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Acetyl-L-Carnitine and NRTI-associated neuropathy in HIV-infection

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Abstract

Objectives—Antiretroviral Toxic Neuropathy is associated with dideoxy-nucleoside reverse transcriptase inhibitor use in HIV, possibly due to mitochondrial toxicity. Acetyl-L-Carnitine (ALC) has been linked to symptomatic improvement in ATN. We present an open-label single-arm pilot study to evaluate change in intra-epidermal nerve fiber (IENF) density and mitochondrial DNA (mtDNA) copies/cell among subjects treated with 3000mg ALC daily.

Methods—Punch skin biopsies were examined at baseline and 24 weeks after therapy. Participants reported neuropathic symptoms using the Gracely Pain Intensity Score. Neurological examinations were completed.

Results—Twenty-one subjects completed the study. ALC was generally well-tolerated. The IENF density did not change among cases completing 24 weeks of ALC therapy, with median (90% confidence interval (CI)) IENF changes of -1.70 ($-3.50, \infty$), $p=0.98$ and 2.15 ($-0.10, \infty$), $p=0.11$ for the distal leg and proximal thigh, respectively. Fat mtDNA copies/cell did not change with therapy. Improvements in neuropathic pain ($p<0.01$), paresthesias ($p=0.01$), and symptoms of numbness ($p<0.01$) were noted. Similarly, improvement was noted on the Gracely Pain Intensity Scores.

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Conclusions—ALC therapy coincided with improvements in subjective measures of pain in this open-label single-arm study. However, changes were not observed in objective measures of IENF density or mtDNA levels, providing little objective support for use of ALC in this setting.

Keywords

fat; HIV; mitochondria; neuropathy

BACKGROUND

HIV-associated sensory neuropathy (HIV-SN), including both distal sensory polyneuropathy (DSP) and antiretroviral toxic neuropathy (ATN) is the most frequent neurological non-opportunistic complication of HIV infection and its treatments (Verma *et al*, 2005). Before widespread use of highly active antiretroviral therapy (HAART) in the US, HIV-SN was reported in 35% of individuals with AIDS (So *et al*, 1988). In the era of HAART, HIV-SN is thought to occur in 10–20% of HIV cases (Marra *et al*, 1998; Moyle and Sadler, 1998); although these rates may over-estimate clinically symptomatic disease in the current era of less toxic antiretroviral therapies. One postulate is that the relationship between toxic antiretroviral medications and the development of symptomatic HIV-SN results from the unmasking of a silent underlying neuropathy by the toxic agent (McArthur *et al*, 2005).

Possibly from their impact on mitochondrial function, specific dideoxy-nucleoside reverse transcriptase inhibitors (d-NRTI) are closely associated with neuropathic symptoms in HIV in most (Blum *et al*, 1996; McArthur *et al*, 2005) but not all studies (Schifitto *et al*, 2002). Neuropathic symptoms contribute to discontinuation of these medications in clinical trials (Moyle and Sadler, 1998). The pathogenesis of HIV-SN involves a length-dependent degeneration of peripheral nerve fibers and the clinical features of ATN are indistinguishable from that of DSP (Verma *et al*, 2005).

The risk for ATN among patients using d-NRTI roughly correlates to the degree to which they reduce mitochondrial DNA (mtDNA) content, supporting the hypothesis that the neuropathic complications of these medications are due to mitochondrial toxicity (Chen *et al*, 1991; Moyle and Sadler, 1998). In one cross-sectional analysis, elevated blood lactate levels correlated with ATN, suggestive of the anaerobic metabolism that would be anticipated with mitochondrial dysfunction (Brew *et al*, 2003). This would be consistent with the ability of NRTIs to interfere with the principal mitochondrial DNA replicase, DNA polymerase- γ and decrease mtDNA levels resulting in altered oxidative phosphorylation, a process also thought to be associated with NRTI-associated lipodystrophy (Kakuda *et al*, 1999; Moyle, 2000). This knowledge has led some to evaluate mitochondrial-directed therapy for these complications.

