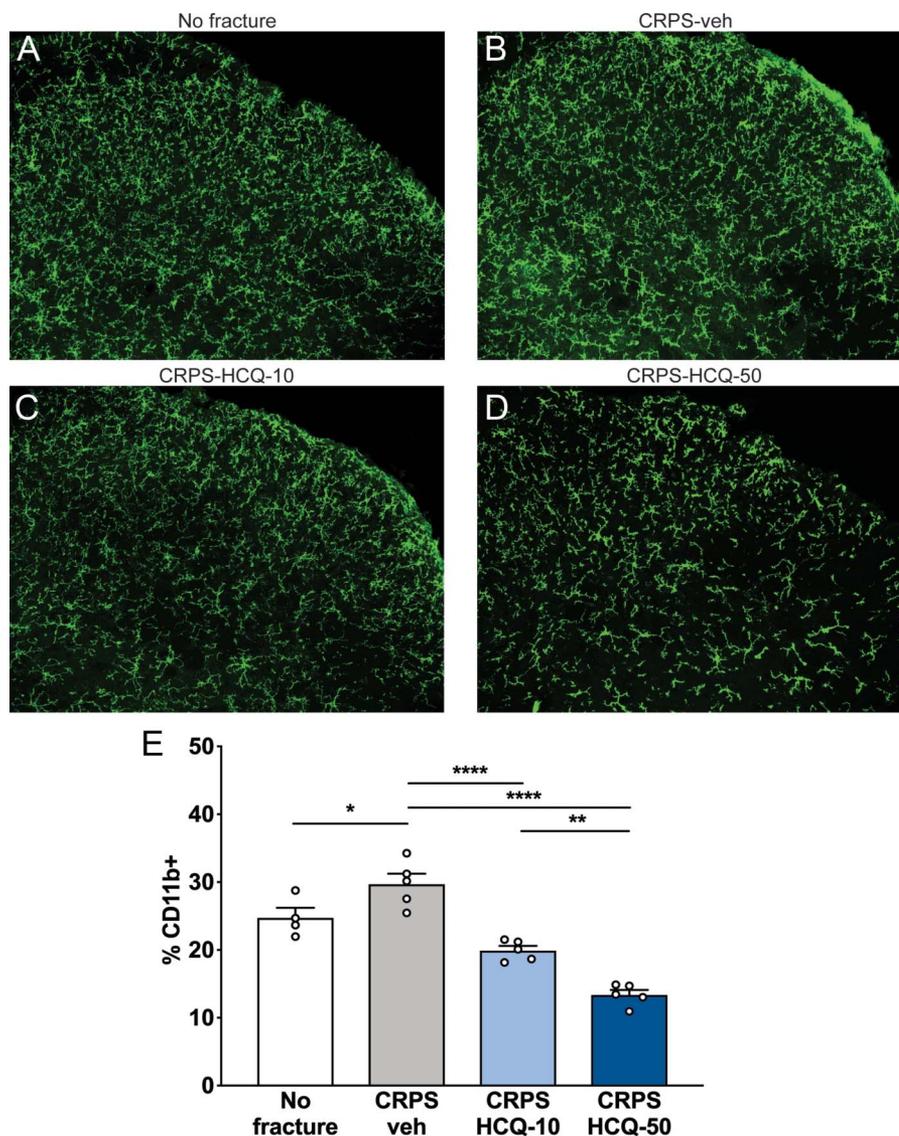
**Figure 4.** HCQ attenuates CRPS signs in mice in the tibial fracture model of CRPS. Gross reductions in edema and erythema are evident for CRPS-HCQ-50 mice between the pretreatment and posttreatment times. CRPS, complex regional pain syndrome; HCQ, hydroxychloroquine.

CRPS-veh mice dropped to  $8 \pm 5\%$  of their baseline mechanical threshold, whereas the CRPS-HCQ-10 and CRPS-HCQ-50 groups decreased to  $48 \pm 12\%$  and  $70 \pm 18\%$  of their baseline mechanical thresholds ( $P < 0.0001$  for CRPS-veh vs CRPS-HCQ-10 and CRPS-HCQ-50) (Fig. 3B), respectively, confirming that HCQ has dose-dependent antiallodynic effects.

Using pretreatment measurements as a baseline, we assessed the benefit of HCQ on paw temperature and edema. At the posttreatment time point, CRPS-veh mice had an  $18 \pm 17\%$  reduction in interpaw temperature difference compared with pretreatment, whereas the CRPS-HCQ-10 group had a  $35 \pm 21\%$  reduction in interpaw temperature difference, and the CRPS-HCQ-50 group had a  $46 \pm 19\%$  reduction in interpaw temperature difference compared with pretreatment (Fig. 3C). Between treatment groups, the % pretreatment temperature difference was statistically significant between CRPS-veh mice

and the CRPS-HCQ-50 group ( $P = 0.040$ ), supporting dose-dependent effects of HCQ on CRPS signs. In addition, vehicle-treated mice had a  $12 \pm 31\%$  reduction in edema compared with pretreatment, whereas the CRPS-HCQ-10 and CRPS-HCQ-50 groups had a  $33 \pm 34\%$  and  $53 \pm 21\%$  reduction in edema, respectively (Fig. 3D). The reduction in edema was significantly different between the CRPS-veh group and the CRPS-HCQ-50 group ( $P = 0.015$ ).

