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Authors

Casselini, Carolina M

Parson, Henri K

Frizzi, Katie E

et al.

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ORIGINAL PAPER

A muscarinic receptor antagonist reverses multiple indices of diabetic peripheral neuropathy: preclinical and clinical studies using oxybutynin

Carolina M. Casselini[1] · Henri K. Parson[1] · Katie E. Frizzi[2] · Alex Marquez[2] · Darrell R. Smith[3] · Lucie Guernsey[2] · Rakesh Nemmani[2] · Alireza Tayarani[2] · Corinne G. Jolivalt[2] · Jessica Weaver[1] · Paul Fernyhough[3,4] · Aaron I. Vinik[1] · Nigel A. Calcutt[2]

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Abstract

Preclinical studies indicate that diverse muscarinic receptor antagonists, acting via the M_1 sub-type, promote neuritogenesis from sensory neurons in vitro and prevent and/or reverse both structural and functional indices of neuropathy in rodent models of diabetes. We sought to translate this as a potential therapeutic approach against structural and functional indices of diabetic neuropathy using oxybutynin, a muscarinic antagonist approved for clinical use against overactive bladder. Studies were performed using sensory neurons maintained in vitro, rodent models of type 1 or type 2 diabetes and human subjects with type 2 diabetes and confirmed neuropathy. Oxybutynin promoted significant neurite outgrowth in sensory neuron cultures derived from adult normal rats and STZ-diabetic mice, with maximal efficacy in the 1–100 nmol/l range. This was accompanied by a significantly enhanced mitochondrial energetic profile as reflected by increased basal and maximal respiration and spare respiratory capacity. Systemic (3–10 mg/kg/day s.c.) and topical (3% gel daily) oxybutynin reversed paw heat hypoalgesia in the STZ and db/db mouse models of diabetes and reversed paw tactile allodynia in STZ-diabetic rats. Loss of nerve profiles in the skin and cornea of db/db mice was also prevented by daily topical delivery of 3% oxybutynin for 8 weeks. A randomized, double-blind, placebo-controlled interventional trial was performed in subjects with type 2 diabetes and established peripheral neuropathy. Subjects received daily topical treatment with 3% oxybutynin gel or placebo for 6 months. The a priori designated primary endpoint, significant change in intra-epidermal nerve fibre density (IENFD) in skin biopsies taken before and after 20 weeks of treatments, was met by oxybutynin but not placebo. Secondary endpoints showing significant improvement with oxybutynin treatment included scores on clinical neuropathy, pain and quality of life scales. This proof-of-concept study indicates that muscarinic antagonists suitable for long-term use may offer a novel therapeutic opportunity for treatment of diabetic neuropathy. *Trial registry number*: NCT03050827.

Keywords Diabetic neuropathy · Epidermal nerve fibres · Muscarinic antagonist · Neuropathic pain · Oxybutynin · Randomized clinical trial

✉ Nigel A. Calcutt
ncalcutt@ucsd.edu

[1] Department of Internal Medicine, Strelitz Diabetes Center, Endocrine and Metabolic Disorders, Eastern Virginia Medical School, Norfolk, VA, USA

[2] Department of Pathology, University of California San Diego, La Jolla, CA, USA

[3] Division of Neurodegenerative Disorders, St Boniface Hospital Albrechtsen Research Centre, R4046 - 351 Taché Ave, Winnipeg, MB R2H 2A6, Canada

[4] Department of Pharmacology and Therapeutics, University of Manitoba, Winnipeg, MB, Canada

Abbreviations

CART	Cardiac autonomic reflex tests
DRG	Dorsal root ganglion
ICC	Intra-class coefficient
EVMS	Eastern Virginia Medical School
HRV	Heart rate variability
IENF(D)	Intra-epidermal nerve fibre (density)
NIS-LL	Neuropathy Impairment Score-lower limb
NRS	Numeric Rating Scale
NSS	Neuropathy Symptom Scale
NTSS-6	Neuropathy Total Symptom Score-6
OCR	Oxygen consumption rate
QOL-DN	Quality of life-diabetic neuropathy

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STZ	Streptozotocin
TNS	Toronto Neuropathy Scale
UCSD	University of California San Diego
UENS	Utah Early Neuropathy Scale

Introduction

The pathogenesis of diabetic neuropathy involves multiple mechanisms that impact a variety of cell types. Hyperglycemia disrupts metabolism in Schwann cells and the vasculature, directly damaging those cells and indirectly impacting the neurons they support [19]. Impaired insulin signaling, secondary to insulin deficiency and/or resistance, is a separate insult that stresses neurons via loss of trophic support [56] while dyslipidemia is emerging as an independent disruptor of neuronal energetic status [39]. Ultimately, metabolic dysfunction of diverse origins promotes the demyelination, axonal degeneration, microvasculopathy and impaired regenerative capacity that are the pathological characteristics of diabetic neuropathy [26, 31].

Preclinical studies have identified diverse pathogenic mechanisms that may contribute to diabetic neuropathy and described efficacy of therapeutic interventions against specific mechanisms [53]. Unfortunately, none of these targeted approaches have translated to clinical use and current treatment options for diabetic neuropathy remain limited to encouraging metabolic control, modification of diet and exercise and, where necessary, symptomatic control of pain [55]. An alternative approach has emphasized promoting neuronal resilience against insults, whatever their origin. Examples include modulation of chaperone proteins to enhance cytoprotective pathways [15] and use of therapeutic cocktails [54]. Antagonists of the muscarinic 1 sub-type receptor (M_1R) also prevent multiple indices of neuropathy in diabetic rodents [8, 23, 24], while efficacy in models of chemotherapy [11, 42] and HIV-associated neuropathy [20] suggests a disease-agnostic neuroprotective and regenerative capacity.

Nerve damage, as measured by reduced density of small sensory nerve fibres in the skin and cornea is increasingly recognized as an early manifestation of diabetic neuropathy [14, 35], while recovery of small fibre density accompanies or precedes improvements in other indices of neuropathy following simultaneous pancreas and kidney transplantation in diabetic patients [3]. Given the ability of muscarinic antagonists to promote neurite outgrowth in vitro and improve corneal and epidermal nerve fibre density in diabetic rodents [8, 23, 24], we investigated whether this neuroregenerative capacity translated to humans with established diabetic neuropathy. We selected the muscarinic antagonist oxybutynin for study as it is in current clinical use to treat overactive bladder [47]. Initial preclinical studies were performed to establish that oxybutynin promoted neurite outgrowth in vitro and prevented structural and functional indices of neuropathy in diabetic rodents. We then performed a proof-of-concept study in subjects with type 2 diabetes and established neuropathy who were treated with topical oxybutynin. The primary endpoint was designated a priori as a statistically significant improvement in small fibres crossing the dermal:epidermal junction, with secondary endpoints encompassing multiple structural and functional indices of diabetic neuropathy.

Materials and methods

Rigor and reproducibility

All studies were performed under protocols approved by the Institutional Animal Care and Use Committees of the University of California San Diego (UCSD) and the University of Manitoba (animals) or the Institutional Review Boards of Eastern Virginia Medical School (EVMS) and UCSD (humans). All cells, animals and tissues were coded and evaluated in randomized sequence (1:1) by investigators unaware of the treatment groups. Group sizes were guided by prior experience and published data for within- and between-group assay variability. Statistical methods and design were selected a priori from approaches in use by the pertinent research fields.

Sensory neuron culture

Sensory neurons were isolated from dorsal root ganglia (DRG) of normal Sprague–Dawley rats or streptozotocin (STZ: Sigma-Aldrich, Canada) diabetic Swiss Webster mice and dissociated as described [8]. Neurons from normal rodents were cultured with 10 mmol/l glucose and 0.1 nmol/l insulin (Sigma-Aldrich, USA) whereas those from diabetic rodents were exposed to 25 mmol/l glucose in the absence of insulin to mimic conditions of murine type 1 diabetes. Cultures were treated with oxybutynin (Sigma-Aldrich, USA) for 1–2 days before assessment of neurite outgrowth or mitochondrial function.

Neurite outgrowth

Neurons were grown on glass cover slips and visualized by exposure to a primary antibody directed against neuron-specific β-tubulin isotype III (1:1000: Sigma-Aldrich, Canada) and CY3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, USA) before viewing via a Carl Zeiss Axioscope-2 fluorescence microscope equipped with an AxioCam camera and Axio-Vision4.8 software [8]. Quantification of total neurite outgrowth was performed

by measuring mean pixel area (ImageJ software) adjusted for the cell body signal and neuronal number [8]. Neurite outgrowth in this system is directly related to an arborizing form of axonal plasticity homologous to collateral sprouting [45].

Mitochondrial respiration

Sensory neurons were obtained as described above. Culture medium was changed to DMEM (pH 7.4) supplemented with 1 mmol/ pyruvate, and 10 mmol/l D-glucose. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were determined using an XF24 Analyzer (Seahorse Biosciences, USA) in which OCR was linear at a neuronal plating density of 2000–4000 cells/well [38]. Oligomycin (1 µmol/), carbonylcyanide-p-trifluoromethoxyphenyl hydrazone (FCCP; 1 µmol/l) and rotenone + antimycin A (both 1 µmol/l) were introduced sequentially to determine basal oxygen consumption, basal ECAR, ATP-linked oxygen consumption, non-ATP-linked oxygen consumption, maximal respiratory capacity and non-mitochondrial oxygen consumption [8]. Cells were lysed and total protein measured. Data are expressed as OCR in pmol/min/mg protein or in mpH/min/normalized unit for ECAR.

