

Cover Page



Universiteit Leiden



The handle <http://hdl.handle.net/1887/25983> holds various files of this Leiden University dissertation.

Author: Swartjes, Maarten

Title: Neuropathic pain and its treatment with ARA 290 and ketamine : overlapping pathways

Issue Date: 2014-06-12

Chapter 8

ARA 290 improves symptoms in patients with sarcoidosis-associated small nerve fiber loss and increases corneal nerve fiber density

Albert Dahan, Ann Dunne, Maarten Swartjes, Paolo Proto,
Lara Heij, Oscar Vogels, Monique van Velzen, Elise Sarton, Marieke Niesters,
Martijn Tannemaat, Anthony Cerami, Michael Brines

Molecular Medicine 2013; 19: 334-345



Introduction

Sarcoidosis is an immune-mediated, inflammatory orphan disease of unclear etiology that can affect virtually any organ of the body¹. In most individuals diagnosed with sarcoidosis the disease is mild with pulmonary and hilar lymph node involvement that resolves within several years whether treated by immune suppression or not. However, in about one third of patients, sarcoidosis evolves into a chronic, progressive disease². In these refractory cases, therapy has generally consisted of immune suppression which has been associated with a variable response rate³.

Recently, it has become apparent that a significant proportion of patients with chronic sarcoidosis report symptoms that suggest abnormal function of the small nerve fibers of the sensory and autonomic nervous systems⁴⁻⁸. Clinical evaluation by skin biopsy, typically of the distal leg, has shown that many of these patients have a demonstrable reduction in intraepidermal nerve fibers^{6,9}. The affected nerves consist of unmyelinated C and lightly myelinated A δ fibers that comprise the sensory and autonomic peripheral nervous systems. Patients having reduced nerve fiber densities typically complain of pain, numbness, and/or dysesthesia, as well as autonomic symptoms that can be extremely variable depending on the organ affected⁵. The neuropathic symptoms in these patients are frequently severe and therefore are major contributors to the poor quality of life of those afflicted¹⁰.

The etiology of the loss of small nerve fibers in sarcoidosis has not been definitively identified, but one prevalent hypothesis is that nerve fiber dropout is the end result of systemic and/or local inflammation¹¹. Neuropathy arising from inflammation can affect both the peripheral nerve endings and the neuronal somata within the dorsal root ganglia of the spinal cord. At the present time, glucocorticoids and other immune suppressants are the principal therapeutic approach to small fiber neuropathy but are often ineffective⁸. In addition to immune modulators as potential disease modifiers, treatment is generally symptomatic, consisting of the analgesics, anti-epileptics, and antidepressants used for other painful neuropathies¹². Thus, there is a clear need for new therapeutics in sarcoidosis-associated small fiber neuropathy.

ARA 290 is an eleven amino acid peptide derived from the structure of erythropoietin (EPO) that possesses potent tissue protective and tissue repair activities¹³. The actions of ARA 290 are mediated through a receptor consisting of a complex formed by the EPO receptor and beta common receptor subunits¹⁴, termed the innate repair receptor (IRR). In preclinical models of neuropathic pain, ARA 290 has demonstrated beneficial effects that include IRR-dependent prevention of the development of allodynia in a peripheral nerve transection model¹⁵ or in an inflammatory neuritis model¹⁶, as well as attenuation of spinal cord inflammation. Also, EPO, and its non-erythropoietic derivatives, e.g., ARA 290, have been shown to support the regrowth

of intra-epidermal nerve fibers in preclinical models of neuropathy arising from toxins¹⁷ or diabetes¹⁸.

An initial open label study of the effects of three intravenous doses of ARA 290 administered over one week on neuropathic pain of patients with sarcoidosis or diabetes showed a 50% improvement without any safety concerns¹⁹. The results of a follow up trial of ARA 290²⁰ administered intravenously three times weekly for 4 weeks to sarcoidosis patients with symptoms of small fiber neuropathy also appeared to be safe and was associated with a significant improvement in the patient reported outcomes of the small fiber neuropathy screening list (SFNSL²¹) and the pain and well-being components of the RAND-36.

Based on these observations, we have conducted the present study to assess the effects of ARA 290 on neuropathic symptoms when given as a daily subcutaneous injection for 28 days. Because of the association of nerve fiber loss with neuropathic symptoms and the potential for ARA 290 to cause nerve fiber regrowth, we hypothesized that ARA 290 administration will improve symptoms and stimulate the regrowth of small nerve fibers. To evaluate this, the nerve fiber densities in the cornea, proximal thigh, and distal leg were assessed. Additionally, cutaneous sensory testing of the face, hand, and foot were determined using quantitative sensory testing, and quality of life assessed with appropriate patient questionnaires. Finally, functional capacity, which is often reduced in chronic sarcoidosis²², was assessed using the 6 Minute Walk Test.

Methods

Rationale for dose selection

Results of a previous study performed in sarcoidosis patients with painful small fiber neuropathy showed that 2 mg of ARA 290 administered intravenously (IV) three times weekly improved neuropathic symptoms. In the current trial we sought to assess the potential of subcutaneous (SC) dosing, as the IV is not practicable in the outpatient setting. Therefore, a crossover pharmacokinetic study was performed using 10 normal volunteers to compare a 2 mg IV dose that was used in the previous study, to 2, 4, or 6 mg of ARA 290 administered SC¹⁹. Results of preclinical and in vitro studies have shown that activation of the IRR requires concentrations of ARA 290 ≥ 1 nM (~ 1.3 ng/mL)¹⁴. Therefore, the area under the curve (AUC) of the pharmacokinetic data was calculated using the trapezoidal rule for the period of time in which the plasma concentrations were > 1.3 ng/mL. The results of this crossover study showed the following median AUCs: 2 mg IV = 65 ng/mL*min, 2 mg SC = 23 ng/mL*min, 4 mg SC = 59 ng/mL*min and 6 mg = 249 ng/mL*min, with only the 6 mg

dose differing significantly from the others ($P < 0.05$; Kruskal-Wallis test). Based on these data, the 4 mg SC group was selected for the daily dosing regimen of this trial.

Study design.

The trial, entitled "Effects of ARA 290 on the regrowth of epidermal nerve fibers in patients with sarcoidosis", was an investigator-initiated, single site, double blind, placebo-controlled trial carried out at the Leiden University Medical Center after receiving Ethics Committee approval. The trial was registered with the International Clinical Trials Registry (NTR3575) and was assigned EudraCT number 2012-001492-37. All study personnel and patients remained blinded as to the treatments until the end of the follow up period (16 weeks from the beginning of dosing).

The primary outcomes were: 1) change in epidermal or corneal nerve fiber density at day 28 versus baseline; 2) change in cutaneous sensitivity of day 28 versus baseline using Quantitative Sensory Testing; and 3) change in visual acuity or retinal edema, at day 28 versus baseline. Secondary outcomes assessed were: 1) change in the Small Fiber Neuropathy Screening List score at day 35 versus baseline; 2) change in Brief Pain Inventory at day 35 versus baseline; and 3) change in distance walked in the 6 Minute Walk Test at day 28 versus baseline.

Patients who satisfied the international consensus statement for diagnosis of sarcoidosis²³ and had symptoms suggestive of neuropathy were recruited after referral by sarcoidosis specialists. The Consolidated Standards of Reporting Trials (CONSORT) flow chart corresponding to this trial is illustrated in Figure 1. After obtaining informed consent, a total of 38 patients (18 females, 20 males) of mean age 49.5 years (range 28-65) satisfying inclusion criteria were enrolled. These patients had a mean duration since sarcoidosis diagnosis of 8.4 years. The baseline characteristics of these patients with respect to the treatment groups are summarized in Table 1. Although all patients were diagnosed as having sarcoidosis, 2 patients also type 2 diabetes mellitus, a condition known to also be associated with SNFLD¹².