Finally, we assessed the effects of HCQ on weight bearing on the injured hind paw. Mice in the CRPS-veh group had a  $5 \pm 8\%$  increase in weight bearing, whereas CRPS-HCQ-10 and CRPS-HCQ-50 mice had a  $6 \pm 6\%$  and  $9 \pm 6\%$  increase in weight bearing compared with pretreatment (Fig. 3E). These results demonstrate a treatment-independent weight-bearing improvement by week 4 after fracture but no statistically significant difference between treatment groups, suggesting that at the doses



**Figure 5.** HCQ decreases microglial activation in CRPS. Images of (A) uninjured, (B) CRPS-veh, (C) CRPS-HCQ-10, and (D) CRPS-HCQ-50 spinal cord dorsal horns at the posttreatment time point. (E) Quantification of the %CD11b+ area reveals a significant increase in the %CD11b+ area after injury ( $*P = 0.038$ , no fracture vs CRPS-veh). CRPS-HCQ-10 and CRPS-HCQ-50 spinal cords exhibited significantly less %CD11b+ area than do CRPS-veh mice ( $****P < 0.0001$  vs CRPS-veh), and this effect is dose dependent ( $**P = 0.004$ , CRPS-HCQ-10 vs CRPS-HCQ-50). All statistics by one-way ANOVA with the Tukey post hoc test. Representative images are shown from  $n = 5$  uninjured,  $n = 5$  CRPS-veh,  $n = 5$  CRPS-HCQ-10, and  $n = 5$  CRPS-HCQ-50 mice at the posttreatment time point, with 3 to 5 spinal cord sections examined per mouse. ANOVA, analysis of variance; CRPS, complex regional pain syndrome; HCQ, hydroxychloroquine; veh, vehicle.

and duration used in this experiment, HCQ did not have discernible impacts on weight bearing. Images of the injured limb at the pretreatment and posttreatment time points demonstrate reduced edema and erythema in the CRPS-HCQ-50 group (Fig. 4).

### 3.5. Hydroxychloroquine abrogates microglial activation with dose dependence in complex regional pain syndrome

Based on studies suggesting that HCQ acts on peripheral and central myeloid lineage cells,<sup>31</sup> we used IHC to assess spinal cord microglial activation (based on the marker CD11b) (Figs. 5A–D). Semiquantitative analysis of the %CD11b+ pixel area in the ipsilateral spinal cord dorsal horn revealed an increase in staining from  $25 \pm 3\%$  CD11b+ in uninjured control mice to  $30 \pm 3\%$  CD11b+ in CRPS-veh mice ( $P = 0.038$ ) (Fig. 5E). By contrast, both HCQ-treated groups demonstrated significantly less % CD11b staining density after HCQ treatment ( $20 \pm 1\%$  CD11b+ in CRPS-HCQ-10 and  $13 \pm 2\%$  CD11b+ in CRPS-HCQ-50;  $P < 0.0001$ ). Moreover, CD11b staining was significantly different between the CRPS-HCQ-10 and CRPS-HCQ-50 groups ( $P = 0.004$ ), suggesting dose-dependent effects.