Induction of diabetes and treatment

Animals were maintained 2–5 per cage under a 12 h light:dark cycle with unrestricted access to water and food (Harlan 5001). Insulin-deficient diabetes was induced in adult male Swiss Webster mice (Charles River Laboratories, Canada) by injection of STZ (90 mg/kg i.p.) in sterile 0.9% NaCl on two consecutive days, with each injection following an overnight fast. Adult female Sprague–Dawley rats (Envigo, USA) received a single injection of STZ (55 mg/kg i.p.) following overnight fast. Female leptin receptor-deficient mice (#000642: BKS.Cg-*Dock7*^{m} + / + *Lepr*^{db}/J, commonly called db/db: The Jackson Laboratory, USA) that model type 2 diabetes and age-matched controls (#000662: C57BL/Ks) were purchased when 4 weeks of age. Non-fasted blood glucose concentration was measured at onset of diabetes and monitored regularly using tail vein blood and a glucose meter (OneTouch Ultra, LifeScan, USA). Only animals with blood glucose values consistently above 15 mmol/l were considered diabetic. Oxybutynin (Sigma-Aldrich, USA) was dissolved in 0.9% sterile saline or hydrogel prior to sub-cutaneous (s.c.) or topical delivery, respectively, and delivered daily for 5 days/week, with topical treatment delivered to the hind paws and left in place for 30 min per session.

Paw sensation

Latency to paw withdrawal from escalating heat was measured using a Hargreaves apparatus (UARD, CA, USA) as described [22]. Floor temperature started 30 °C and increased at 1 °C/s until stopping after 20 s to prevent tissue damage. Paws were tested 3 times, at 5-min intervals, with the median designated as withdrawal latency. Paw sensitivity to pressure applied to the plantar surface via von Frey filaments was measured in unrestrained rats [22].

Density of sensory nerve profiles in skin and cornea

Intra-epidermal nerve fibres (IENF) and nerves of the papillary dermis were identified in plantar paw skin by immunostaining with anti-PGP9.5 antibody (#7863–0504, AbD Serotec, UK). Nerve profiles were counted from glass slides by light microscopy and expressed relative to length of dermal:epidermal border [22]. Corneal nerves of isoflurane-anesthetized mice were imaged using a corneal confocal microscope (HRT3 with Rostock corneal module; Heidelberg Engineering, Germany). A volume scan of 40 consecutive images (384 × 384 pixels with 1 µm lateral resolution at 2 µm depth intervals) was collected between the corneal epidermis and stroma. Nerve occupancy was measured in 3 consecutive images of the sub-basal nerve plexus and 10 consecutive images of the superficial stroma by overlay of an 8 × 8 grid [22].

Clinical trial design

A randomized, double-blind, placebo-controlled interventional trial (NCT03050827, clinicaltrial.gov) was performed at the Strelitz Diabetes Center, EVMS, in subjects with type 2 diabetes and established peripheral neuropathy. After providing informed consent, subjects were randomized (1:1) to receive oxybutynin or placebo (Fig. 1). The primary endpoint, designated a priori, was within-group change from baseline of IENF density (IENFD) measured in 3 mm punch biopsies at the proximal leg after 0 and 20 weeks of treatment. Secondary endpoints designated a priori were within-group change from baseline of clinical neuropathy, quality of life (QOL) and pain scores, sudomotor function, cardiac autonomic function and corneal nerve morphometrics.

Test agent

A gel, consisting of 3% (w:v) oxybutynin and an inactive base of water, ethyl alcohol, ethoxy diglycol, propylene glycol and hydroxypropylcellulose, was produced by a compounding pharmacist. Participants applied oxybutynin or placebo (inactive base) once daily to one of the stomach, calves/top of feet or upper arms in a rotating fashion to avoid

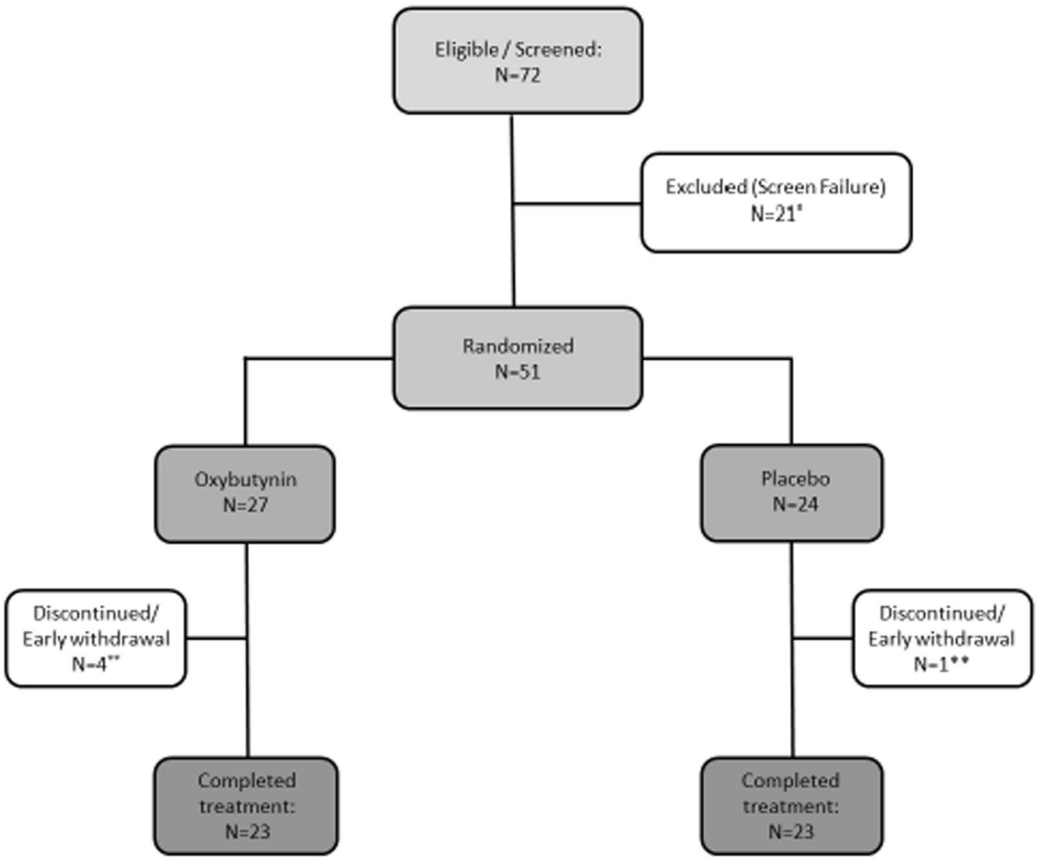

Fig. 1 Recruitment of type 2 diabetic subjects into the exploratory randomized clinical study. Eligible participants were assigned a number in recruitment order and randomized in a 1:1 ratio by an individual unconnected to the study using a web site (www.randomizer.org) without stratification. A subject blinding/randomization form was completed and placed in a sealed envelope carrying the patient number to allow emergency breaking of individual blinds without compromising the remaining data. Envelopes remained in a restricted access cabinet until final database lock occurred. *Main reasons for screen failure were uncontrolled diabetes, advanced chronic kidney disease and uncontrolled thyroid disease. **All 5 participants who opted for early withdrawal cited patient-perceived lack of efficacy

adverse skin reactions. This treatment regime was necessary for off-label use of an approved drug.

Inclusion and exclusion criteria

Subjects (males and females aged 30–80 years of varying ethnicity) were recruited from patients attending the EVMS diabetes clinic and the greater Hampton Roads, VA, area. Inclusion criteria were diagnosis of type 2 diabetes of > 2 years according to published guidelines [48] and confirmed diagnosis of diabetic peripheral neuropathy according to the Toronto Consensus guidelines [49]. Exclusion criteria were type 1 diabetes, clinically significant neuropathy of non-diabetic origin, lower extremity foot ulcers or amputation, uncontrolled or untreated hypothyroidism, liver or kidney abnormalities, stable (> 6 months) use of antioxidants or drugs known to reduce oxidative stress, urinary retention or clinically significant benign prostatic hyperplasia, uncontrolled glaucoma, gastric retention or severe gastroparesis and prior/current use of anticholinergics.

Clinical assessment of neuropathy and neuropathy scores

Assessment was performed at baseline and week 20 of treatment by recording a complete medical history, demographics, vital signs, height and weight, ECG and physical examination. Clinical assessment of neuropathy was performed using (i) the Total Neuropathy Score (TNS) comprising the Neuropathy Symptom Score (NSS) and the Neuropathy Impairment Score (NIS) [50], (ii) the Neuropathy Impairment Score of the Lower Limbs (NIS-LL) [7] and (iii) the Utah Early Neuropathy Scale (UENS) [44].

Pain scores

Pain was assessed by (i) the Numeric Rating Scale (NRS) to record pain over the previous 24 h where 0 is no pain and 10 the worst pain imaginable [16] and (ii) the Neuropathy Total Symptom Score 6 (NTSS-6) to assesses intensity and frequency of different pain modalities [4, 9].