Study inclusion criteria required meeting three thresholds: 1) spontaneous pain level ("pain now" of the Brief Pain Inventory) > 5 (scale 0-10); 2) small fiber neuropathy screening list score (SFNSL) > 22 (out of 84 possible), or pain < 5 and SFNSL > 37 ; and 3) pain defined as distal extremity pain plus one of the following: dysesthesia, burning/painful feet worsening at night, or intolerance of sheets/clothes touching the legs or feet. Additional inclusion criteria were: age between 18 to 65 years (inclusive), a body mass index (BMI) between 18 and 30 kg/m² (inclusive), and the ability to read and understand the written consent form, complete study-related procedures, and communicate with the study staff. Exclusion criteria were: abnormal blood pressure, history of alcoholism or illicit drug use, positive pregnancy test, refusal to use acceptable contraception throughout the study period (unless surgically sterilized

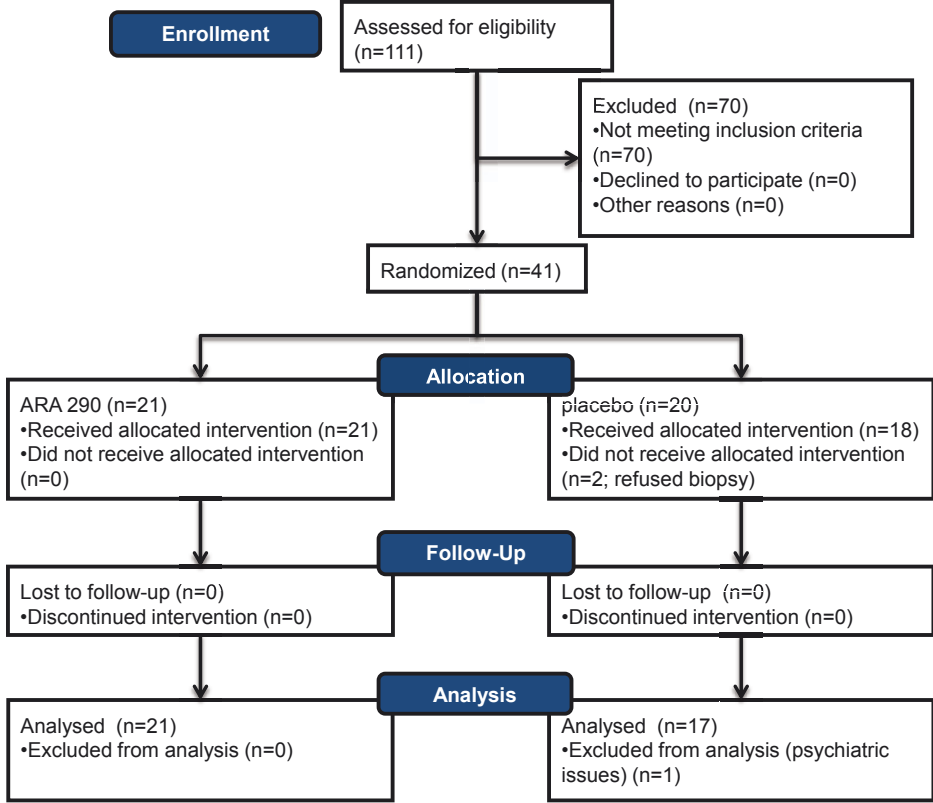


Figure 1: CONSORT flow diagram.

or post-menopausal), vaccination or surgery within the prior 3 months, or use of anti-TNF therapy in the prior 6 months.

Safety was assessed by questioning the patient weekly during ARA 290 administration and throughout the 12 week follow up for the occurrence of adverse events. Additionally, the patients were examined at three occasions during the active treatment phase of the study: baseline, 2 weeks, and 4 weeks at the end of dosing. Additionally, blood was drawn for routine hematology and chemistry at these times points. Finally, serum was obtained for determination of possible anti-ARA 290 antibodies.

Patient Questionnaires

Questionnaires were administered at the screening visit and then weekly during the dosing and follow-up period of 3 months (total 16 weeks). Questionnaire data were also obtained approximately 6 months following the end of the follow up period (i.e., 9 months from end of dosing) to assess durability of any effects. The Brief Pain Inventory Short Form, consisting of pain intensity and pain interference sections, was

Table 1. Baseline Patient Characteristics.

	ARA 290 (n=21)	Placebo (n=17)
Years since diagnosis of sarcoidosis (mean $\pm$ SEM)	7.1 $\pm$ 1.2	9.9 $\pm$ 2.4
Concomitant medical treatment n (%)		
NSAIDS	5 (23.8)	8 (47.1)
Neurological/psychological drugs	5 (23.8)	6 (35.3)
Oral corticosteroids	6 (28.6)	7 (41.2)
Opioids	6 (28.6)	2 (11.8)
Systemic immune suppressants (methotrexate or azathioprine)	7 (33.3)	3 (17.7)
Prior TNF- α antagonist treatment (n=yes)	2 (9.5)	0
SFNSL		
Total score	43.9 $\pm$ 2.9	42.8 $\pm$ 3.2
Autonomic component	20.6 $\pm$ 2.0	20.8 $\pm$ 1.5
Pain component	23.3 $\pm$ 1.2	22.9 $\pm$ 1.2
BPI		
Mean score (Pain now; range 0-10)	5.0 $\pm$ 0.4	5.3 $\pm$ 0.5
Pain interference (Maximum 70)	32.1 $\pm$ 1.9	36.5 $\pm$ 2.9
6 Minute Walk		
Test actual (meters)	468 $\pm$ 18	479 $\pm$ 26
Test predicted (meters) ^a	700 $\pm$ 12	683 $\pm$ 15
Nerve Fiber Density		
Corneal nerve fiber area (μm^2)	1576 $\pm$ 94	1304 $\pm$ 104
Normal corneal nerve fiber area ^b	3134 $\pm$ 119	
Ankle IENFD (number/mm)	5.3 $\pm$ 0.5	4.6 $\pm$ 0.4
Normal sex and age adjusted ankle IENFD ^c	9.9 $\pm$ 0.3	9.8 $\pm$ 0.3
Proximal thigh IENFD (number/mm)	10.8 $\pm$ 0.7	11.1 $\pm$ 0.9
Normal proximal thigh IENFD ^d	21.1 $\pm$ 0.2	21.0 $\pm$ 0.1
Laboratory Markers		
High sensitivity C-reactive protein (mg/L)	1.5 $\pm$ 0.2	2.9 $\pm$ 1.1
Angiotensin converting enzyme ^e	47.4 $\pm$ 6.1	53.6 $\pm$ 8.0
Number with elevated ACE n (%)	5 (23.8)	6 (35.3)

^aPredicted 6 minute walk test was calculated using formula from ref³².

^bdata calculated from³⁰.

^cNormal sex-age adjusted ankle IENFD is from ref²⁶.

^dNormal proximal thigh IENFD is from ref²⁷.

^e(normal: 23-67 nmol/min/mL)

administered in the validated Dutch language format. The Small Fiber Neuropathy Screen List (SFNSL) is a questionnaire developed specifically in Dutch patients with sarcoidosis to assess pain and autonomic dysfunction consistent with small nerve fiber loss and damage²¹. In addition to the total score, the questionnaire was divided

into an autonomic component (questions 2-5, 9, 11-16) and a pain component (questions 1, 6-8, 17-21) to assess those dimensions of the patients' neuropathic symptoms.

Quantitative Sensory Testing

Small nerve fiber and large fiber cutaneous sensory function was assessed using Quantitative Sensory Testing of the face, hand, and foot using a Medoc Advanced Medical Systems TSA-II device (Ramat Yishai, Israel), following the published protocol of the German Research Network on Neuropathic Pain²⁴. Normative data was

Table 2: Results of baseline quantitative sensory testing. Patients in the ARA 290 and placebo groups showed functional impairment of both small nerve fibers (A δ and C) as well as larger sensory nerve fibers (A β). Data are expressed as number of patients deviating beyond the 95% confidence interval of a sex- and age-matched normal population. Test sites of face, hand, and foot are pooled for calculation of percentages. "Decrease" indicates a loss of function; "Increase" indicates a gain in function compared to a normal population. For example, a decreased CDT means that a patient required a lower temperature stimulus than normal to determine that an object was cold, i.e., a decrease in sensitivity.