### 3.6. Hydroxychloroquine decreases central but not peripheral cytokine secretion in complex regional pain syndrome

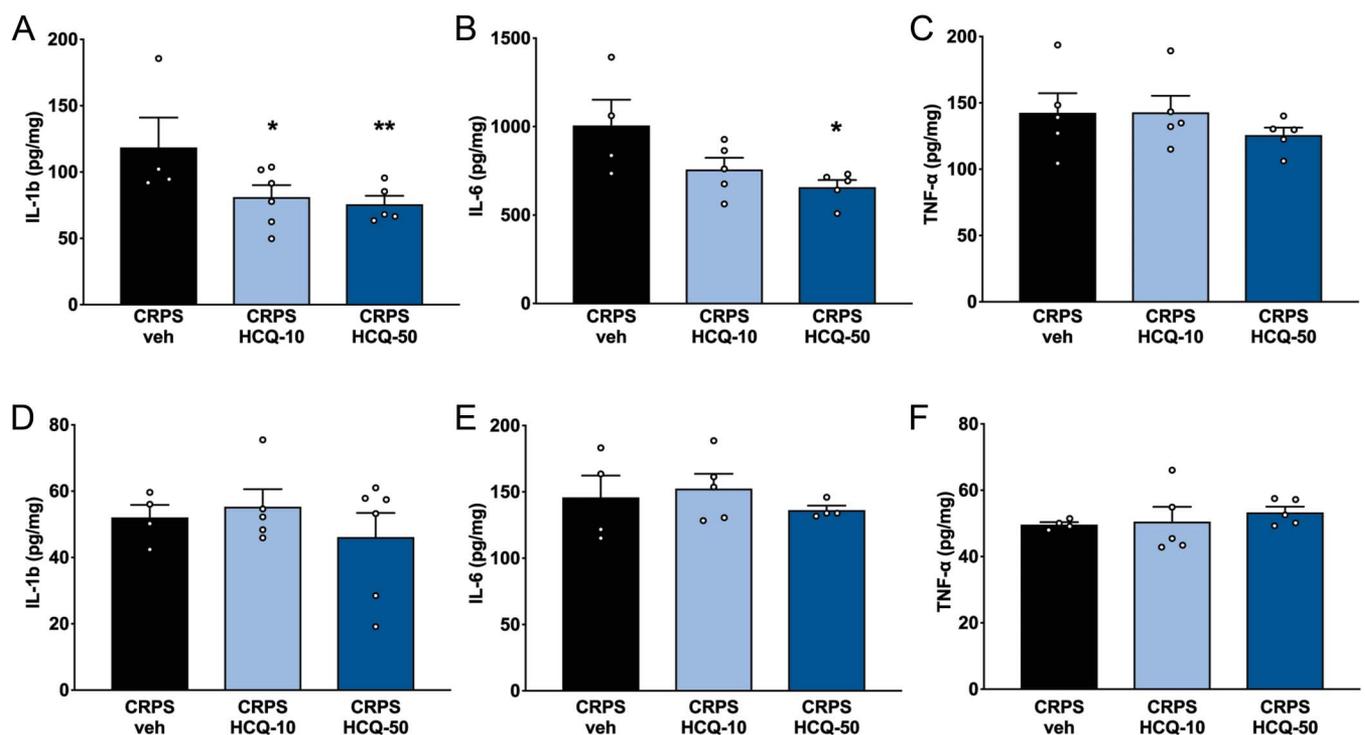
Because of the decreased CD11b IHC staining, we next sought to evaluate whether HCQ decreases proinflammatory cytokines associated with microglial activation centrally and macrophage stimulation peripherally. We quantified IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in the spinal cord and paw skin at the posttreatment time point. We

observed a significant, dose-independent  $\sim 40\%$  reduction in spinal IL-1 $\beta$  among mice in both HCQ-treated groups, compared with CRPS-veh mice (Fig. 6A;  $P = 0.015$  and  $P = 0.004$  for CRPS-HCQ-10 and CRPS-HCQ-50, respectively). We also observed a 35% reduction in spinal IL-6 in the CRPS-HCQ-50 group ( $P = 0.040$ ), with no reduction in the CRPS-HCQ-10 group (Fig. 6B), suggesting dose-dependent effects. We did not observe any effect of HCQ on TNF- $\alpha$  levels (Fig. 6C). Interestingly, there were no discernible peripheral changes in cytokines after HCQ administration (Figs. 6D–F), suggesting HCQ primarily acts centrally in the CRPS model.

## 4. Discussion

Here, we report the results of a retrospective cohort study of off-label use of HCQ in the treatment of 7 patients with CRPS. Our positive preliminary experience using HCQ in the clinic motivated the reporting of our findings and investigations into the mechanism of HCQ in CRPS using a clinically relevant rodent model of CRPS.

Autoinflammation and microglial activation are not new concepts in the study of persistent pain.<sup>28</sup> Preclinical studies note microglial activation in multiple pain models,<sup>17,47</sup> which promotes central sensitization,<sup>19,30</sup> a process linked to pain states like CRPS.<sup>7</sup> To date, clinical studies using microglia-targeted agents such as minocycline and propentofylline have had limited success.<sup>13,18</sup> These agents may have failed because they are nonspecific or because microglial differences exist between mice and humans. Hydroxychloroquine, however, has a significant history of clinical use for immune/inflammatory conditions such as SLE and rheumatoid arthritis. Therefore, treating a primary pain condition such as CRPS, with



**Figure 6.** HCQ modulates central spinal cord but not peripheral paw skin cytokine levels in CRPS. (A) A significant dose-independent reduction in IL-1 $\beta$  secretion in the lumbar spinal cord occurs with HCQ treatment ( $*P = 0.015$  and  $**P = 0.004$  vs CRPS-veh by one-way ANOVA with the Tukey post hoc test). (B) CRPS-HCQ-50 decreases IL-6 secretion compared with CRPS-veh mice ( $*P = 0.04$  by one-way ANOVA with the Tukey post hoc test). (C) HCQ treatment has no effect on central TNF- $\alpha$  secretion. Quantification of (D) IL-1 $\beta$ , (E) IL-6, and (F) TNF- $\alpha$  in the dorsal paw skin at the posttreatment time point reveals no changes in cytokine secretion in HCQ-treated vs vehicle-treated mice. ANOVA, analysis of variance; CRPS, complex regional pain syndrome; HCQ, hydroxychloroquine; veh, vehicle.