Acetyl-L-Carnitine (ALC) is a transport molecule for fatty acids across the mitochondrial membrane via the "Carnitine Shuttle". Given the postulated mitochondrial basis of NRTI-associated lipodystrophy and ATN, supplementation with ALC has received considerable interest. In one study, ALC blood levels differed by HIV-SN status (Famularo *et al*, 1997), in contrast to another study which failed to identify relationships between total, free, or ALC levels and pain, intra-epidermal nerve fiber (IENF) density, or quantitative sensory testing in HIV patients (ACTG 291) (Simpson *et al*, 2001). However, blood levels may not be representative of tissue or intracellular levels (De Simone *et al*, 1994). In an open-label treatment study, ALC was associated with a 100% increased in mean immunostaining area for small sensory fibers of epidermis using punch skin biopsies and computer-assisted quantitative immunohistochemical techniques (Hart *et al*, 2004). Change in epidermal nerve fiber density counts (as we report herein) was not reported. A more recent placebo-controlled study by the same group using intramuscular ALC twice daily for 14 days followed by oral supplementation

failed to identify benefit in an intent-to-treat analysis; although secondary analysis among a selected subset of individuals was reported to be associated with reduction in pain levels (Youle, 2007). A lack of clarity on clinical outcomes despite broad use by patients prompted our pilot study to evaluate IENF density and mtDNA changes during 6 months of oral ALC supplementation.

METHODS

ACTG 5157 was a 24-week, open-label, dose-escalation pilot study of ALC for the treatment of d-NRTI-associated ATN. The study aimed to enroll a target of 36 subjects with primary endpoints of safety and tolerability as well as to evaluate potential increases in epidermal nerve fiber density associated with ALC treatment. Secondary objectives aimed to evaluate changes in mtDNA copies/cell in fat tissue attached to the epidermis in the biopsies obtained and to evaluate signs and symptoms of HIV-SN. Enrollment was discontinued after 27 participants due to slow accrual associated with new availability of NRTIs in the US and the subsequent decreased use of 'neurotoxic' d-NRTIs. Participants who were enrolled received ALC 500mg twice daily during the first 7 days, increased to 1000mg daily for days 8–14, then 1500mg twice daily for the remainder of the 24-week period.

Patient Population

The study enrolled individuals with clinician-diagnosed ATN at sites with neurological AIDS expertise. ATN was defined as bilateral neuropathic pain in the lower extremities for at least 2 weeks and neurological findings of one of the following: (1) diminished ankle reflexes, (2) decreased vibratory sense (perceptions of vibration < 10 seconds at the great toe with a tuning fork struck hard enough to be audible), (3) diminution of pin or temperature sensation in the lower extremities, or (4) contact allodynia in the feet. Participants were required to be taking HAART with at least one of the following NRTIs: stavudine (d4t), didanosine (ddI) or zalcitabine (ddC) and could not have other conditions or history of conditions that in the opinion of the site neurologist confounds the diagnosis of ATN (e.g. Diabetes mellitus, vitamin B12 deficiency, compression neuropathy, or previous treatment with agents known to be associated with neuropathy). Subjects taking analgesic therapy were required to be on a stable dose for the 60 days prior to study enrollment and not to change their analgesic treatment during the study.

Study Outcomes

We employed the Gracely Pain Intensity Scale to quantify pain, rating discomfort from 0 (no pain sensation) to 20 (extremely intense pain). Participants were required to document pain diaries twice daily (morning and evening) for seven days prior to study visits. Mean pain scales were defined as the mean score of all non-missing entries.

Three millimeter punch skin biopsies were obtained in pairs from the proximal lateral thigh and the distal lateral leg 10 cm above the lateral malleolus at baseline and after 24 weeks of therapy. A standard Accupunch device was used. Subcutaneous adipose tissue was carefully separated from the epidermis at the time of biopsy.

IENF Density Quantification

Epidermal biopsies were placed in 2% PLP fixative for 12–24 hrs at 4°C. They were rinsed with 0.08M Sorrenson's phosphate buffer and cryoprotected with 20% glycerol/0.08M Sorrenson's PO4. Frozen biopsies were then sectioned at 50µM on a Microm HM440 freezing sliding microtome. These sections were immunohistochemically stained with antibodies directed against PGP9.5 (Chemicon/Millipore AB1761ASR, 1:2000, Temecula, CA). Overnight incubation of primary antibody was followed by biotinylated goat anti-rabbit

secondary antibody at 1:100 dilution (BA1000, Vector labs, Burlingame, CA). Sections were then incubated in 1:100 dilution of avidin biotin complex from Vector's Vectastain kit (PK6100) and labeled with Vector SG substrate (SK4700). We mounted the immunostained sections on gelatin subbed slides, eosin stained, dehydrated and cover-slipped with Permount mounting media (Fisher Scientific).