Variable	Nerve fibers involved	ARA 290 (n=21)		Placebo (n=17)	
		Change	Number of patients (%)	Change	Number of patients (%)
Cold detection threshold	A δ & C	Decrease	19 (91)	Decrease	11 (65)
Warm detection threshold	A δ & C	Decrease Increase	17 (81) 1 (5)	Decrease	13 (77)
Thermal sensory limen	A δ & C	Decrease Increase	4 (19) 2 (10)	Decrease	4 (24)
Paradoxical heat sensation	A δ	Decrease	8 (38)	Decrease	7 (41)
Cold pain threshold	A δ & C	Increase	3 (14)	-	0
Heat pain threshold	C	Decrease Increase	3 (14) 5 (24)	Decrease	1 (6)
Mechanical detection threshold	A β	Decrease	11 (52)	Decrease	10 (59)
Mechanical pain threshold	A β	Decrease Increase	11 (52) 4 (19)	Decrease Increase	4 (24) 2 (12)
Mechanical pain sensitivity	A β + C	Decrease Increase	2 (10) 5 (24)	Decrease	1 (6)
Dynamic mechanical allodynia	A β	Increase	11 (52)	Increase	3 (18)
Windup ratio	A β & C	Increase	4 (19)	Increase	2 (12)
Vibration detection threshold	A β	Decrease	20 (95)	Decrease	15 (88)
Pressure pain threshold	A δ & C	Decrease Increase	3 (14) 10 (48)	Decrease Increase	1 (6) 7 (41)

obtained from Rolke et al.²⁴. Baseline data for each patient group is summarized in Table 2. To arrive at the percentages shown in this table, the three regions tested were pooled, i.e., each patient's data was considered abnormal if the results were more than 2 standard deviations from the normative population mean in at least one of the locations evaluated.

Skin Biopsy

Skin biopsies were obtained at baseline and after 28 days from the proximal thigh (20 cm below the anterior superior iliac spine) and the distal leg (10 cm above the lateral malleolus) using a disposable punch biopsy (3 mm) and processed following established guidelines²⁵. After fixation of the biopsy specimens, free floating 50 µm thick sections were cut and stained using rabbit anti-protein gene product 9.5 antibody (Dako Netherlands BV, Netherlands) and visualized using a goat anti-rabbit Alexa fluor 488 antibody (Invitrogen, Life Technologies, Grand Island, NY). A minimum of 3 sections selected from each end and the middle of each biopsy specimen were evaluated using a Leica M5500 fluorescence microscope (Leica Microsystems, Rijswijk, The Netherlands), at magnification 1000x. The nerve fibers were counted manually. Images of the sections were recorded using Leica Application Suite, magnification 400x and the length of the epidermal-dermal junction measured using ImageJ (NIH, Bethesda, MD, USA). Sex and age dependent normative data of nerve fiber density used for the distal leg were those of Lauria et al.²⁶ and for the thigh from Umapathi et al.²⁷. All measurements and counting was performed by the same individual who was blinded to treatment modality. Technical problems during tissue preparation resulted in the loss of 2 placebo biopsies of the lower leg, one ARA 290 biopsy of the thigh, and three placebo biopsies of the thigh.

Corneal Confocal Microscopy

Corneal nerve fiber density was determined by corneal confocal microscopy carried out using the Rostock Cornea Module with the Heidelberg Retina Tomograph III using established methodology²⁸. Briefly, following the application of a topical anesthetic, the sterile objective of the confocal microscope was placed on the apex of the cornea as determined by the characteristic orientation of the nerve fibers in a superior-inferior direction. Using the automatic scan feature of the device, confocal images of graduated depth in the plane of the cornea were acquired. The field of view of each image was 0.4 mm by 0.4 mm. Images containing sensory nerve fibers within the sub-basal layer between Bowman's layer and the basal epithelium were further analyzed. Collected images were subjected to automated analysis employing a custom macro written for FIJI, a public-domain image analysis program, version 1.47e²⁹. This macro maps all neurites in the image on the basis of their brightness

and tubeness. The area covered by the mapping is then expressed as a percentage of total image area. For each patient, the ten images with the highest nerve fiber density were averaged to generate a representative sample for that patient for that eye. Since the variation between eyes of different patients was similar to the variation between eyes of individual patients (standard deviation of the mean neurite area between patients=562; standard deviation of the difference between eyes of individual patients=501), each eye was treated as an independent sample. The automated analysis was validated by comparison of 78 randomly selected images in which total neurite length in each image was determined by manually outlining individual neurites. Linear regression analysis showed an excellent goodness of fit (95% confidence interval of the slope: 0.99 – 1.19; $R^2=0.76$; $P<0.0001$) between the computer-generated nerve fiber area and the manually measured total nerve fiber length for each image. Both the automated analyses and the manual measurements were performed by a researcher blinded to the treatment modality. The Shapiro-Wilk test showed that at baseline the corneal nerve fiber area data were not distributed normally, therefore non-parametric statistical analysis was performed to determine if a significant treatment effect was observed.

Normative data were calculated from corneal confocal data previously reported³⁰ obtained from 22 healthy volunteers (M/F: 9/13) age 49 ± 2.7 by determining the mathematical relationship between corneal nerve fiber area and corneal nerve fiber length. The results showed that a normal corneal nerve fiber area is $3134 \pm 119 \mu\text{m}^2$.

6 Minute Walk Test

The 6 Minute Walk Test (6 MWT), the distance in meters walked in 6 minutes, was conducted following American Thoracic Society guideline³¹. Normal 6 MWT values were calculated using the regression equation developed from data obtained from a healthy, older normal Dutch population by Troosters et al.³²

Ophthalmologic tests

To assess for possible retinal edema, optical coherence tomography was carried out to quantitate retinal thickness using the Zeiss CIRRUS1 system that includes normative values.

Visual acuity was carried out under standard uniform lighting conditions for patients wearing corrective lenses, if any, using a SLOAN ETDRS chart and scoring system.

Statistical analysis

Statistical analysis was performed using JMP (SAS, Inc, Cary, NC). Parametric and non-parametric tests, linear modeling, and analysis of covariance were carried out where appropriate. P-values <0.05 (two tailed) were considered to be significant.

Results

Safety

No medically significant deviations were noted in the general blood chemistry or hematology assessments. There was no pain or local irritation surrounding the site of the injection into the upper leg or lower abdomen. No serious adverse events were encountered during the dosing period or within the 12 weeks of follow up. Three adverse events judged to be moderate were noted in the placebo group that resolved spontaneously (diarrhea, irritability, and light-headedness). One patient receiving ARA 290 suffered a moderate adverse event consisting of a long term weight loss of 14 kg over several months that stabilized thereafter. Verification of the patient's medical history showed that the weight loss began before entering the study. The etiology of the weight loss was undetermined and persisted after administration of ARA 290 ceased. Multiple, mild adverse events were recorded, all of which spontaneously resolved and none were judged by the investigators as likely to be associated with administration of the study drug. All doses of ARA 290 were administered daily for the full 28 period. One placebo patient suffering from diarrhea discontinued dosing for the last week of the study. No anti-ARA 290 antibodies were detected in any of the post-exposure serum samples.

Primary Endpoints

Nerve Fiber Density

Corneal nerve fibers

The baseline corneal nerve fiber area showed that the patient population exhibited about a 50% reduction compared to normal controls (Figure 2; Table 1). Following 28 days of dosing, the ARA 290 group exhibited a significant increase in the median nerve fiber area over baseline of 14.5%, corresponding to an absolute median increase of $185 \mu\text{m}^2$ ($P=0.022$; Wilcoxon signed rank test). In contrast, the placebo group had a non-significant decrease in median nerve fiber area over baseline of -5.3% and an absolute median decrease of $64 \mu\text{m}^2$ ($P=0.462$). Figure 2C illustrates the corneal nerve density of two normal individuals compared to two ARA 290 patients who showed the best responses.