Quality of life questionnaires

All participants completed the Norfolk Quality of Life-Diabetic Neuropathy questionnaire (Norfolk QOL-DN) [51], previously used as an outcome measure in interventional trials against diabetic neuropathy [5].

Sudomotor function

Sudomotor function was evaluated using the Sudoscan device (Impeto Medical, France), previously shown to be sensitive to change after interventions in subjects with type 2 diabetes [10].

Cardiac autonomic reflex tests (CART) and heart rate variability (HRV)

CART and HRV were measured using the cardio-respiratory monitoring software ANSAR (ANX 3.0 software; ANSAR Group, Inc., USA). Valsalva and standing from sitting position maneuvers were also performed [46] and the following indices recorded: resting heart rate and blood pressure; E/I ratio (during deep breathing manoeuvre), Valsalva ratio during the Valsalva manoeuvre and 30:15 ratio during standing from sitting manoeuvre. Frequency domain measures were total spectral power, low-frequency power and high frequency power. Time domain measures were SD of beat-to-beat variability and root mean square of successive R–R intervals.

Nerve conduction

Nerve conduction velocities, latencies and amplitudes of peroneal motor and sural sensory nerves in the non-dominant lower extremity [9] were measured using the XLtek Neuromax device (XLtek, Inc., Canada).

Corneal confocal microscopy

Imaging was performed using the Rostock Cornea Module of a HRTIII (Heidelberg Engineering, Germany). Two to 3 images per eye were analyzed using the CCMetrics tool (University of Manchester, UK) and parameters calculated as mean of both eyes. Images were assessed by 2 independent observers with intraclass correlation coefficients (ICC's) of 0.82 for corneal nerve fibre density, 0.79 for corneal nerve branch density, and 0.86 for corneal nerve fibre length.

Skin biopsies

Skin biopsies were collected from the lateral aspect of the non-dominant lower leg, 10 cm below the mid-patellar border using a 3 mm punch. This site was chosen to avoid including a preponderance of subjects with pre-treatment IENF values of 0, as attempting to promote regeneration where nerves may already be dead seemed unwise. Biopsies were processed to paraffin blocks, cut and stained with antibody to PGP9.5 (Chemicon®, Millipore Sigma, MA USA). Slides were de-identified and IENF counted from glass slides at site 1 (EVMS) by a blinded independent observer (JW) in at least 3 sections per biopsy using Image Pro Plus Software (Media Cybernetics, MD, USA). IENFD was quantified as the number of nerve fibres crossing the dermal–epidermal junction per length of the junction [28]. De-identified slides were sent to site 2 (UCSD) where IENFD was calculated by another blinded observer (KF) using the same method. ICC between the observers was 0.74. To examine whether treatment also impacted the density and morphology of epidermal fibre terminal arborizations, sprout density (epidermal fibre fragments + branch points) per length of the junction was also measured at site 2. Nerves surrounding incidental sweat glands were viewed by Image Pro Plus Software and quantified at site 2 using both automated and manual methods [17].

Fasting blood chemistries

HbA_{1c}, lipid profile (total serum cholesterol, HDL, LDL, and triglycerides), creatinine, thyroid stimulating hormone, alanine aminotransferase, aspartate aminotransferase, fasting serum glucose, C-peptide, C reactive protein and adiponectin were assayed at a CLIA-certified laboratory (Sentara Laboratories, USA) along with vitamin B_{12} and the rapid plasma regain tests to exclude other causes of neuropathy.

Power calculations

Powering of the clinical study was based on variance data obtained in prior studies demonstrating efficacy of topiramate on IENFD in diabetic subjects [6]. A sample size of 20/group reflects the ability to detect a 20% deviation following treatment and was sufficient to identify therapy-induced improvements in NTTS-6 score using a similar study design [9].

Data analysis

Data are reported as group mean ± SEM or 95%CI for continuous variables and *n* (%) for categorical variables. For preclinical data, between-group comparisons were made by one or two-way ANOVA followed by the Holm–Sidak post hoc test (Prism, Graphpad, USA). For clinical data, normal distribution of each variable was confirmed by a normality test to ensure all appropriate assumptions were met for each statistical test. For categorical data, Chi-square or Fisher's exact tests were used to compare differences in baseline characteristics between groups. For continuous baseline data, parametric (two-sample Student's *t* test) and non-parametric tests (Wilcoxon signed-rank test) were used, depending on sample size and distribution. Two-sample Student's *t* test was used to compare treatment effect between groups for all outcomes. Repeated measures multivariate ANOVA (MANOVA) was used to analyse within-group change from baseline to endpoint for all primary and secondary outcomes. Where overall significance was attained, post hoc analysis was applied. Statistical analyses were performed using JMP Pro 10 software (SAS Institute Inc., USA), with risk of Type I error set at $\alpha = 0.05$.

Results

Neurite outgrowth in vitro

Oxybutynin (1.0–10^4 nmol/l) significantly ($p < 0.05$ vs 0.1nnmol/l) increased neurite outgrowth from sensory neurons of normal rats, with efficacy diminishing at higher concentrations (Fig. 2a). Oxybutynin (10–100 nmol/l) also increased neurite outgrowth in sensory neurons from STZ-diabetic mice ($p < 0.01$ vs 0 nmol/l: Fig. 2b).

Mitochondrial respiration in vitro

Oxybutynin (100 nmol/l) for 3 h significantly ($p < 0.05$ vs control) increased basal respiration, maximal respiration and spare respiratory capacity, but not coupling efficiency or respiratory control ratio (Fig. 2c–h). Increased mitochondrial respiration was accompanied by extracellular acidification (Supplementary Fig. 1), which can arise from glycolytic or non-glycolytic sources, and subsided 6–9 h after exposure.

Paw sensation in diabetic rodents

STZ-diabetic mice developed paw heat hypoalgesia after 8 weeks of diabetes (Fig. 3a) that persisted during 3–6 weeks of systemic oxybutynin treatment (3–10 mg/kg/day s.c.). Response latency of oxybutynin-treated groups was significantly ($p < 0.01$) faster than vehicle-treated diabetic mice by week 9 of treatment, while remaining significantly ($p < 0.05$) slower than controls. Similarly, db/db mice exhibited paw heat hypoalgesia that was significantly ($p < 0.001$ vs db/db mice treated with vehicle) diminished after 8 weeks of treatment with topical oxybutynin (50 µl of 3% in hydrogel, 30 min/day), although values remained significantly ($p < 0.01$) higher than controls (Fig. 3b). Efficacy did not extend to the untreated contralateral paw (Fig. 3c).

STZ-diabetic rats developed tactile allodynia (Fig. 3d) that persisted for 24 h following a single topical delivery of oxybutynin (50 µl of 3% in hydrogel for 30 min) to the paw, whereas a single injection of gabapentin (100 mg/kg i.p.) produced transient alleviation. Repeated topical delivery of oxybutynin (50 µl of 3% in hydrogel, 30 min/day) to the paw of STZ-diabetic rats with established allodynia (Fig. 3e) resulted in gradual alleviation of allodynia that was significantly ($p < 0.05$ or less) different from pre-treatment values after 2–5 weeks of treatment and persisted after treatment withdrawal. Efficacy was also detected in the contralateral untreated paw ($p < 0.05$ or less from pre-treatment).

Sensory nerve pathology

At study end, vehicle-treated db/db mice had significantly ($p < 0.01$ vs control) reduced paw IENFD, whereas in those treated with topical oxybutynin (50 µl of 3% in hydrogel, 30 min/day) values were not different from either controls or the untreated diabetic group (Fig. 4a). A similar pattern was seen for nerve profiles in the papillary dermis (Fig. 4b) and nerve occupancy within the corneal sub-basal nerve plexus (Fig. 4c) and stroma (Fig. 4d). Representative images of paw skin are shown in Supplemental Fig. 2.