The intraepidermal nerve fibers were counted along the length of the 3mm punch biopsies. In each biopsy the counts were enumerated in four sections. The length of the epidermis along the upper margin of the stratum corneum was measured with Bioquant software (R&M Biometrics, Nashville, TN). The IENF density was derived and expressed as the number of fibers per millimeter of epidermal length (fibers/mm) using counting rules previously described (McArthur *et al*, 1998; Stocks *et al*, 1996). Individual nerve fibers were counted as they crossed the dermal-epidermal junction, and epidermal nerve fragments that do not cross the basement membrane but in the epidermis were also included in the quantification as previously described and validated (Ebenezer *et al*, 2007; Kennedy *et al*, 2005; Lauria *et al*, 2005). The immunostaining was all performed in a central laboratory and microscopic evaluations were performed by one individual blinded to the timing of the biopsies. The outcome measure is a linear density of unmyelinated nerve fibers within the epidermis and a normative range has been established from previous work (McArthur *et al*, 1998) (Figure 1). The intra-observer IENF density quantitation reliability was assessed in 10% of biopsies and found to be 93%.

MtDNA Quantification

Subcutaneous adipose tissue from lateral and distal thigh was stored in RNAlater (Ambion, Inc., Austin, TX) at -70°C until assayed. Total DNA was isolated using QIAamp DNeasy Tissue kit (Qiagen Inc., Valencia, CA). DNA integrity was examined by agarose gel electrophoresis. Intact DNA was assayed for mtDNA copies/cell using mitochondrial primers that amplify a region of mtDNA (90 bp) that encodes for NADH dehydrogenase subunit 2 and genomic primers specific for the region of the genome encoding the Fas Ligand (98 bp) by real-time PCR (Gerschenson *et al*, 2005; Simpson *et al*, 2006). The PCR reactions were assayed with the Lightcycler FastStart DNA Master Plus SYBR Green I mix in the LightCycler real-time instrument (Roche Diagnostic Corporation, Indianapolis, IN). Each sample and standard was run in duplicate (20 μL reaction volume) containing: 1X SYBR Green master mix, 0.5 μM of each primer, and 10 ng sample of DNA. PCR cycling conditions were: denaturation for 1 cycle 95°C for 10 min, amplification for 40 cycles 95°C for 10 sec, 58°C for 5 sec, and 72°C for 5 sec. A melt curve was performed from 65°C at a $0.3^{\circ}\text{C}/\text{sec}$ ramp rate with continuous acquisition. The results were then analyzed with Lightcycler Version 4.0 software. Mitochondrial DNA copies per cell were calculated using the formula (mtDNA copies/gDNA copies) $\times 2 =$ mtDNA copies per cell.

Regulatory and Statistical Considerations

All participants signed IRB-approved consent forms. We employed Wilcoxon signed-rank tests (symmetric data) and sign tests (non-symmetric data) to evaluate IENF density changes. Given the pilot nature of the protocol, the primary analyses for the IENF outcomes consisted of one-sided, 0.10 level exact confidence intervals (CI). Two-sided, 0.10 level confidence intervals were utilized for all other outcomes. Adjustment for multiple testing was not employed. Analyses were completed based on the intent-to-treat principal with analyses of all registered participants. Missing data for the IENF density was imputed using the median value of the non-missing measurements of study participants at the same week. A complete case analysis was also performed. The study was powered to detect a mean IENF density change of two nerve fibers assuming that the standard deviation of the change from baseline was four nerve fibers using a one-sided test at the 0.10 level of significance. Under these assumptions, thirty evaluable participants provided 92% power.