Intra-epidermal nerve fibers

Similar to the corneal nerve fiber area, at baseline the mean intra-epidermal nerve fiber densities of the proximal and distal leg were significantly reduced by approximately 50% in both treatment groups, compared to the median of age- and sex-matched normal controls ($P<0.0001$; Table 1). The mean ratio of IENFD of the proximal thigh to the distal leg was 3.9 ± 1.5 SEM, with no patient having a ra-

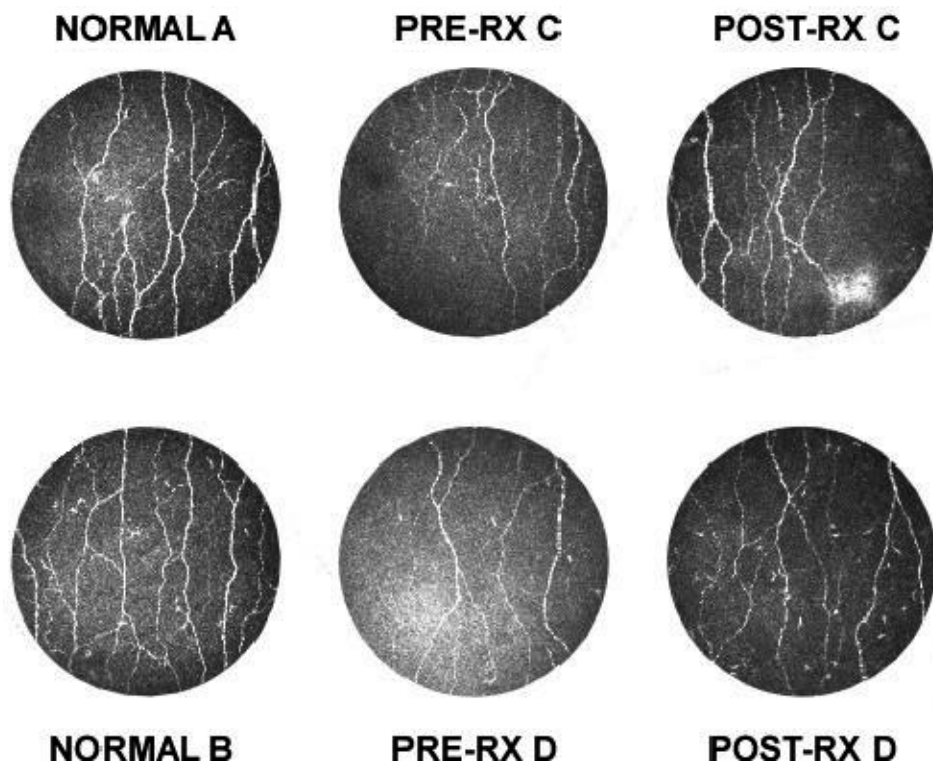


Figure 2: ARA 290 administration is associated with an increase in corneal nerve fiber area. Examples of the distribution and density of corneal nerve fibers obtained via corneal confocal microscopy performed on two normal individuals A and B (Left panels). Examples of corneal nerve density obtained from two sarcoidosis patients show a decreased density at baseline (Middle panel: Pre-RX) and an increase when reimaged after 28 days of ARA 290 administration (Right panel: Post-RX).

tio < 0.9. The patients in this study, therefore, suffered from a peripheral neuropathy characterized by a length-dependent loss of epidermal nerve fibers. IENFD of the proximal leg was not significantly correlated to that of the distal leg (Pearson's correlation coefficient = 0.20; $P = 0.22$).

Following 28 days of dosing, the ARA 290 group exhibited a mean increase in IENFD in the distal leg of 0.38 ± 0.48 fibers/mm (7.2% of baseline; $P = \text{ns}$), compared to the placebo group with a mean reduction of nerve fiber density of 0.06 ± 0.42 fibers/mm (1.3% of baseline; $P = \text{ns}$). The thigh IENFD at 28 days showed a mean decrease of 0.49 ± 0.53 fibers/mm for the ARA 290 group (-2.3% of baseline; $P = \text{ns}$) and the placebo group had a mean decrease of 1.24 ± 0.88 fibers/mm (-5.7% of baseline; $P = \text{ns}$).

Cutaneous Sensitivity

Baseline QST data showed that as a group the patients with sarcoidosis and painful neuropathy exhibited findings consistent with both small fiber ($A\delta$ and C) and large fiber ($A\beta$) dysfunction (Table 2). Most patients exhibited a reduced ability to determine cold (CDT; 79% of the study group) or warm temperatures (WDT; 79%), and to detect vibratory stimuli (VDT; 92%). Fifty five percent of the patients also experienced a reduced ability to detect graded mechanical stimuli elicited by von Frey fibers (MDT) or pain caused by graded pin prick (MPT) or to pressure (PPT). A minority of patients in each treatment group also exhibited abnormalities in a variety of the other sensory modalities tested as summarized in Table 2.

Following 28 days of daily dosing, the cold pain threshold (CPT), hot pain threshold (HPT), and the thermal sensory limen (TSL) significantly increased in the ARA 290 group, as illustrated in Figure 3 which summarizes data obtained from the hand testing location. In contrast, there were no changes noted in the placebo group.

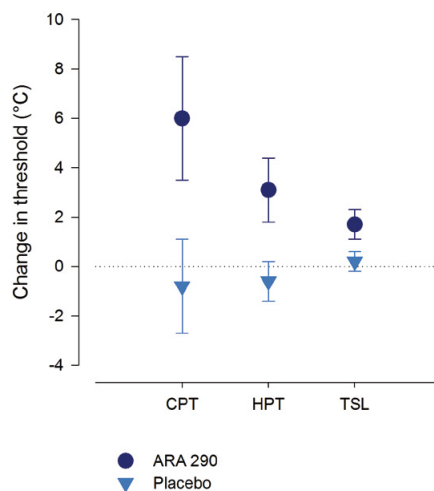


Figure 3: ARA 290 administration increases the threshold for thermal pain and decreases thermal sensitivity in the hand. The cold pain threshold (CPT), heat pain threshold (HPT), and thermal sensitivity limen (TSL) of most patients were within normal limits at baseline (Table 1). Following ARA 290 administration, the mean threshold for determining a painful cold ($P=0.027$; paired t test compared to baseline) or hot ($P=0.032$) stimulus increased, whereas the placebo group remained unchanged ($P=ns$). Similarly, the thermal sensory limen (the temperature threshold at which they can discriminate a hot or cold stimulus) increased in the ARA 290 post exposure ($P=0.008$). This decreased thermal sensitivity could correspond to reduced symptoms of temperature-induced allodynia. Post ARA 290 treatment, the CPT, HPT, and TSL remained within the normal range. The normative means (in $^{\circ}C$) for CPT, HPT, and TSL were 9.7 ± 0.5 , 44.8 ± 0.2 , and 3.0 ± 0.1 respectively. Similar smaller changes were noted for the face, as well as a non-significant trend for the foot (data not shown).

Although a decreased sensitivity was noted for these sensory modalities, the population means at baseline and after ARA 290 dosing remained within the normal range. Similar, but smaller changes were noted in the face test location, while a non-significant trend was noted at the foot testing site (data not shown). Additionally, the mean cold detection (CDT) and warm detection (WDT) thresholds also decreased (i.e., decreased sensitivity) at the hand and face sites, but the changes were not quite large enough to be statistically significant (data not shown). No changes were observed in any other sensory modality within the QST battery.

Retinal thickness and visual acuity

Baseline average thickness of the macula and central macula, and retinal nerve fibers of both eyes were normal in all patients and did not significantly change over the 28 day observation period (data not shown). Visual acuity at baseline obtained with corrective lenses was normal except for one patient in the ARA 290 group (data not shown). The visual acuity of this patient, and that of all other patients, did not change following ARA 290 exposure.