Clinical recruitment and baseline characteristics

Between July 2015 and March 2017, 72 subjects were screened, of whom 21 failed due to uncontrolled diabetes, advanced chronic kidney disease or uncontrolled thyroid disease. Of the 51 subjects enrolled and randomized to either placebo (24 participants) or oxybutynin (27 participants), five subjects (4 on oxybutynin and 1 on placebo) withdrew during the trial due to perceived lack of benefit (Fig. 1). Demographics and baseline characteristics of the 46 subjects (23 of each group) who completed the study are shown (Table 1). There were no significant differences in any measured parameter between the groups assigned to placebo or oxybutynin. Of all baseline peripheral neuropathy characteristics, Total NIS-LL and proximal leg IENFD of subjects with skin biopsies suitable for quantification ($n = 19$ per group) were significantly different between the two groups ($p < 0.05$ and $p < 0.01$, respectively) while Total NIS, TNS, UENS and corneal nerve fibre density also trended towards

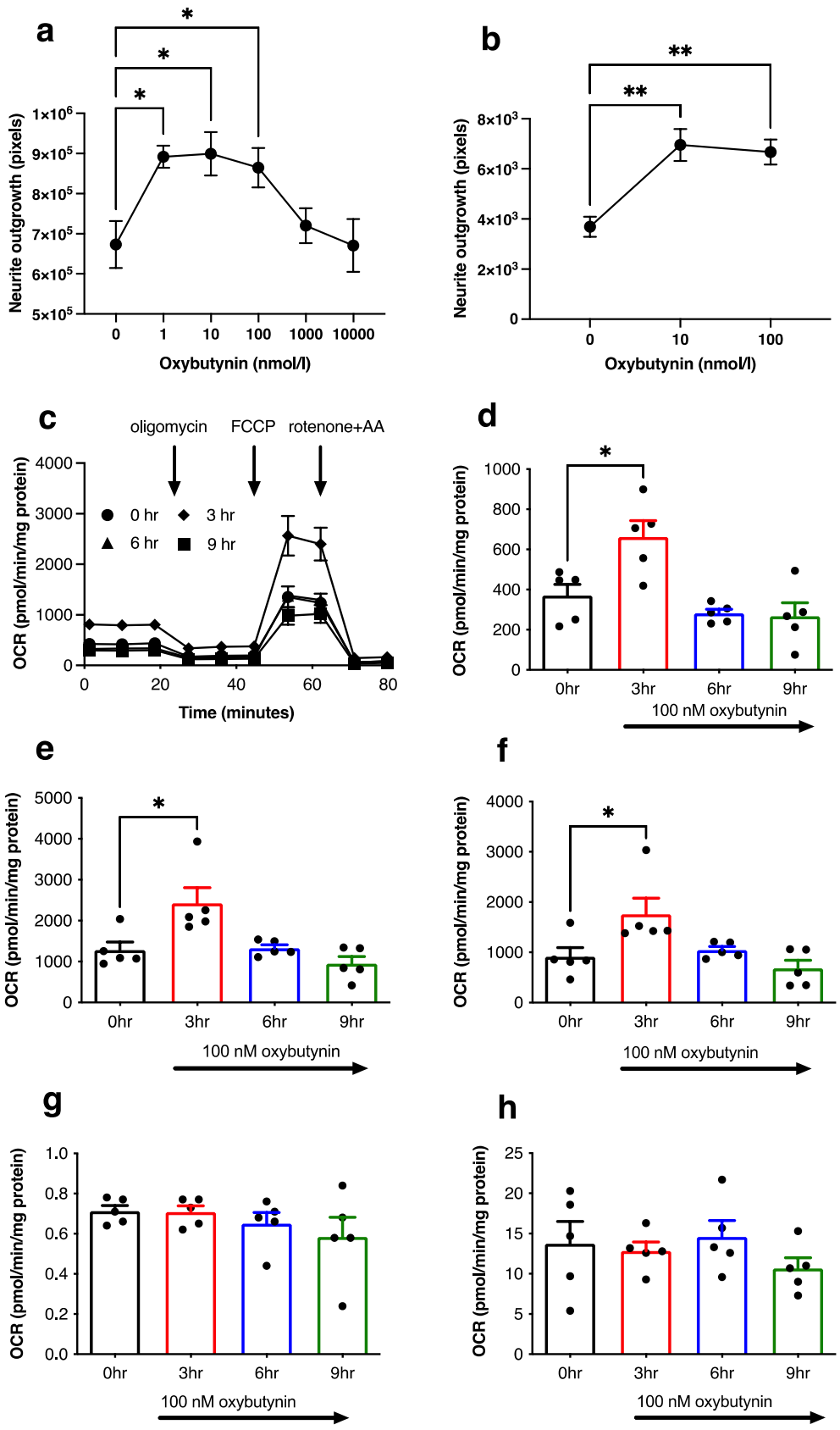

Fig. 2 Oxybutynin enhanced neurite outgrowth and mitochondrial function. Sensory neurons derived from the DRG of normal adult rats (**a**) or STZ-diabetic mice (**b**) were exposed to oxybutynin for 2 days and neurite density quantified. DRG from normal adult rats were exposed to oxybutynin for 3, 6 or 9 h before OCR was measured under basal conditions and following sequential addition of manipulators of respiratory chain function (**c**) to allow calculation of basal respiration (**d**), maximal respiration (**e**), spare respiratory capacity (**f**), coupling efficiency (**g**) and respiratory control ratio (**h**). Data are group mean ± SEM of $N = 6$ (panel a) or 5 (all other panels) replicates. Statistical analysis by one-way ANOVA with the Holm–Sidak post hoc test. $*p < 0.05$, $**p < 0.01$

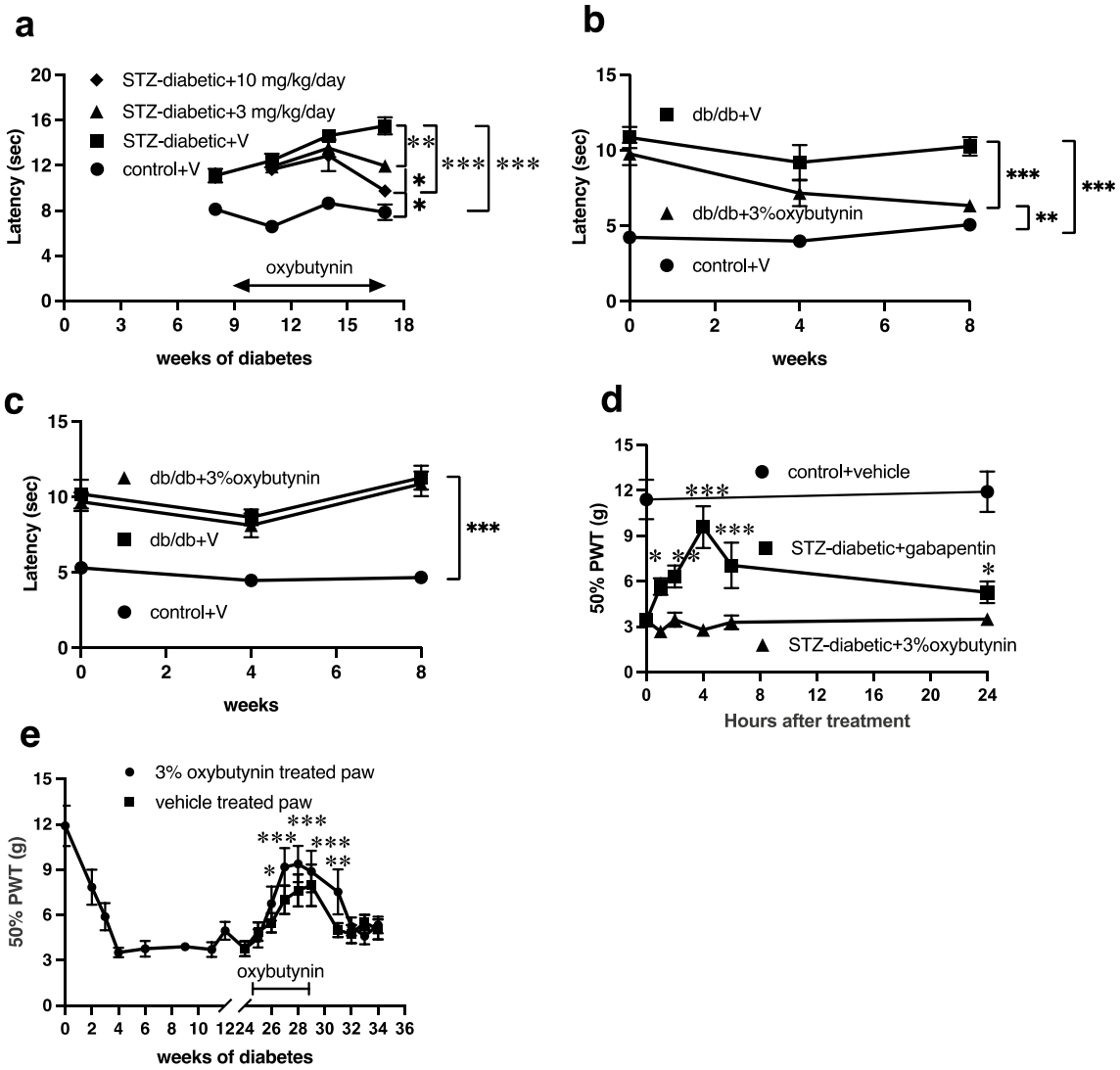

Fig. 3 Oxybutynin reverses heat hypoalgesia and tactile allodynia. **a** Paw withdrawal latency from heat in male STZ-diabetic Swiss Webster mice treated with systemic vehicle (V) or oxybutynin (3–10 mg/kg/day s.c.) between weeks 9–17 of diabetes. **b** Paw withdrawal latency from heat in female db/db mice treated with vehicle or topical oxybutynin (3%) applied 30 min/day to the right paw for 8 weeks. **c** Untreated contralateral paw of mice described in b. **d** 50% paw withdrawal threshold (PWT) of female Sprague Dawley control and STZ-diabetic rats following a single treatment with topical oxybutynin (3% to the hind paw for 30 min) or gabapentin (100 mg/kg ip). **e** 50% PWT of female Sprague–Dawley rats before (week 0) and after onset of STZ-induced diabetes and following repeated daily delivery of topical oxybutynin (3% to the hind paw for 30 min) for 5 weeks. Data are group mean ± SEM of $N = 7$–10/group. Statistical analysis on terminal measurements (**a**–**c**) by one-way ANOVA with the Holm–Sidak post hoc test. Statistical analysis within each group versus pre-treatment values (0 h in 3d, 24 wk in **e**) by repeated measures ANOVA with the Holm–Sidak post hoc test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

more extreme neuropathy in the group assigned to oxybutynin treatment (Table 2).