RESULTS

Twenty-seven subjects entered the study between November of 2004 and September of 2006 with 21 subjects completing the study (Figure 2). Twenty-three participants reached the maximum protocol dose, most (21/23) of whom reached this dose within the standard titration phase. One death occurred following renal failure, which was deemed to be unassociated with the study medication. Additionally, seven subjects discontinued treatment prematurely for the following reasons: participant entered rehabilitation facility or hospital for unrelated illness (2); worsening neuropathy and diarrhea (1); fearful that medication would cause kidney stones (1); anxiety and fear of pill burden/multiple somatic complaints after first dose (1); not able to attend the clinic (1); not believing treatment was helping (1). Three participants requested a dose reduction, but subsequently tolerated the maximal study dose. Two patients temporarily stopped their ARV (for nine and seventeen days) for non-study related issues; and one patient stopped ARV at week 2, restarting a non-dideoxynucleoside-based regimen at week 12. Most study participants were male (23/27, 85%). The population was diverse and included individuals self-identified as Caucasian (13/27, 48%), Black (10/27, 37%), and Hispanic (4/27, 15%). The median age was 46 years. Most (22/27, 81%) denied previous or current IV drug abuse.

Participants reported, on average, 5.3 years (range: 0.4 – 11 years) of d-NRTI use. The median (Q1, Q3) baseline CD4 lymphocyte count was 313 (238, 549) cells/mm³. By study design, all participants had plasma HIV RNA levels of less than 10,000 copies/ml with 20/27 (74%) having undetectable levels (≤ 50 copies/ml). Participants reported a median (Q1, Q3) baseline pain intensity of 10.9 (6.4, 14.0).

At baseline, the median (Q1, Q3) IENF density in the distal leg and proximal thigh were 7.3 (3.6, 12.7) and 12.0 (7.9, 19.1) respectively (table 1). The IENF density did not change significantly among participants completing 24 weeks of ALC therapy, with median (CI) of IENF changes of -1.70 ($-3.50, \infty$), $p=0.98$, and 2.15 ($-0.10, \infty$), $p=0.11$, for the distal leg and proximal thigh, respectively. Intent to treat analyses utilizing imputation of the median of non-missing values resulted in changes of -1.10 ($-2.70, \infty$), $p=0.83$, and 1.80 ($0.70, \infty$), $p=0.04$. Exploratory analyses suggested that detectable baseline plasma HIV RNA (>50 copies/ml) ($p=0.03$) correlated with greater improvement in distal leg IENF density and that older age ($p=0.01$) and Caucasian race ($p=0.04$) correlated with greater improvement in the proximal thigh IENF density.

Participants reported improvement in the median 24-week change in neuropathic pain ($p<0.01$); paresthesias ($p=0.01$) and symptoms of numbness ($p<0.01$). Similarly, improvement was noted on the Gracely Pain Intensity Scores [median, CI] for both morning [$-1.29, p=0.01$, ($-5.07, -0.79$)] and evening [$-0.14, p=0.04$, ($-5.50, -0.43$)] scores and both scores combined [$-0.79, p=0.02$, ($-5.25, -0.68$)] (Figure 3). On the neurological examination, improvement in vibratory sensation was noted ($p=0.02$).

Statistically significant changes in mtDNA copies/cell with treatment were not observed in subcutaneous adipose tissue. The median (Q1, Q3) baseline mtDNA copies/cell in the distal ($n=18$) and proximal ($n=16$) leg were 825 (655, 1409) and 1106 (658, 1750), respectively while the changes in mtDNA (median, p -value, CI) were [29 copies/cell, $p=0.93$, ($-329.5, 339.0$)] and [230 copies/cell, $p=0.45$, ($-509, 679$)] respectively.

DISCUSSION

This study reports the safety and tolerability of ALC in HIV participant while assessing preliminary data for efficacy in neuropathy. In generally, ALC was well-tolerated in this open-label, single-arm study among participants with neuropathy receiving d-NRTI with most

participants who discontinued treatment doing so for reasons not associated with ALC. Furthermore, most subjective measures of neuropathy symptoms including pain, paresthesias, and numbness, improved over the course of the study. This was accompanied by a modest improvement in vibration sensation on the neurological examination. While these findings are consistent with that reported by another group (Youle and Osio, 2007), the results should be interpreted with caution as our study did not include a placebo arm, these outcomes were secondary, and not the primary endpoint, and our statistical approach did not adjust results for multiple comparisons due to the pilot nature of the study. It is not known if the reported change in these subjective measures differs from that which would have been observed in a placebo arm, should one have been employed.