Secondary Endpoints

Small Fiber Neuropathy Screening List

Baseline scores of the SFNSL developed specifically for sarcoidosis patients showed that the treatment groups were very symptomatic and well-matched with mean baseline values of 43.9 and 42.8 for the ARA 290 and placebo groups respectively (not significantly different; t-test). When evaluated at week 5 (i.e., one week following the end of dosing), the ARA 290 group showed a mean reduction in the SFNSL score of 12.2 ± 1.9 (median of 13.0; ~ 28% reduction from baseline) compared to 3.8 ± 2.1 (median 1.0; ~ 9% reduction from baseline) for placebo (difference between groups: $P=0.005$; t test). Construction of proportional responders curves (Figure 4A) showed that the percentage of patients receiving ARA 290 having symptomatic improvement in the SFNSL score was greater than the placebo group at each response level. For example, 81% of the ARA 290 patients exhibited at least a 2 point improvement in the SFNSL score, compared to only 47% if patients within the placebo group. This response profile was substantially maintained during 12 week follow up period at which time the mean score reduction from baseline for the ARA 290 group was 9.7 ± 1.8 (median 11.0) and for placebo was 4.1 ± 1.9 (median 3.0; difference between groups: $P=0.037$; t-test), in contrast to no significant change for the placebo group. The proportional responder curves at 16 weeks (12 weeks following the end of dosing) were similar to that observed immediately following treatment (Figure 4B). Follow up at 6 months after the study observation period (i.e., 9 months following the termination of dosing) was possible for 19/21 of the ARA 290 patients and all of the

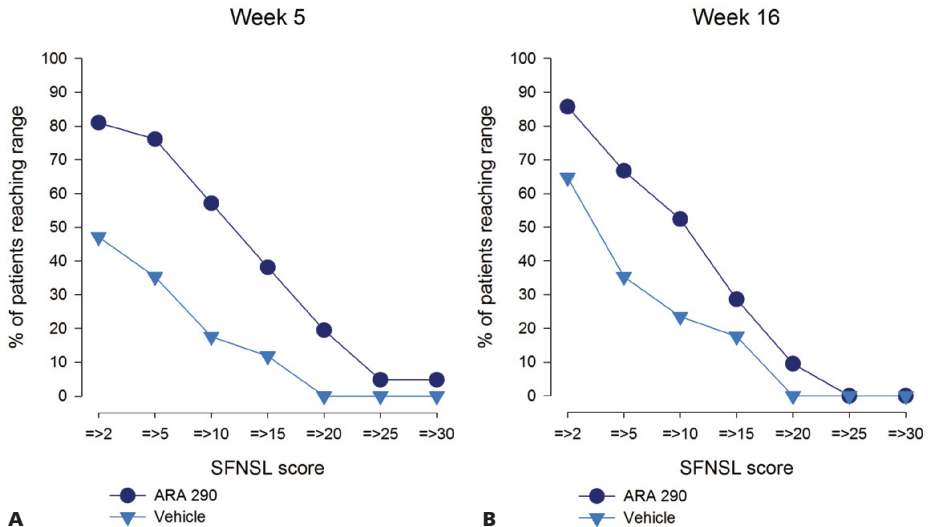


Figure 4: Evaluation of efficacy using the Small Fiber Neuropathy Screening List shows a sustained improvement in the ARA 290 treatment group compared to placebo. A: One week following the end of dosing a larger percentage of patients in the ARA 290 group attained a specified range of score improvement over a broad range of responses. B: This difference was largely maintained at the end of the sixteen weeks of follow-up.

placebo patients and was notable for a mean SFNSL score of 38.1 ± 3.2 SEM versus 43.7 ± 3.2 , respectively. This represented a significant improvement over baseline for the ARA 290 group (5.2 ± 1.9) compared to the placebo group (-0.9 ± 2.0 ; $P=0.036$). With respect to the autonomic component of the SFNSL, the ARA 290 group demonstrated a significant improvement in the autonomic score when compared to the placebo group with mean improvements of 6.0 ± 1.1 and 1.2 ± 1.3 respectively ($P=0.009$; t-test). These correspond to a 29% change from baseline for the ARA 290 group compared to a 6% improvement in the placebo group. A significant difference was observed in the pain component with the ARA 290 group having a mean improvement of 6.2 ± 1.1 points (27% of baseline) compared to the placebo group with a mean improvement of 2.6 ± 1.3 points (12% of baseline; $P=0.032$; t-test).

Brief Pain Inventory

Pain intensity

One week following the last injection (i.e., on day 35), the average Brief Pain Inventory pain intensity score was reduced $\sim 9\%$ from baseline in both treatment groups with a mean decrease of -3.4 (out of a maximum of 40). This represented a significant improvement for both the active and placebo arms with respect to baseline ($P=0.01$; t-test), but with no significant difference between the treatment groups. The individual pain intensity scores were notable for a similar reductions in

“most pain” (–1.2; $P=0.003$), “average pain” (–1.1; $P=0.004$), and “pain now” (–1.0; $P=0.03$), whereas “least pain” did not change from baseline (–0.2; $P=0.54$).

Pain Interference

In contrast to the mean pain intensity scores that were significantly improved in both groups by week 5, the mean change in the BPI pain interference score differed significantly between the treatment groups by the third week of dosing ($P<0.02$; Figure 5A). Specifically, while the baseline values of the two groups were not different, the ARA 290 group dropped from a mean score of 32.1 ± 2.3 at baseline to 20.6 ± 2.7 by the week following dosing (a 36% reduction from baseline). This compares to a change in the placebo group from a baseline of 36.5 ± 2.5 to 30.8 ± 3.1 (a 16% change from baseline). Proportional responder analysis (Figure 5B) illustrates that the ARA 290 group exhibited about a 20% greater proportion of responders across the response spectrum up to an improvement total of 20 points. At the time of evaluation 9 months after dosing, 2 patients in the ARA 290 group were lost to follow up. For the remaining patients, the pain interference score did not significantly differ from the baseline values. Specifically, the ARA 290 group mean was 30.0 ± 2.2 and the placebo group was 33.9 ± 2.9 .

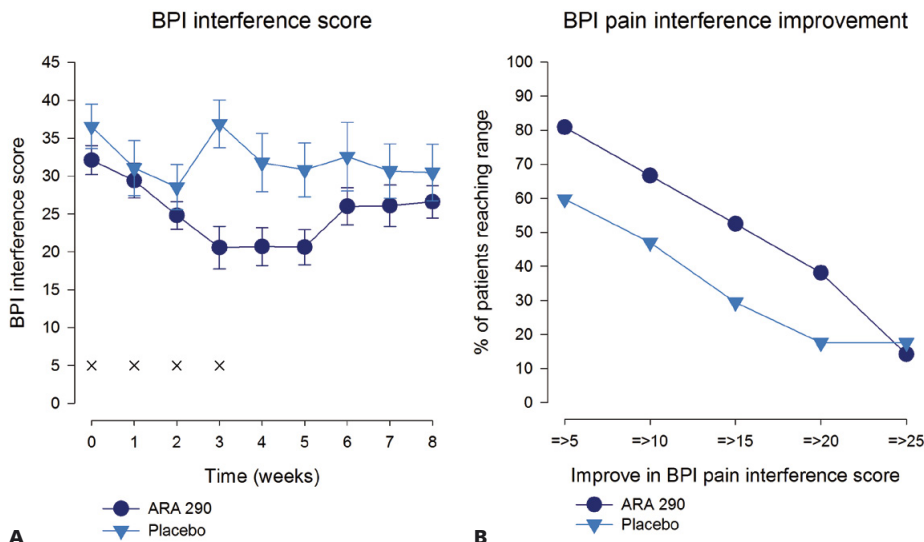


Figure 5: ARA 290 treatment improves the Brief Pain Inventory pain interference score. A: Weekly pain interference scores significantly decline over the 4 weeks of daily dosing for the ARA 290 compared to the placebo group. X=treatment with either ARA 290 or vehicle. B: A proportional responder display illustrates that the ARA 290 group responded to a larger extent at all levels of improvement.