autoimmune and autoinflammatory components, seems less of a mechanistic stretch, particularly given research investigating exosomal miRNA signatures in patients with CRPS.<sup>40</sup> Further elucidation of HCQ's mechanism might yield the exciting opportunity for selecting patients for HCQ therapy based on inflammatory signatures. Moreover, based on our preclinical findings of attenuated microglial activation after HCQ, we posit that HCQ functions in CRPS at least in part through microglial modulation, suggesting subtype-independent autoinflammation in CRPS.

It is interesting that we did not observe peripheral changes in cytokines after HCQ administration. This is important because each patient receiving HCQ had chronic CRPS, more associated with a cold, centrally mediated subtype<sup>11</sup> more likely to benefit from a medication with central activity,<sup>45</sup> a property HCQ has been proven to possess through the measurement of HCQ concentrations in brain biopsy specimens.<sup>10</sup> A lack of peripheral cytokine changes may also explain why unweighting, a measure of spontaneous pain, was not affected by HCQ treatment. That HCQ acts centrally in our CRPS model supports its use in patients with chronic CRPS. Although CRPS can be universally difficult to treat, it is commonly more difficult in chronic cases. Therefore, our study provides evidence supporting further investigation of HCQ in the CRPS population most lacking in treatment options.

As mentioned, treating inflammatory conditions with HCQ has a long history of success. Koch et al.<sup>31</sup> demonstrated potential for HCQ use in multiple sclerosis when they administered HCQ to lipopolysaccharide-stimulated human microglia and observed reduced proinflammatory cytokine secretion, delayed symptom onset, and reduced Iba1 staining in a mouse model of experimental autoimmune encephalitis. This provides perhaps the most direct support for our current findings, showing attenuation of autoinflammation by HCQ. In addition, in patients with SLE, those taking HCQ for under 1 year are at greater risk for flares than those taking it for more than 1 year.<sup>2</sup> In patients with RA, HCQ has been shown to be significantly better than placebo and, importantly, with limited toxicity when patients are maintained at doses <5 mg/kg to protect against retinopathy, the primary concern for most HCQ prescribers.<sup>36,44</sup> Since experimental use of HCQ began in patients with COVID-19 in early 2020, the risk of cardiac dysrhythmias with HCQ treatment has been reported.<sup>9</sup> Before this resurgence in interest, however, there were only scattered case reports of HCQ-induced QTc prolongation in patients with rheumatic conditions treated with HCQ.<sup>39</sup> QTc prolongation in critically ill patients with COVID-19 is therefore likely attributable to other factors, including heart failure, polypharmacy, electrolyte abnormalities, and cardiac ischemia.<sup>9</sup> We anticipate that with continued COVID-19-related interest in HCQ,<sup>25,34</sup> we will better understand potential toxicities and ways to optimize administration of HCQ, which may inform future randomized controlled trial protocols for studying HCQ for CRPS.

Limitations of the case series include the retrospective nature, small sample size, and uncontrolled nature of the patient cohort. Nonspecific treatment effects that might have contributed to the apparent benefit are legion but bear repeating: (1) secular trends, (2) regression to the mean, and (3) placebo effect. We acknowledge that CD11b also labels infiltrating macrophages in the spinal cord; however, we have evidence using the microglia-specific marker Tmem119<sup>5</sup> that 90% to 99% of CD11b+ cells in the spinal cord are also Tmem119+ after tibial fracture (unpublished). We therefore ascribe increased CD11b+ immunoreactivity to microglia.

In sum, we present for the first time a potential benefit of HCQ for patients with chronic, refractory CRPS and preclinical evidence that HCQ functions in CRPS at least in part through microglial modulation. The safety, tolerability, and low cost of

HCQ make its continued study in further feasibility studies, followed by well-designed, prospective, blinded, controlled trials imperative.

## Disclosures

The authors have no conflicts of interest to declare.

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E.S. Haight performed behavioral studies. E.S. Haight performed histology. E.S. Haight performed immunoassays. E.M. Johnson and V.L. Tawfik performed patient chart review. E.S. Haight, E.M. Johnson, and V.L. Tawfik wrote and edited the manuscript. I.R. Carroll and V.L. Tawfik provided clinical care. E.S. Haight, I.R. Carroll, and V.L. Tawfik conceived the project, and V.L. Tawfik supervised all experiments and clinical analysis.

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