In contrast, we were not able to demonstrate a change in IENF density among subjects treated with 3000mg ALC daily in divided doses, a primary aim of the study. However, the study had low power due to a small sample size and higher variability of the IENF changes than expected (observed SD of changes = 7.45 for the distal leg). Only 18 subjects had both baseline and week 24 biopsy data necessary to evaluate the primary endpoint. With only 18 evaluable participants and the observed SD of changes, the power to detect an increased density of 2/mm is only 44%. The statistically significant finding in IENF density at the proximal thigh noted only when we imputed the median value of non-missing data for missing values may be spurious given that it was not supported by other objective study measures and it was not accompanied by findings in the distal leg, the site more likely to represent changes with HIV-SN. Notably, 9/27 (33%) of observations were imputed. No effects were seen in the analyses of observed data (table 1).

MtDNA levels in subcutaneous adipose tissue were also unaffected by treatment. Since ALC would have enhanced carnitine transported and increased β -oxidation, this may have not had direct effects on the NRTI induced mtDNA depletion (Alesci and Gerschenson, 2005). In addition to the small sample size and short duration of treatment, it is recognized that fat may not be the optimal tissue to evaluate PN. Additionally, NRTIs are not the sole etiological cause of PN, HIV polypeptides like HIV-1, HIV gp120, and Tat (1–72) can also, alone or in combination with nucleoside analogs cause both mitochondrial and microtubule morphological and ultra-structural changes in dorsal root ganglion neurons. Further, infected macrophages produce factors that impair development of neuronal precursor cells, and soluble viral protein R (Vpr) is one of the factors which has the ability to suppress axonal growth (Kitayama *et al*, 2008; Robinson *et al*, 2007).

Since the launch of this study coincided with the new availability of tenofovir and abacavir in the US, bias may have been present in participant selection, possibly selecting for milder disease. We observed that many clinicians opted to switch antiretroviral medications if neuropathic symptoms existed. Individuals choosing to enroll may have had fewer antiretroviral options (e.g. multiple antiretroviral resistance mutations) associated with more advanced HIV disease, which could have coincided with more established neuropathy or permanent damage. In such cases, it is reasonable to consider that they may have had less potential for regression of neuropathology.

There are important distinctions in the approach employed in this work compared to previously published work where robust changes in mean epidermis immunostaining was reported and the sample size was similar (n=21)(Hart *et al*, 2004). Our technique employed IENF counts to evaluate nerve regeneration rather than mean area of immunostaining. This technique should not be influenced by other morphological changes in nerve tissue. Crush artifacts, which were noted in several of our biopsies, may have affected the precision of the quantification. Other distinctions from previously published work include our restriction of ATN to patients exposed

to d-NRTIs rather than all NRTIs and, possibly, a longer duration of ATN in our study (medial NRTI exposure of 4.4 years).

In summary, ALC was safe and well-tolerated in HIV participants with ATN. Subjective improvement was noted over the course of this non-blinded single-arm study; however, we are unable to confirm that such improvement is greater than would be expected by placebo, should one have been employed. Objective measures of improvement in IENF density and mtDNA were not noted. Study power may have influenced our ability to identify these changes.

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Abbreviations used

ACTG	AIDS Clinical Trials Group
ALC	acetyl-L-carnitine
d-NRTI	dideoxy-nucleoside reverse transcriptase inhibitor
DSPN	distal symmetric peripheral neuropathy
HAART	highly active antiretroviral therapy
IENF	intra-epidermal nerve fiber, MtDNA, Mitochondrial DNA