Six minute walk test (6 MWT)

The 6 MWT is a measure of functional exercise capacity. Both groups had approximately the same baseline 6MWT distance (Table 1), that was significantly less than normal. Using a normative prediction formula for a normal population with the same approximate age spread³², the patients in this study at baseline exhibited a mean reduction of 219 meters ($P < 0.0001$; 95% confidence interval of -186 to -253 meters) in the actual distance walked in 6 minutes from a predicted value of 693 meters. Following 28 days of daily dosing, the 6 MWT showed that the ARA 290 group increased the distance walked by a mean of 18.7 meters, whereas the placebo group's performance fell by a mean of -15.1 meters (difference between groups: $P = 0.049$; t test). A proportional responder analysis (Figure 6) illustrates that about half of the patients in both treatment groups had an improved their 6 minute walk distance by up to 12 meters. However, for an increase from greater than 25 meters, only 12% in the placebo group improved, compared to 52% of the ARA 290 group. Substantial percentages of the ARA 290 group exhibited even larger increases in the 6 minute walk distance, whereas none of the placebo patients did. A 6 MWT was repeated at 9 months following dosing (2 ARA 290 patients and 1 placebo patient were lost to follow up). The mean change from baseline in the ARA 290 group was 8.3 ± 13.3 meters and for placebo was -12.9 ± 13.9 , neither of which constituted a significant change from baseline ($P = \text{ns}$; t test).

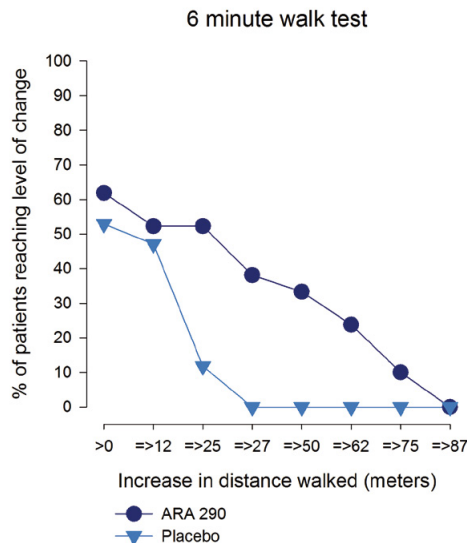


Figure 6: ARA 290 increases the distance patients can walk in 6 minutes. Similar to the results of symptom questionnaires, patients receiving ARA 290 performed better at all levels of response in the 6 Minute Walk Test.

Discussion

Sarcoidosis complicated by small fiber neuropathy is a chronic disease characterized by the loss of small nerve fibers with associated pain, decreased temperature sensitivity, thermal allodynia, and pronounced autonomic dysfunction that severely degrades quality of life. All patients included in this trial had painful neuropathic symptoms consistent with SFN that were unresponsive to the standard therapies for chronic sarcoidosis that they had received and many continued on immune suppression and symptom-directed therapy throughout the trial.

The principal hypothesis to be tested in this study was whether exposure to ARA 290, a molecule demonstrating tissue protective, anti-inflammatory, and reparative activities in numerous preclinical models, would stimulate nerve fiber regrowth with associated improvements in pain and other sensory symptoms, and autonomic function. To accomplish this, the trial was designed to focus on the assessment of objective endpoints such as small nerve fiber quantification using both skin biopsy and corneal confocal microscopy and to relate these findings to semi-objective sensory testing using QST which directly assesses the effects of potential changes in cutaneous innervation. The 6 MWT was also included as a simple semi-objective test that requires the integration of complex sensory stimuli of the lower limbs and good exertion by the patient. Finally, patient reported outcomes were included for subjective assessments of pain and the degree to which pain interfered with activities of daily living, as well as symptoms of autonomic dysfunction which could also be potentially related to changes in nerve fiber density.

Baseline nerve fiber data from this study have been analyzed³⁰ and these show that corneal nerve quantification (density and length) correlates well with the IENFD of the distal, but not the proximal lower limb when adjusted for the covariates of gender and age. Further, at baseline the corneal nerve fiber density (and length) is inversely related to the BPI pain interference score and therefore has relevance for the symptoms that the patients report. Previous work performed in patients with diabetes has also shown a good correspondence between corneal nerve quantification and nerve fiber counts performed in the distal leg³³, thereby confirming the usefulness of corneal nerve assessment in patients with symptoms of small fiber neuropathy.

The results of nerve fiber assessment following 28 days of dosing show that the corneal nerve fiber density improved significantly in the ARA 290 group when compared to the placebo group at the end of 28 days of dosing. In contrast, no change was observed in the IENFD obtained from the proximal thigh, although a trend was observed for the distal leg biopsy site. Notably, a recent study carried out in a diabetic population has reported positive effects of treatment on corneal nerve

fiber density, although over a longer time scale with no change in the skin biopsy nerve density of the distal extremity³³. In this study, patients with type 1 diabetes were followed after curative therapy by pancreas transplantation. Twelve months (but not 6 months) after normalization of blood glucose concentrations, a significant increase in corneal nerve fiber density was documented, whereas no changes were observed in the IENFD of the distal leg or in the results of Quantitative Sensory Testing. Additionally, Boyd et al.³⁴ were able to demonstrate a change in skin biopsy nerve densities following drug administration. These investigators studied type 2 diabetic patients with small fiber neuropathy following 12 weeks of administration of the anti-epileptic drug topiramate and documented an increase in cutaneous nerve fiber length at multiple biopsy sites and in nerve fiber density in the proximal leg. It would be of interest to know what assessment of the corneal nerve fibers would have shown.

Prior study³⁵ of re-innervation following experimental denervation using capsaicin application to the skin of diabetics with neuropathy or normal individuals has shown that the natural rate of regrowth of sensory nerve fibers is slow in normal individuals and very slow in patients with diabetes. In contrast, regrowth of autonomic fibers is appreciably faster (40-50 days to return to baseline density) than sensory fibers (140-160 days for normalization)³⁶. Similar experiments have not been performed on corneal nerve fibers, but the results of a preclinical model shows that rapid regeneration (days to weeks) occurs following mechanical injury³⁷. It is possible that the cornea is an especially useful location to evaluate potential nerve regrowth. Corneal confocal microscopy has the benefit that it is a non-invasive technique that can be repeated many times in the same patient and thus is well-suited for longitudinal interventional studies.

As a group, QST showed that the majority of the patients in this study had significantly increased cold, warm, and vibratory detection thresholds. For patients with sensitivity to cold or heat, this could translate into less pain during activities of daily living. Previous study of patients with diabetic neuropathy has reported similar findings in patients that specifically complained of pain³⁸. Since thermal sensory function depends upon small fiber function, the admission criterion of neuropathic pain may have specifically selected patients that possess a high degree of fiber loss. This possibility was confirmed by the intra-epidermal and corneal nerve fiber assessments that showed a marked reduction in the mean number of small fibers innervating cutaneous and corneal sites compared to a normal population.

It is currently unclear what sensory changes may be associated with the axon regeneration that occurs during the short time frame of this clinical trial, as the results of few relevant studies have been reported. Clinical studies performed using nerve growth factor (NGF) show that a single injection into normal individuals produces

both mechanical and thermal hypersensitivity at the site of injection which is rapid, reaching a maximum by 21 days and 3 days respectively³⁹. Hypersensitivity has been observed at injection site in longer term clinical trials with repeated injections carried out on patients with neuropathy, e.g., diabetic polyneuropathy⁴⁰. As mentioned above, no injection site pain was noted following ARA 290 administration in the current study. The observation of reduced thermal thresholds associated with ARA 290 that occur at several sites examined that were remote to the injection site are suggestive a predominantly central effect in contrast to the peripheral effect previously observed for NGF.

On the basis of preclinical work it also appears that changes in responsiveness may occur within the time frame of the present clinical study. Tanelian and Scott³⁷ studied a rabbit model in which they produced corneal nerve fiber injury by a small punch biopsy and subsequently used electrophysiological methods to directly determine the behavior of regenerating small nerve fibers to cold stimuli. Their findings document electrophysiological changes that returned to normal by 30 days after injury. If similar changes occur in patients during the early period of regrowth, we would expect to observe changes in thermal thresholds to the extent that axon sprouting has occurred. However, no assessments were carried out during the period of dosing that can provide relevant information. Additionally, the questionnaires administered do not provide information that is helpful in determining thermal sensory thresholds. It will be important to add these assessments in future trials. However, it is highly likely that any changes that might occur in the sensory system as a result of effects of 28 days of dosing with ARA 290 would not have reached a steady state.