Adverse events

The most frequently reported adverse events (< 5% participants) were associated with mild anticholinergic reactions such as dry eye/mouth and minor application site reactions. Of the two serious adverse events reported, comprising one incidence of stroke (placebo) and one of hyponatremia (oxybutynin), neither was considered treatment related by the Data and Safety Monitoring Board. No adverse events caused withdrawal from the study.

Change from baseline

Of all metabolic parameters, only plasma C-reactive protein concentrations in the oxybutynin treated group were

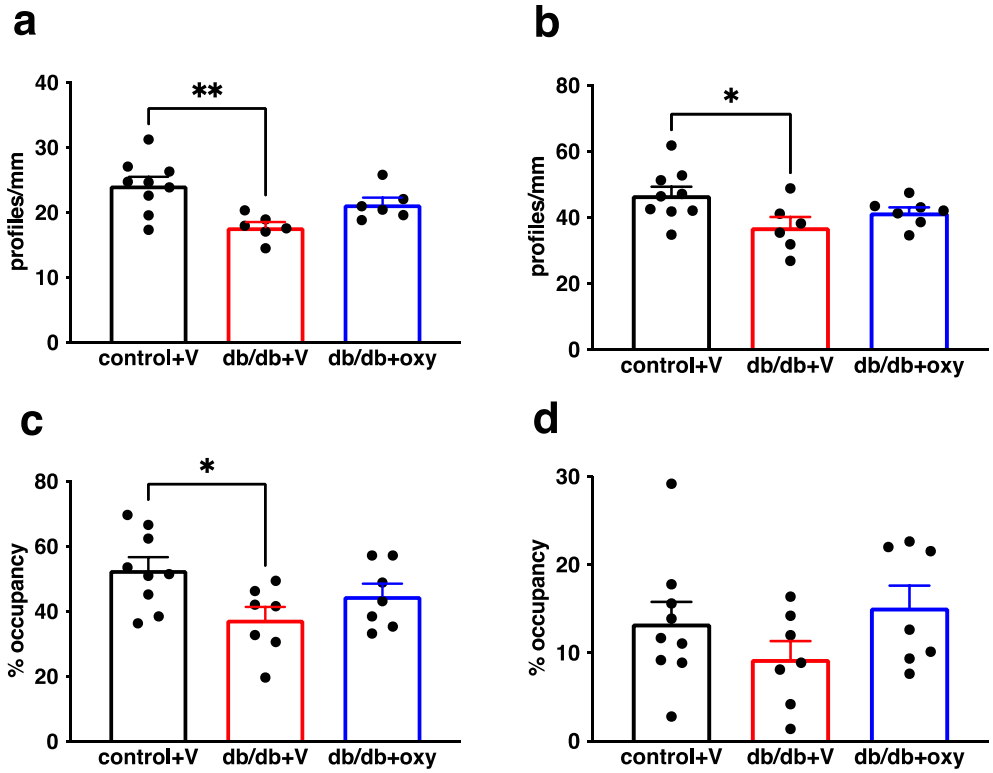

Fig. 4 Oxybutynin preserves small sensory fibre density. Quantification of nerve density in the epidermis (**a**) and papillary dermis (**b**) of hind paw plantar skin and in the corneal sub-basal nerve plexus (**c**) and stroma (**d**) of female control mice and db/db mice treated with vehicle (V) or topical 3% oxybutynin (oxy) applied 30 min/day to the right paw for 8 weeks. Data are group mean ± SEM of $N = 7$–10/group. Statistical analysis by one-way ANOVA with the Holm–Sidak post hoc test. *$p < 0.05$ vs vehicle-treated db/db mice

significantly ($p < 0.05$) changed compared to within-group baseline values (Table 3). The designated primary endpoint was change in proximal leg IENFD at study end compared to baseline (Fig. 5a, b). Placebo treatment (Fig. 5c) did not significantly alter IENFD (4.27 ± 0.38 vs 4.48 ± 0.63/mm of $n = 18$ readable pairs). In contrast, the oxybutynin treated group (Fig. 5d), showed a significantly ($p < 0.01$; paired t test) higher IENFD at study end compared to baseline (1.97 ± 0.49 vs 3.02 ± 0.74/mm of $n = 19$ readable pairs). The same pattern was observed when slides were read at a second independent site, with no change in IENFD in the placebo-treated group (3.00 ± 0.34 vs 3.82 ± 0.46/mm: Fig. 5e) but a significant ($p < 0.001$) increase above baseline in the oxybutynin treated group (1.90 ± 0.43 vs 3.54 ± 0.67/mm: Fig. 5f). Sprout density also showed no significant change in the placebo group (1.67 ± 0.22 vs 1.87 0.22/mm: Fig. 5g) whereas there was a significant ($p < 0.001$) increase above baseline in the oxybutynin-treated group (1.29 ± 0.32 vs 2.54 ± 0.54/mm: Fig. 5h).

Amongst the secondary end points oxybutynin, but not placebo, produced significantly improved clinical neuropathy scores (Table 4) in the NIS total score ($p < 0.05$), the NTSS-6 total score ($p < 0.02$) and its aching ($p < 0.02$) and lancinating ($p < 0.001$) pain components and in the NRS score for pain in the feet and legs ($p < 0.01$). The NTTS-6 score for burning pain also trended lower ($p = 0.056$). Both the QOL-DN total score and its physical/large fibre subcomponent showed significant ($p = 0.002$ and 0.001, respectively) improvements. There was no change in values for either group amongst measures of large fibre neve conduction, cardiac autonomic function, sudomotor function, corneal nerve structure (Table 5) or sweat gland innervation (Supplementary Fig. 3).

Discussion

We previously identified muscarinic antagonists as potential therapeutics for peripheral neuropathy using an unbiased in vitro drug screening approach [8]. The unanticipated neuritogenic property was isolated to agents that bind the M_1R sub-type and blocked by knockout or disruption of the M_1R. Potential mechanisms by which M_1R antagonism drives neuritogenesis include signalling through the CaMKKβ/AMPK/PGC-1α pathway to enhance mitochondrial function [8, 42] and regulation of the tubulin cytoskeleton to facilitate

Table 1 Baseline characteristics

	Oxybutynin ($n=23$)	Placebo ($n=23$)	p value
Age (years)	61.2 ± 1.7	62.3 ± 2.0	0.669
Gender F/M (%)	12/11 (52/48)	14/9 (61/39)	0.766
Ethnicity AA/C (%)	9/14 (39/61)	13/10 (57/43)	0.372
Diabetes duration (years)	15.59 ± 2.13	12.19 ± 1.83	0.116
# (%) receiving treatment[a]	11 (48%)	10 (44%)	0.723
Weight (kg)	106 ± 5	102 ± 5	0.266
BMI (kg/m^2)	35.23 ± 1.30	35.95 ± 1.94	0.62
Body fat %	38.41 ± 1.28	38.85 ± 2.14	0.57
Waist (cm)	117 ± 3	114 ± 4	0.23
Waist/hip ratio	0.99 ± 0.02	0.97 ± 0.02	0.167
Sitting SBP (mmHg)	139.52 ± 4.22	131.39 ± 3.63	0.076
Sitting DBP (mmHg)	81.13 ± 1.90	77.30 ± 2.50	0.115
Sitting HR (bpm)	76.70 ± 2.42	78.30 ± 3.72	0.641
Glucose (mmol/l)	10.7 ± 1.2	8.9 ± 0.7	0.187
Cholesterol (mmol/l)	4.98 ± 0.28	4.42 ± 0.23	0.133
Triglycerides (mmol/l)	2.74 ± 0.48	1.68 ± 0.21	0.082
HDL (mmol/l)	1.32 ± 0.10	1.47 ± 0.12	0.363
LDL (mmol/l)	2.54 ± 0.22	2.17 ± 0.18	0.193
C Peptide (nmol/l)	1.22 ± 0.17	1.21 ± 0.15	0.951
HbA_{1C} (mmol/mol)	64 ± 3	63 ± 2	0.731
HbA_{1C} (%)	8.05 ± 0.36	7.89 ± 0.33	0.731
Adiponectin (µg/ml)	7.30 ± 1.71	7.77 ± 1.92	0.855
Insulin (uIU/mL)	13.99 ± 2.16	20.29 ± 3.54	0.101
Creatinine (µmol/l)	84.87 ± 4.42	76.03 ± 3.54	0.121
Thyroid-stimulating hormone (mcU/mL)	1.60 ± 0.22	1.80 ± 0.23	0.539
Vitamin B12 (pg/mL)	991 ± 133	717 ± 103	0.111
C reactive protein (ng/mL)	0.90 ± 0.24	0.60 ± 0.21	0.357

Baseline characteristics for subjects who completed the study presented as group mean ± SEM %. Statistical analysis by Students' *t* test for continuous variables and Fisher's exact test for categorical variables

F/M female/male, *AA/C* African American/Caucasian, *BMI* body mass index, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *HR* heart rate, *HDL* high-density lipoprotein, *LDL* low-density lipoprotein

[a]Treatment for pain included SSRIs, SNRIs, pregabalin, gabapentin or tricyclic antidepressants

axonal transport [40, 41]. In the present study, oxybutynin, an antagonist of M_1, M_2 and M_3 receptor subtypes, was also neuritogenic, with maximal potency and magnitude of response consistent with that of the M_1R specific antagonist muscarinic toxin (MT-7) [8]. Neuritogenesis was accompanied by increased mitochondrial basal, maximal and spare respiratory capacity. The ability of oxybutynin to promote neurite outgrowth is pertinent as it is approved for clinical use and thus amenable for use investigating whether promoting neurite outgrowth in vitro predicts efficacy in restoring epidermal fibres and other disorders in patients with peripheral neuropathy.