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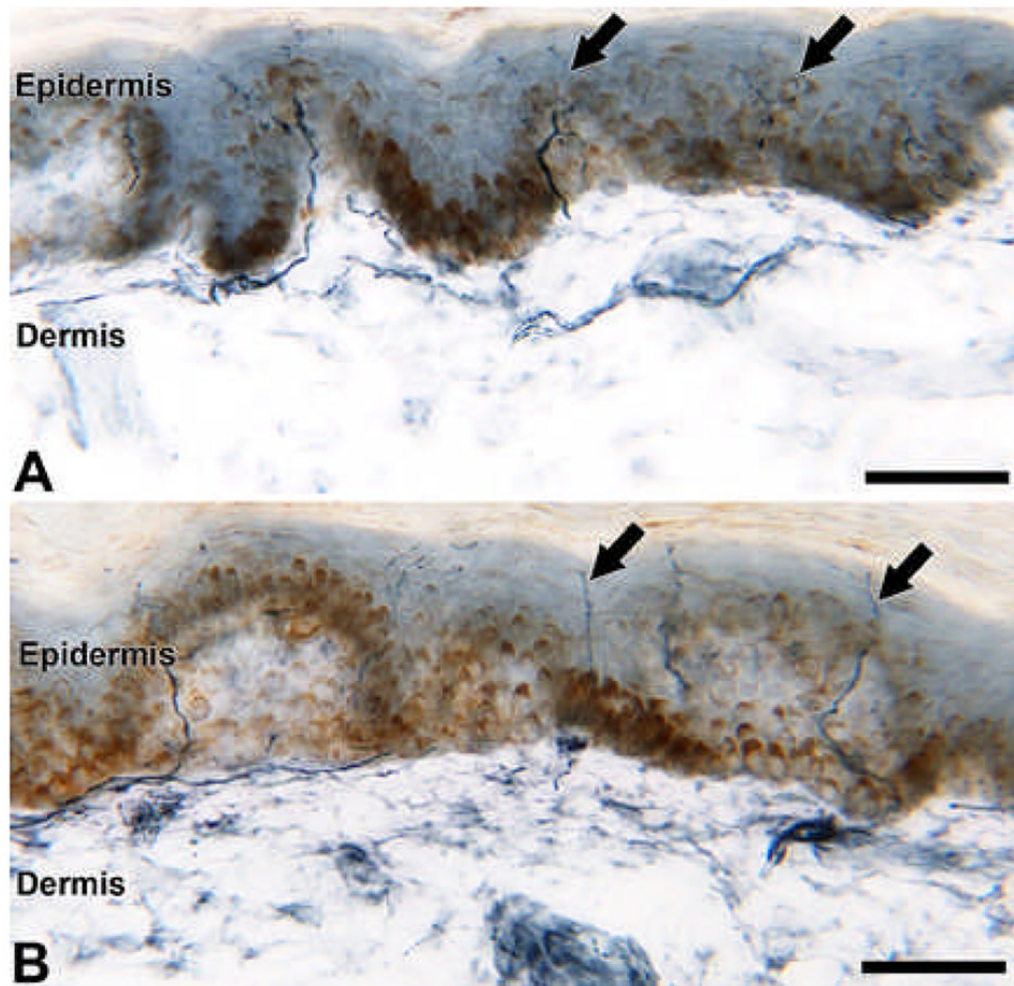


Figure 1. Photomicrograph of skin biopsies stained with PGP9.5 to demonstrate intraepidermal nerve fibers [scale bar 50 μm]. (A) proximal thigh site, with density of fibers at 17/mm; (B) distal leg, with density of fibers 16.9/mm