The results of this study show that ARA 290 administration to patients with painful small fiber neuropathy is associated with a significant improvement in patient-reported symptoms, compared to patients receiving placebo, without any evident adverse events attributable to the drug. The changes in level of discomfort as assessed by the SFNSL following 4 mg ARA 290 administered daily SC was remarkably similar to what was observed in the previous blinded trial in which 2 mg ARA 290 was administered three times weekly by the IV route²⁰. In the prior trial, approximately 80% of the patients in the active arm exhibited some improvement and ~ 40% showed improvement of ~ 50% over baseline. In contrast, while about 45% of the patients in the placebo arm showed some improvement, only ~ 12% showed a 50% improvement. Daily administration of 4 mg ARA 290 administered subcutaneously was well tolerated without any evident adverse effects. Also similar to the previous blinded trial, a large proportion of the change in SFNSL score was attributable to questions that are relevant to autonomic symptoms. Finally, it is remarkable how sustained the response to ARA 290 appears to be. This may reflect the growth of small nerve fibers as the corneal confocal nerve fiber data reveals.

Self-assessment of pain intensity using the BPI showed that similar to the first blinded trial²⁰, both groups improved equally, indicating a significant placebo effect on this dimension. In contrast, assessment of to what extent the level of pain interfered with activities of daily living, mood, and enjoyment of life showed that patients that received ARA 290 had an immediate reduction in mean score reaching a nadir that was significantly different from placebo by the end of the dosing period. This result suggests that ARA 290 is having a complex activity that extends beyond the sensation of pain to include effects on activities of daily living.

The 6 minute walk test was originally developed to assess functional exercise capacity (i.e., a measure of the ability to engage in physically demanding activities) in patients with chronic cardiopulmonary diseases. Since its introduction, the 6MWT has been used to evaluate functional capacity in a wide range of diseases and in healthy normal individuals³² and has been used as a means to assess the effects of therapeutic interventions. Studies evaluating patients with chronic sarcoidosis have observed that about 50% of these patients have a markedly impaired baseline 6MWT^{22,41}. In the current study, we found that all of the patients had a reduction in expected walk distance, some very severe. The reason for the higher prevalence in this patient population is not clear, but could arise from the fact that the patients were selected for the presence of neuropathic symptoms which involved the feet, which could contribute to a poor performance on a walk test due to sensory deficits and pain.

At the end of dosing, the ARA 290 group had improved a mean of ~19 meters while the placebo group had declined by ~15 meters, about a 4% improvement and 3% decrease of baseline respectively. Although only about half of the patients improved in both groups (Figure 6), the improvement in distance walked in the 6MWT was limited in the placebo group to less than 37 meters, whereas almost one quarter of the ARA 290 patients improved the distance walked by up to 75 meters. A minimally clinically significant difference (MCSD) has not been established for sarcoidosis patients with painful neuropathy, but for patients with cardiopulmonary disease, the MCSD has been determined to be as low as 25 meters⁴².

The most prevalent form of SFN occurs in patients with pre-diabetes or diabetes, and in this group retinal edema and visual acuity changes are very common. Additionally, another major clinical manifestation of chronic sarcoidosis is ocular inflammation, especially uveitis that often affects the retina². Alternatively, a recent study has shown that patients with neurosarcoidosis frequently have macular edema even in the absence of ocular symptoms⁴³. It was of interest, therefore, to evaluate retinal thickness and visual acuity pre- and post-dosing. At baseline, there were no significant abnormalities observed in the optical coherence tomographic evaluation of either retinal or optic nerve head thickness. Similarly, almost all patients had good

visual acuity at baseline. Therefore, retinal abnormalities and visual acuity impairment do not appear to be a common feature of sarcoidosis complicated by SFN.

The patients included in this trial all had longstanding sarcoidosis with mean time of diagnosis of 8.3 years. They all had failed existing therapy for neuropathy including the use of anti-inflammatory agents (NSAIDs, glucocorticoids, and methotrexate principally), as well as anti-epileptics and antidepressants. About 30% of the patients were using a variety of these drugs during the conduct of this trial. Due to the small numbers of patient studied it is not possible to evaluate synergistic effects with any of these agents. It will be interesting to assess for this possibility in future trials with ARA 290.

The principal limitations of this study are that only patients with pain were studied and these patients did not have known active sarcoid involvement of any other organ. Circulating markers of inflammation were not significantly elevated and presumptive markers of active sarcoidosis, e.g., angiotensinogen converting enzyme levels, were only mildly increased in a minority of patients. Small nerve fiber loss is also well known to occur without associated painful symptoms, e.g., in the prediabetic state⁴⁴. It will be of interest to determine whether corneal nerve fiber density is also abnormal in this patient group.

In conclusion, ARA 290 is the first drug that exhibits the ability to induce small nerve fiber regeneration in the cornea without serious side effects, showing a potential of true disease modification, not just symptom improvement. In addition, this trial design using the combination of objective and subjective endpoints offers insight into correlations with patient reported outcomes, and may provide a blueprint for superior trial design for future pain studies. Most importantly, the results of this study can provide some hope for sarcoidosis patients suffering from small nerve fiber loss and damage that ARA 290 could substantially improve their quality of life.

References

1. Iannuzzi MC, Rybicki BA, Teirstein AS: Sarcoidosis. *New England Journal of Medicine* 2007; 357: 2153-65
2. Chen ES, Moller DR: Sarcoidosis - Scientific progress and clinical challenges. *Nature Reviews Rheumatology* 2011; 7: 457-67
3. Baughman RP, Nagai S, Balter M, Costabel U, Drent M, du Bois R, Grutters JC, Judson MA, Lambiri I, Lower EE: Defining the clinical outcome status (COS) in sarcoidosis: results of WASOG Task Force. *Sarcoidosis Vasculitis and Diffuse Lung Diseases* 2011; 28: 56-64
4. Bakkers M, Faber CG, Drent M, Hermans MCE, Van Nes SI, Lauria G, De Baets M, Merkies ISJ: Pain and autonomic dysfunction in patients with sarcoidosis and small fibre neuropathy. *Journal of Neurology* 2010; 257: 2086-90
5. Heij L, Dahan A, Hoitsma E: Sarcoidosis and pain caused by small-fiber neuropathy. *Pain Research and Treatment* 2012; 2012:
6. Hoitsma E, Marziniak M, Faber CG, Reulen JPH, Sommer C, De Baets M, Drent M: Small fibre neuropathy in sarcoidosis. *The Lancet* 2002; 359: 2085-6
7. Judson MA: Small fiber neuropathy in sarcoidosis: Something beneath the surface. *Respiratory Medicine* 2011; 105: 1-2
8. Tavee J, Culver D: Sarcoidosis and Small-fiber neuropathy. *Current Pain and Headache Reports* 2011; 15: 201-6
9. Bakkers M, Merkies ISJ, Lauria G, Devigili G, Penza P, Lombardi R, Hermans MCE, Van Nes SI, De Baets M, Faber CG: Intraepidermal nerve fiber density and its application in sarcoidosis. *Neurology* 2009; 73: 1142-8
10. Hoitsma E, De Vries J, van Santen-Hoeufft M, Faber CG, Drent M: Impact of pain in a Dutch sarcoidosis patient population. *Sarcoidosis Vasculitis and Diffuse Lung Diseases* 2003; 20: 33-9
11. Üçeyler N, Kafke W, Riediger N, He L, Necula G, Toyka KV, Sommer C: Elevated proinflammatory cytokine expression in affected skin in small fiber neuropathy. *Neurology* 2010; 74: 1806-13
12. Tavee J, Zhou L: Small fiber neuropathy: A burning problem. *Cleveland Clinic Journal of Medicine* 2009; 76: 297-305
13. Brines M, Patel NSA, Villa P, Brines C, Mennini T, De Paola M, Erbayraktar Z, Erbayraktar S, Sepodes B, Thiernemann C, Ghezzi P, Yamin M, Hand CC, Xie Qw, Coleman T, Cerami A: Nonerythropoietic, tissue-protective peptides derived from the tertiary structure of erythropoietin. *Proceedings of the National Academy of Sciences USA* 2008; 105: 10925-30
14. Brines M, Cerami A: The receptor that tames the innate immune response. *Molecular Medicine* 2012; 18: 486-96
15. Swartjes M, Morariu A, Niesters M, Brines M, Cerami A, Aarts L, Dahan A: ARA290, a peptide derived from the tertiary structure of erythropoietin, produces long-term relief of neuropathic pain. An experimental study in rats and β -common receptor knockout mice. *Anesthesiology* 2011; 115: 1084-92
16. Pulman KG, Smith M, Mengozzi M, Ghezzi P, Dilley A: The erythropoietin-derived peptide ARA290 reverses mechanical allodynia in the neuritis model. *Neuroscience* 2012;