The translational potential of oxybutynin was augmented by studies in diabetic rodents, with systemic or topical delivery to the paw reversing tactile allodynia and loss of paw heat sensation in models of type 1 and type 2 diabetes. Reduced nerve density in the skin and cornea of type 2 diabetic mice was attenuated by oxybutynin. Higher doses or longer durations of treatment may be required for more complete efficacy, as occurs using more selective M_1R antagonists in models of diabetes, chemotherapy-induced peripheral neuropathy and HIV neuropathy [8, 20, 23, 24, 42]. In contrast to repeated treatment strategies, a single application of oxybutynin to the paw of STZ-diabetic rats was without impact on established allodynia. This, along with the persistent alleviation of allodynia after treatment cessation, suggests modification of the painful neuropathy phenotype rather than acute anti-allodynic actions. As topical oxybutynin impacted corneal nerve density and alleviated allodynia (but not heat hypoalgesia) in the untreated contralateral paw, systemic distribution of effective concentrations of drug may occur beyond the application site. Oxybutynin applied to the skin of mice achieves peak plasma concentrations within 4 h and remains detectable for at least 24 h [34].

Table 2 Baseline neuropathy

	Oxybutynin ($n=23$)	Placebo ($n=23$)	p value
Skin biopsy			
Proximal leg IENFD ($n=19, 18$)	1.97 ± 0.49	4.13 ± 0.39	**0.01**
Neuropathy scores			
NSS	5.34 ± 0.59	4.44 ± 0.41	0.213
NIS Total (Motor + Sensory)	8.18 ± 0.95	5.94 ± 0.70	0.061
TNS (NSS + NIS)	12.99 ± 1.06	10.37 ± 0.75	0.059
NIS-LL Total (Motor + Sensory)	11.91 ± 1.19	8.26 ± 0.85	**0.044**
UENS Total	10.98 ± 1.13	8.13 ± 0.93	0.058
Pain scores			
NTSS-6	7.96 ± 1.34	5.30 ± 1.06	0.127
NRS—Pain feet & legs (0–10)	2.68 ± 0.77	2.55 ± 0.75	0.642
QOL-DN			
Total score	29.39 ± 4.95	21.26 ± 3.64	0.269
Physical/large fibre function	18.39 ± 3.53	13.22 ± 2.74	0.301
Activities of daily living	2.74 ± 0.65	1.83 ± 0.44	0.31
Symptoms	5.00 ± 0.70	4.22 ± 0.57	0.156
Small fibre function	2.00 ± 0.61	0.55 ± 0.22	0.17
Autonomic function	1.34 ± 0.34	1.22 ± 0.33	0.713
Cardiac autonomic function measures (CARTs & HRV)			
E/I ratio	1.09 ± 0.02	1.09 ± 0.02	0.92
Valsalva	1.18 ± 0.04	1.25 ± 0.06	0.303
Postural	1.06 ± 0.01	1.12 ± 0.03	0.071
Base SDNN	21.24 ± 20.3	25.05 ± 1.77	0.165
Base RMSSD	14.57 ± 1.89	17.38 ± 1.75	0.282
Base LFA	0.66 ± 0.15	0.98 ± 0.27	0.297
Base RFA	0.47 ± 0.12	0.67 ± 0.19	0.368
Base LFA/RFA	1.90 ± 0.39	1.67 ± 0.25	0.619
Sudomotor function			
Feet mean ESC	58.42 ± 4.37	55.96 ± 4.46	0.695
Hand mean ESC	54.42 ± 3.85	49.95 ± 4.27	0.442
Electrophysiology			
Peroneal ankle latency (ms)	5.38 ± 0.20	5.37 ± 0.27	0.973
Peroneal ankle amplitude (mV)	2.01 ± 0.28	2.20 ± 0.31	0.64
Peroneal BF latency (ms)	14.34 ± 0.40	14.11 ± 0.50	0.712
Peroneal BF amplitude (mV)	1.50 ± 0.21	1.41 ± 0.22	0.775
Peroneal BF C.V. (m/s)	35.81 ± 0.87	36.18 ± 0.89	0.766
Peroneal F-wave latency (ms)	54.58 ± 2.46	51.63 ± 2.58	0.413
Sural latency (ms)	3.62 ± 0.15	3.83 ± 0.18	0.383
Sural amplitude (μV)	4.49 ± 0.58	5.94 ± 1.16	0.275
Sural conduction velocity (m/s)	38.55 ± 1.59	37.19 ± 1.75	0.571
IVCCM			
CNFD (fibres/mm^2)	16.36 ± 1.16	20.20 ± 1.63	0.088
CNBD (branches/mm^2)	33.15 ± 6.76	37.85 ± 6.33	0.417
CNFL (mm/mm^2)	14.47 ± 1.64	16.46 ± 1.49	0.236

p values in bold font indicate $p < 0.05$

Data are presented as mean ± SEM. (%). Statistical analysis by Students' t test for continuous variables and Fisher's Exact test for categorical variables

Table 3 Change in metabolic parameters after 20 weeks of treatment

	Oxybutynin ($n=23$)			Placebo ($n=23$)		
	Baseline	20 weeks	p value	Baseline	20 weeks	p value
Weight (kg)	106 ± 5	106 ± 5	0.996	102 ± 5	100 ± 5	0.825
BMI (kg/m^2)	35.23 ± 1.30	35.33 ± 1.28	0.956	35.95 ± 1.94	35.58 ± 1.91	0.891
Body Fat %	38.41 ± 1.28	38.87 ± 1.21	0.795	38.85 ± 2.14	38.25 ± 1.74	0.828
Waist/hip ratio	0.99 ± 0.02	0.99 ± 0.02	0.751	0.97 ± 0.02	0.97 ± 0.02	0.996
Sitting SBP (mmHg)	139.5 ± 4.2	138.0 ± 4.4	0.809	131.4 ± 3.6	127.8 ± 3.5	0.477
Sitting DBP (mmHg)	81.13 ± 1.90	82.13 ± 1.69	0.696	77.30 ± 2.50	76.35 ± 1.99	0.766
Sitting HR (bpm)	76.70 ± 2.42	76.04 ± 2.80	0.861	78.30 ± 3.72	78.90 ± 3.32	0.911
Glucose (mmol/l)	10.7 ± 1.2	10.0 ± 1.0	0.645	8.9 ± 0.7	10.7 ± 1.3	0.223
Cholesterol (mmol/l)	4.98 ± 0.28	4.81 ± 0.25	0.703	4.42 ± 0.23	4.38 ± 0.23	0.808
Triglycerides (mmol/l)	2.74 ± 0.48	3.11 ± 0.53	0.533	1.68 ± 0.21	1.76 ± 0.23	0.815
HDL (mmol/l)	1.32 ± 0.10	1.28 ± 0.10	0.748	1.47 ± 0.12	1.47 ± 0.12	0.995
LDL (mmol/l)	2.54 ± 0.22	2.47 ± 0.21	0.809	2.17 ± 0.18	2.15 ± 0.19	0.956
C Peptide (nmol/l)	1.22 ± 0.17	1.59 ± 0.25	0.222	1.21 ± 0.15	1.29 ± 0.24	0.765
HbA_{1C} (mmol/mol)	64 ± 3	64 ± 3	0.966	63 ± 2	67 ± 3	0.480
HbA_{1C} (%)	8.05 ± 0.36	8.03 ± 0.38	0.966	7.89 ± 0.33	8.26 ± 0.42	0.480
Adiponectin (µg/ml)	7.30 ± 1.71	6.67 ± 1.33	0.774	7.77 ± 1.92	7.75 ± 1.67	0.992
Insulin (µIU/mL)	13.99 ± 2.16	22.85 ± 3.81	0.066	20.29 ± 3.52	23.09 ± 5.01	0.503
Creatinine (mg/dl)	84.87 ± 4.42	83.10 ± 5.30	0.793	76.03 ± 3.54	77.79 ± 3.53	0.661
C Reactive protein (ng/mL)	0.90 ± 0.24	0.43 ± 0.07	**0.036**	0.60 ± 0.21	0.61 ± 0.21	0.896
HOMA2%B (%)	56.6 ± 9.7	71.5 ± 9.1	0.104	82.9 ± 13.8	68.1 ± 11.4	0.196
HOMA2%S (%)	69.81 ± 11.02	52.96 ± 10.6	0.435	57.56 ± 9.80	49.51 ± 9.17	0.45
HOMA2 IR	2.88 ± 0.65	3.55 ± 0.60	0.613	2.96 ± 0.50	3.70 ± 0.75	0.642
QUICKI index	0.31 ± 0.01	0.29 ± 0.01	0.352	0.30 ± 0.01	0.29 ± 0.01	0.346

Data aer presented as mean ± SEM. Within-group statistical analysis by repeated measures MANOVA

p value in bold font indicates $p < 0.05$

The selective M_1R antagonist pirenzepine also shows efficacy distant to application site in diabetic mice, suggesting systemic distribution [23]. Efficacy of oxybutynin against indices of degenerative neuropathy and neuropathic pain in models of both type 1 and type 2 diabetes, efficacy as a topical preparation both locally and systemically, efficacy in both sexes and reversal of established indices of neuropathy encouraged an exploratory study to determine whether oxybutynin could reverse neuropathy in diabetic subjects.