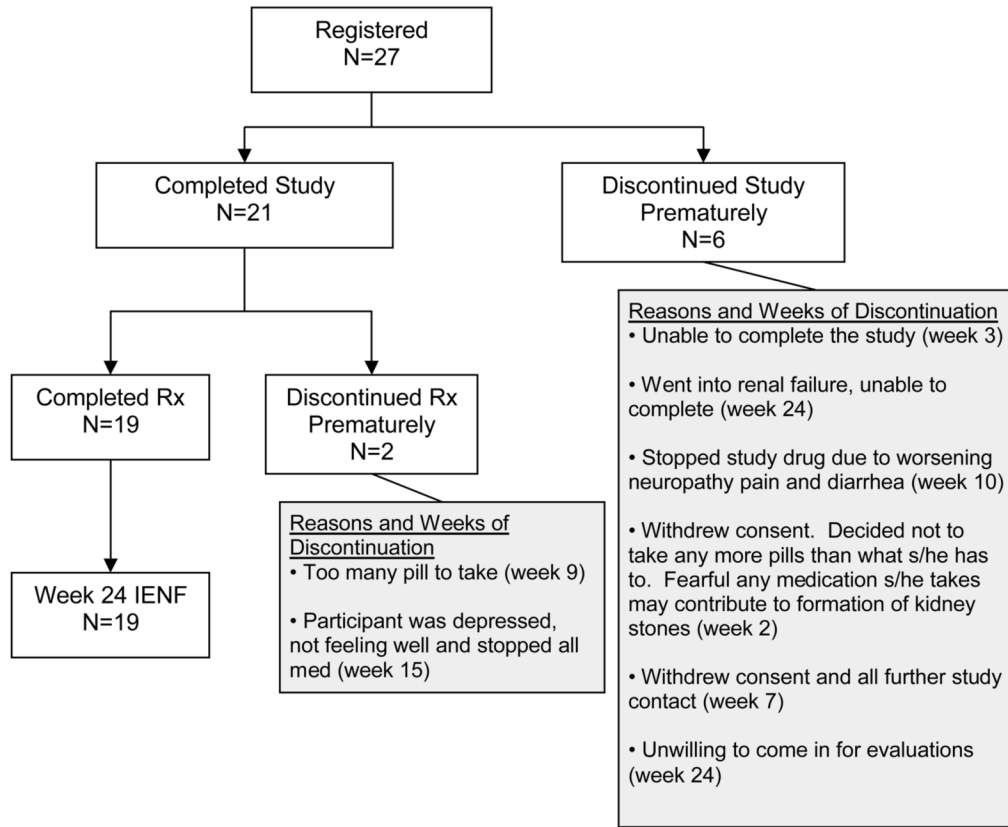


Figure 2.
CONSORT Diagram of study enrollment

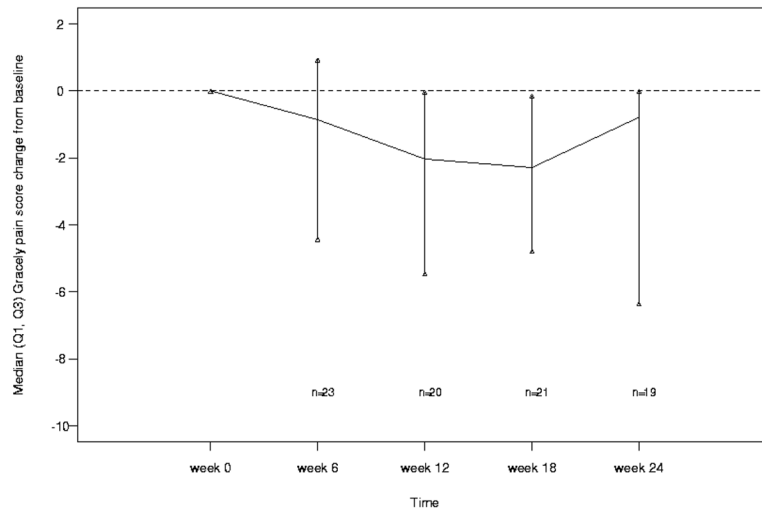


Figure 3. Median (Q1, Q3) Gracely Pain Score (Morning and Evening Combined) Change from Baseline

Table 1
Baseline, Week 24, and 24-Week Change in IENF Density (enf/mm)

	Distal Leg	Proximal Thigh
Baseline		
N	26	26
Median (Q1, Q3)	7.3 (3.6, 12.7)	12.0 (7.9, 19.1)
Week 24		
N	19	19
Median (Q1, Q3)	7.3 (5.4, 11.2)	17.7 (6.7, 26.9)
24-Week Change		
Observed data		
N	18	18
Median (Q1, Q3)	-1.7 (-4.6, 0.0)	2.2 (-1.4, 12.4)
<i>p</i> -value (90% CI)	0.98 (-3.5, 4) ^b	0.11 (-0.1, 4) ^c
Imputation ^d		
N	27	27
Median (Q1, Q3)	-1.1 (-4.0, 3.1)	1.8 (-1.4, 12.4)
<i>p</i> -value (90% CI)	0.83 (-2.7, 4) ^c	0.04 (0.7, 4) ^c

^a: Missing values imputed by the median values of the non-missing values at the corresponding week and biopsy location

^b: Sign test and 90% 1-sided exact CI

^c: Wilcoxon signed-rank test and 90% 1-sided exact CI