17. Bianchi R, Brines M, Lauria G, Savino C, Gilardini A, Nicolini G, Rodriguez-Menendez V, Oggioni N, Canta A, Penza P: Protective effect of erythropoietin and its carbamylated derivative in experimental Cisplatin peripheral neurotoxicity. *Clinical Cancer Research* 2006; 12: 2607-12
18. Bianchi R, Buyukakilli B, Brines M, Savino C, Cavaletti G, Oggioni N, Lauria G, Borgna M, Lombardi R, Cimen B, Comelekoglu U, Kanik A, Tataroglu C, Cerami A, Ghezzi P: Erythropoietin both protects from and reverses experimental diabetic neuropathy. *Proceedings of the National Academy of Sciences USA* 2004; 101: 823-8
19. Niesters M, Swartjes M, Heij L, Brines M, Cerami A, Dunne A, Hoitsma E, Dahan A: The erythropoietin analog ARA 290 for treatment of sarcoidosis-induced chronic neuropathic pain. *Expert Opinion on Orphan Drugs* 2012; 1: 77-87
20. Heij L, Niesters M, Swartjes M, Hoitsma E, Drent M, Dunne A, Grutters J, Vogels O, Brines M, Cerami A, Dahan A: Safety and efficacy of ARA290 in sarcoidosis patients with symptoms of small fiber neuropathy: a randomized, double blind, pilot study. *Molecular Medicine* 2013; 18: 1430-6
21. Hoitsma E, De Vries J, Drent M: The small fiber neuropathy screening list: Construction and cross-validation in sarcoidosis. *Respiratory Medicine* 2011; 105: 95-100
22. Marcellis RGJ, Lenssen AF, Elfferich MDP, De Vries J, Kassim S, Foerster K, Drent M: Exercise capacity, muscle strength and fatigue in sarcoidosis. *European Respiratory Journal* 2011; 38: 628-34
23. Costabel U, Hunninghake GW: ATS/ERS/WASOG statement on sarcoidosis. *European Respiratory Journal* 1999; 14: 735-7
24. Rolke R, Magerl W, Campbell KA, Schalber C, Caspari S, Birklein F, Treede R: Quantitative sensory testing: a comprehensive protocol for clinical trials. *European Journal of Pain* 2006; 10: 77
25. Lauria G, Hsieh ST, Johansson O, Kennedy WR, Leger JM, Mellgren SI, Nolano M, Merkies IS, Polydefkis M, Smith AG: European Federation of Neurological Societies/Peripheral Nerve Society Guideline on the use of skin biopsy in the diagnosis of small fiber neuropathy. Report of a joint task force of the European Federation of Neurological Societies and the Peripheral Nerve Society. *European Journal of Neurology* 2010; 17: 903-e49
26. Lauria G, Bakkers M, Schmitz C, Lombardi R, Penza P, Devigili G, Smith AG, Hsieh S, Mellgren SI, Umapathi T: Intraepidermal nerve fiber density at the distal leg: a worldwide normative reference study. *Journal of the Peripheral Nervous System* 2010; 15: 202-7
27. Umapathi T, Tan WL, Tan NC, Chan YH: Determinants of epidermal nerve fiber density in normal individuals. *Muscle & Nerve* 2006; 33: 742-6
28. Schneider CA, Rasband WS, Eliceiri KW: NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* 2012; 9: 671-5
29. Tavakoli M, Rayaz A: Corneal confocal microscopy: a novel non-invasive technique to quantify small fibre pathology in peripheral neuropathies. *Journal of Visualized Experiments* 2011;
30. Brines M, Swartjes M, Tannemaat MR, Dunne A, van Velzen M, Proto P, Hoitsma E, Petropoulos I, Chen X, Niesters M, Dahan A, Malik R, Cerami A: Corneal nerve quantification predicts the severity of symptoms in sarcoidosis patients with painful neuropathy. *Technology* 2013; 1-7

31. Crapo RO, Casaburi R, Coates AL, Enright PL, MacIntyre NR, McKay RT, Johnson D, Wanger JS, Zeballos RJ, and Bittner V: ATS statement: guidelines for the six-minute walk test. 2002. American Thoracic Society 1740 Broadway, New York, NY 10019-4374 USA.
32. Troosters T, Gosselink R, Decramer M: Six minute walking distance in healthy elderly subjects. *European Respiratory Journal* 1999; 14: 270-4
33. Quattrini C, Tavakoli M, Jeziorska M, Kallinikos P, Tesfaye S, Finnigan J, Marshall A, Boulton AJ, Efron N, Malik RA: Surrogate markers of small fiber damage in human diabetic neuropathy. *Diabetes* 2007; 56: 2148-54
34. Boyd AL, Barlow PM, Pittenger GL, Simmons KF, Vinik AI: Topiramate improves neurovascular function, epidermal nerve fiber morphology, and metabolism in patients with type 2 diabetes mellitus. *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy* 2010; 3: 431
35. Polydefkis M, Hauer P, Sheth S, Sirdofsky M, Griffin JW, McArthur JC: The time course of epidermal nerve fibre regeneration: studies in normal controls and in people with diabetes, with and without neuropathy. *Brain* 2004; 127: 1606-15
36. Gibbons CH, Wang N, Freeman R: Capsaicin induces degeneration of cutaneous autonomic nerve fibers. *Annals of Neurology* 2010; 68: 888-98
37. Tanelian DL, Monroe SCOT: Altered thermal responsiveness during regeneration of corneal cold fibers. *Journal of Neurophysiology* 1995; 73: 1568-73
38. Sorensen L, Molyneaux L, Yue DK: The level of small nerve fiber dysfunction does not predict pain in diabetic neuropathy: a study using quantitative sensory testing. *The Clinical Journal of Pain* 2006; 22: 261-5
39. Rukwied R, Schley M, Forsch E, Obreja O, Dusch M, Schmelz M: Nerve growth factor-evoked nociceptor sensitization in pig skin in vivo. *Journal of Neuroscience Research* 2010; 88: 2066-72
40. Apfel SC, Schwartz S, Adornato BT, Freeman R, Biton V, Rendell M, Vinik A, Giuliani M, Stevens JC, Barbano R: Efficacy and safety of recombinant human nerve growth factor in patients with diabetic polyneuropathy. *Journal of the American Medical Association* 2000; 284: 2215-21
41. Baughman RP, Sparkman BK, Lower EE: Six-minute walk test and health status assessment in sarcoidosis. *Chest Journal* 2007; 132: 207-13
42. Gremeaux V, Troisgros O, Benaïm S, Hannequin A, Laurent Y, Casillas JM, Benaïm C: Determining the minimal clinically important difference for the six-minute walk test and the 200-meter fast-walk test during cardiac rehabilitation program in coronary artery disease patients after acute coronary syndrome. *Archives of Physical Medicine and Rehabilitation* 2011; 92: 611-9
43. Eckstein C, Saidha S, Sotirchos ES, Byraiah G, Seigo M, Stankiewicz A, Syc SB, Sharma S, Calabresi PA, Pardo CA: Detection of clinical and subclinical retinal abnormalities in neurosarcoidosis with optical coherence tomography. *Journal of Neurology* 2012; 259: 1390-8
44. Papanas N, Vinik AI, Ziegler D: Neuropathy in prediabetes: does the clock start ticking early? *Nature Reviews Endocrinology* 2011; 7: 682-90