Oxybutynin is widely used to treat overactive bladder due to combined anti-muscarinic, spasmolytic and local anaesthetic actions [29]. Topical formulations [30] reduce anticholinergic side effects and avoid first pass metabolism of oxybutynin [2, 43]. We performed a single site, randomized, double-blind, placebo-controlled interventional trial of topical oxybutynin in subjects with type 2 diabetes and established diabetic peripheral neuropathy. Oxybutynin had no effect on metabolic parameters of diabetes, other than a currently inexplicable reduction of plasma C-reactive protein levels and thus is unlikely to have exerted effects via improved diabetic state. Randomization inadvertently produced an oxybutynin-assigned group with significantly lower IENFD and trends to lower corneal nerve morphometric parameters, perhaps reflecting their greater sensitivity to distal small fibre neuropathy compared to other biometric or subjective assays [3, 12]. This was mitigated by our a priori decision to use within-group change from baseline as the primary analysis. The finding that IENFD and epidermal sprouting improved significantly only in the oxybutynin-treated group indicates that our preclinical assays successfully predicted clinical efficacy. Despite an almost doubled IENF density, oxybutynin did not fully restore normal values [27], perhaps reflecting the severity of permanent IENF loss in this cohort prior to treatment or sub-optimal location, duration and/or dose of oxybutynin. It is notable that both regrowth from the dermis and arborization of epidermal fibres were increased. Prior studies in diabetic subjects with neuropathy have reported that supervised exercise or simultaneous pancreas and kidney transplantation improve measures of epidermal arborization, but not regrowth from the dermis [3, 25]. In contrast, prior pharmacological interventions directed at specific pathogenic mechanisms such as an antioxidant cocktail [37], GLP-1 receptor agonists [21]

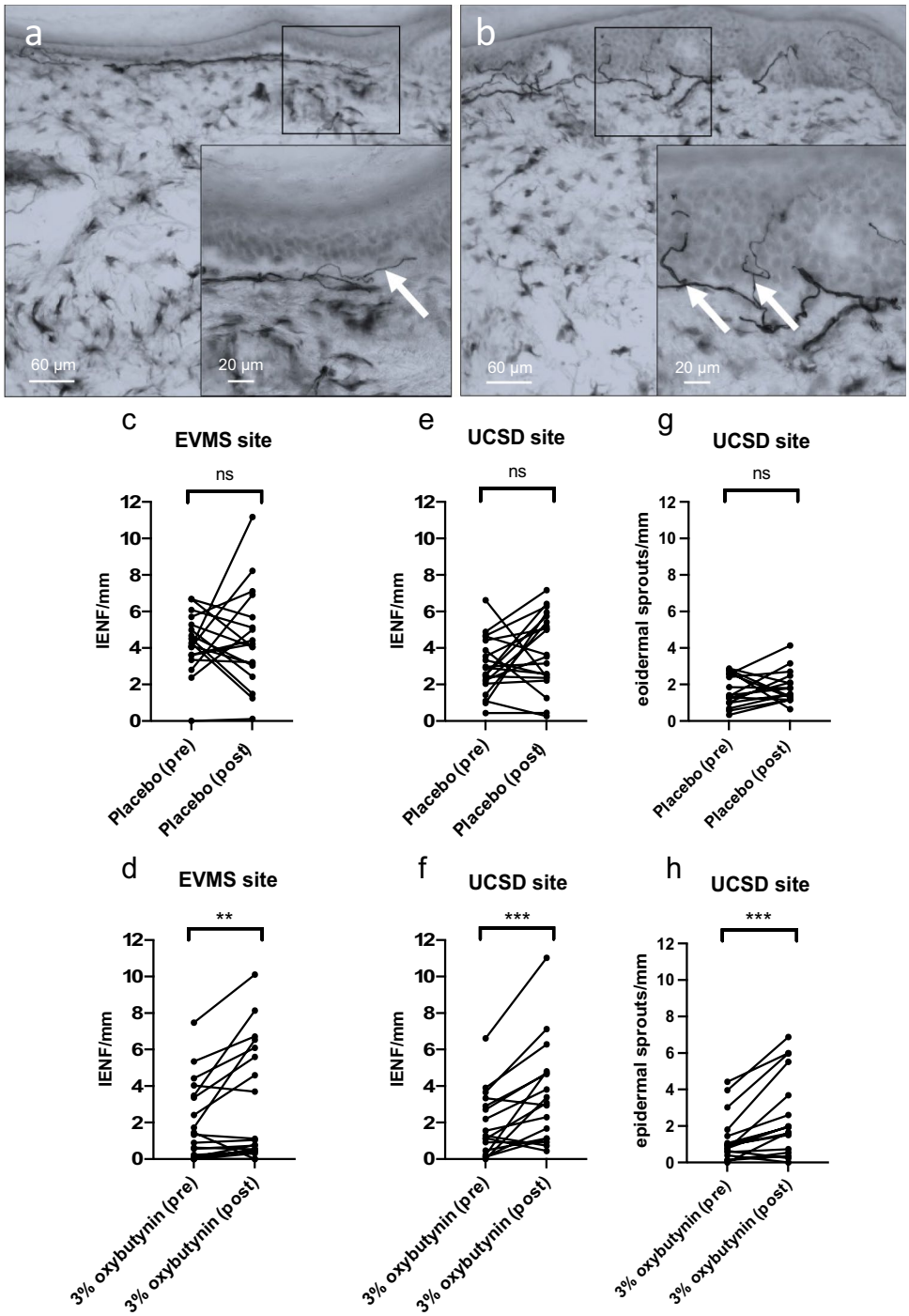

Fig. 5 Topical oxybutynin increases sensory innervation in diabetic subjects. Representative images of PGP9.5 immunostained IENF nerves in human skin biopsies collected at baseline (**a**) and after 5 months of treatment with topical 3% oxybutynin (**b**). Fibres crossing the dermal–epidermal interface are indicated by white arrows. Change in IENFD, as quantified at the primary (EVMS: **c**–**d**) and secondary (UCSD: **e**–**f**) analysis sites in subjects treated with topical placebo or 3% oxybutynin. Also, change in epidermal fibre sprouts, as quantified at the secondary (UCSD: **g**–**h**) analysis site in subjects treated with topical placebo or 3% oxybutynin. Data are individual subject values in biopsies collected at onset (pre) and after 5 months of treatment (post). Statistical analysis within each group versus pre-treatment values by paired *t* test. $^{**}p < 0.01$, $^{***}p < 0.001$. Bar = 20 or 60 µm

and growth factors derived from mesenchymal stem cells [18] did not improve IENFD in diabetic subjects.

Multiple secondary endpoints were assessed, although the clinical study was not specifically powered for them. Of the biometric endpoints, there was no significant impact of topical oxybutynin on any component of autonomic function, sweat gland nerve fibre density, large fibre nerve conduction or corneal nerve morphometry. This contrasts with our preclinical data where topical oxybutynin was effective at protecting corneal nerve density in db/db mice and also published preclinical studies in which systemic or topical muscarinic antagonists dose-dependently increased large fibre nerve conduction and corneal nerve density in diabetic mice [8, 23]. Notably, the non-selective muscarinic antagonist atropine prevents corneal nerve loss in diabetic mice only when applied to the eye, not to the paw [23], suggesting dependence on local drug concentrations. Efficacy of topical oxybutynin in driving epidermal re-innervation may reflect

Table 4 Change in clinical neuropathy after 20 weeks of treatment

	Oxybutynin ($n=23$)			Placebo ($n=23$)		
	Baseline	20 weeks	p value	Baseline	20 weeks	p value
Clinical neuropathy scores						
NSS	5.34 ± 0.59	6.10 ± 0.78	0.697	4.44 ± 0.41	4.95 ± 0.46	0.522
NIS total (Motor + Sensory)	8.01 ± 0.87	6.09 ± 0.76	**0.049**	5.94 ± 0.70	5.44 ± 0.59	0.425
TNS (NSS + NIS)	13.17 ± 1.00	12.19 ± 1.12	0.351	10.37 ± 0.75	10.39 ± 0.73	0.993
NIS-LL total (Motor + Sensory)	11.48 ± 1.12	9.35 ± 0.91	0.152	8.26 ± 0.85	8.13 ± 0.85	0.874
UENS total score	10.98 ± 1.13	8.85 ± 0.78	0.165	8.13 ± 0.93	8.34 ± 0.99	0.803
NTSS-6 total score	7.61 ± 1.33	5.54 ± 1.26	**0.021**	5.30 ± 1.06	4.01 ± 0.91	0.371
NTSS-6 pain quality: Aching	1.51 ± 0.30	0.89 ± 0.29	**0.018**	0.67 ± 0.21	0.82 ± 0.25	0.644
Lancinating	1.45 ± 0.29	0.62 ± 0.27	**0.009**	0.94 ± 0.29	0.38 ± 0.17	0.088
Burning	1.23 ± 0.29	0.72 ± 0.26	0.056	0.65 ± 0.25	0.45 ± 0.19	0.543
Prickling	1.45 ± 0.24	1.10 ± 0.24	0.265	1.19 ± 0.21	0.98 ± 0.21	0.491
Numbness	1.84 ± 0.26	1.44 ± 0.31	0.315	1.22 ± 0.27	0.92 ± 0.22	0.341
Allodynia	0.82 ± 0.26	0.51 ± 0.26	0.151	0.48 ± 0.22	0.65 ± 0.23	0.429
NRS—pain feet and legs	2.68 ± 0.77	0.56 ± 0.39	**0.003**	2.55 ± 0.75	1.25 ± 0.51	0.191
QOL-DN						
Total score	29.39 ± 4.95	17.29 ± 3.10	**0.002**	21.26 ± 3.64	16.41 ± 3.13	0.113
Physical/large fibre function	18.39 ± 3.53	10.18 ± 2.12	**0.001**	13.22 ± 2.74	10.32 ± 2.30	0.155
Activities of daily living	2.74 ± 0.65	1.53 ± 0.44	0.163	1.83 ± 0.44	1.27 ± 0.42	0.192
Symptoms	5.00 ± 0.70	3.47 ± 0.61	0.078	4.22 ± 0.57	3.59 ± 0.52	0.109
Small fibre function	2.00 ± 0.61	1.12 ± 0.38	0.264	0.55 ± 0.22	0.27 ± 0.14	0.101
Autonomic function	1.34 ± 0.34	1.00 ± 0.30	0.553	1.22 ± 0.33	0.96 ± 0.28	0.388

Data are presented as mean ± SEM. Within-group statistical analysis by repeated measures MANOVA

p values in bold font indicate $p < 0.05$

high local concentrations insufficient to impact more distant nerve terminals in places such as dermal sweat glands, skeletal muscle and the cornea. To address this, future studies could assess efficacy of increased topical dose or alternative routes of delivery. While nerve conduction slowing and corneal nerve depletion are sensitive biomarkers of onset and progression of peripheral neuropathy [36], they may not always reliably detect drug efficacy at local sites in the skin.

Of the physician and patient reported outcomes studied, significant improvements in NIS and NTSS-6 total scores of the oxybutynin-treated group were largely driven by alleviation of lancinating, aching and burning pain. Pain relief was confirmed using the NRS scale while significant improvement in the Norfolk QOL-DN scores was driven largely by impact on large fibre function. Our clinical study population was not selected specifically for pain and baseline scores indicate only mild to moderate pain. Clinical trials of analgesics targeting painful diabetic neuropathy generally pre-select patients with moderate to severe pain and whether oxybutynin alleviates more severe neuropathic pain awaits investigation. There is clearly not a simple association between loss of sensory nerve fibres and pain in diabetic neuropathy—equivalent IENF loss is found in patients with painless neuropathy [33]. Further, oxybutynin, like other antimuscarinics [8], did not act as an acute analgesic in diabetic rodents. Notably, the time course of alleviation of allodynia paralleled onset of efficacy against indices of degenerative neuropathy. It is possible that muscarinic antagonists drive the gradual correction of a broad neuropathy phenotype, of which pain is one of many diverse manifestations. A similar concurrence has recently been reported following use of topical capsaicin, which initially evokes acute sensory loss and IENF depletion [32] but ultimately provokes a regenerative response with increased IENFD and reduced pain [1].

Use of oxybutynin in this proof-of-concept study was driven by availability for short-term off-label use in a topical formulation that minimizes side effect profile [47]. Long-term oxybutynin use is likely to be problematic due to concerns that accumulated exposure to CNS-penetrating anticholinergics can impede cognition—particularly in the elderly or those compromised by other diseases [13, 52]. We do not propose or encourage use of oxybutynin for long-term treatment of diabetic neuropathy and await clinical studies of selective, CNS-impermeable, M_1R specific/selective antagonists with more restricted side effect profiles. Nevertheless, this study has demonstrated that a therapeutic concept identified using an in vitro screening program and supported

Table 5 Change in secondary neuropathy measures after 20 weeks of treatment

	Oxybutynin ($n=23$)			Placebo ($n=23$)		
	Baseline	20 weeks	p value	Baseline	20 weeks	p value
Cardiac autonomic function						
E/I Ratio	1.09 ± 0.02	1.09 ± 0.02	0.936	1.09 ± 0.02	1.10 ± 0.02	0.752
Valsalva ratio	1.18 ± 0.04	1.19 ± 0.04	0.719	1.25 ± 0.06	1.18 ± 0.03	0.332
Postural (30:15) ratio	1.06 ± 0.01	1.08 ± 0.02	0.378	1.12 ± 0.03	1.10 ± 0.02	0.433
SDNN	21.24 ± 20.3	29.43 ± 4.85	0.127	25.05 ± 1.77	26.24 ± 2.90	0.718
RMSSD	14.57 ± 1.89	22.86 ± 4.85	0.119	17.38 ± 1.75	18.24 ± 2.37	0.769
LFA	0.66 ± 0.15	0.82 ± 0.22	0.545	0.98 ± 0.27	1.70 ± 0.55	0.223
RFA	0.47 ± 0.12	0.83 ± 0.20	0.131	0.67 ± 0.19	1.29 ± 0.57	0.271
LFA/RFA ratio	1.90 ± 0.39	1.65 ± 0.35	0.625	1.67 ± 0.25	1.52 ± 0.22	0.274
Sudomotor function						
Feet Mean ESC (μS)	58.42 ± 4.37	57.18 ± 4.70	0.849	55.96 ± 4.46	54.40 ± 5.57	0.827
Hand Mean ESC (μS)	54.42 ± 3.85	48.55 ± 3.53	0.268	49.95 ± 4.27	42.75 ± 4.60	0.258
Electrophysiology						
Peroneal ankle latency (ms)	5.38 ± 0.20	5.12 ± 0.18	0.336	5.37 ± 0.27	5.51 ± 0.31	0.744
Peroneal ankle amplitude (mV)	2.01 ± 0.28	1.91 ± 0.29	0.81	2.20 ± 0.31	2.17 ± 0.37	0.944
Peroneal BF latency (ms)	14.34 ± 0.40	14.26 ± 0.43	0.884	14.11 ± 0.50	14.32 ± 0.69	0.809
Peroneal BF amplitude (mV)	1.50 ± 0.21	1.37 ± 0.21	0.674	1.41 ± 0.22	1.75 ± 0.30	0.381
Peroneal BF velocity (m/s)	35.81 ± 0.87	35.01 ± 0.81	0.51	36.18 ± 0.89	36.08 ± 1.36	0.951
Peroneal F-wave latency (ms)	54.58 ± 2.46	53.06 ± 3.99	0.739	51.63 ± 2.58	48.73 ± 3.36	0.511
Sural latency (ms)	3.62 ± 0.15	3.74 ± 0.12	0.524	3.83 ± 0.18	3.69 ± 0.13	0.542
Sural amplitude (μV)	4.49 ± 0.58	5.66 ± 1.13	0.358	5.94 ± 1.16	6.47 ± 0.94	0.723
Sural velocity (m/s)	38.55 ± 1.59	37.70 ± 1.05	0.662	37.19 ± 1.75	39.81 ± 1.38	0.244
Corneal nerve						
CNFD (fibres/mm^2)	16.36 ± 1.16	16.88 ± 1.30	0.769	20.20 ± 1.63	20.20 ± 1.75	0.990
CNBD (branches/mm^2)	33.15 ± 6.76	31.60 ± 5.79	0.863	37.85 ± 6.33	37.72 ± 4.51	0.855
CNFL (mm/mm^2)	14.47 ± 1.64	14.05 ± 1.57	0.855	16.46 ± 1.49	15.67 ± 1.22	0.724

Data are presented as mean ± SEM. Within-group statistical analysis by repeated measures MANOVA

by efficacy against appropriate endpoints in rodents can translate to demonstrable benefits in humans with diabetic neuropathy.

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Author contributions The original project was conceived by NAC and PF. NAC, PF, CMC, HKP and AIV contributed to the design of the study. Data acquisition was performed by CMC, HKP, KEF, AM, DRS, RN, LG, AT, and JW. All authors contributed to data analysis and the original draft of the manuscript. NAC, PF, CMC, HKP, KEF and CGJ were involved in reviewing and editing the manuscript. All authors have approved the final manuscript. NAC serves as the guarantor of the work, with full access to all study data, and is responsible for the integrity and accuracy of the data analysis.

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Data availability Data are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest NAC and PF are co-founders of, and hold equity in WinSanTor Inc., a company which is developing products related to the research described in this paper. The terms of this arrangement have been reviewed and approved by the University of California, San Diego in accordance with its conflict-of-interest policies. The other authors declare that there are no relationships or activities that might bias, or be perceived to bias, their work.

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