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Omega-3 supplementation is neuroprotective to corneal nerves in dry eye disease: a pilot study

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ABSTRACT

Purpose: To investigate whether oral, long-chain omega-3 (ω-3) essential fatty acid (EFA) supplementation, for three months, induces changes to the central corneal sub-basal nerve plexus in dry eye disease and whether nerve alterations correlate with clinical findings.

Methods: This prospective, comparative study involved the final 12 participants enrolled in a randomised, double-masked, placebo-controlled clinical trial of 60 participants with moderate dry eye disease. Participants received either placebo (olive oil 1500mg/day; n=4) or ω-3 EFA supplements (~1000mg/day eicosapentaenoic acid + ~500mg/day docosahexaenoic acid; n=8) for 90 days. The main outcome measure was the mean change in central corneal sub-basal plexus nerve parameters between days one and 90, quantified using *in vivo* confocal microscopy. Secondary outcomes included mean change in tear osmolarity, corneal dendritic cell density and basal epithelial cell density.

Results: Compared with baseline, the reduction in OSDI score and tear osmolarity at day 90 were greater in the ω-3 EFA group than the placebo group (OSDI: ω-3 EFA, mean±SEM: -15.6±2.8 versus placebo: -2.8±4.1 units, $t(5)=2.6$, p=0.04; osmolarity: ω-3 EFA: -22.63±5.7 versus placebo: -8±2.7 mOsmol/L, $t(9)=2.3$, p=0.04). At day 90, corneal total nerve branch density (CTBD: 91.1±8.6 versus 45.1±13.4 branches/mm^2, $F_{1,10}=14$, p=0.004) and corneal nerve branch density on the main fibre (CNBD: 63.4±6.5 versus 27.9±11.5 branches/mm^2, $F_{1,10}=6$, p=0.03) were higher in the ω-3 EFA group compared with placebo. Relative to day 1, CNBD (branches/mm^2) increased at day 90 in the ω-3 EFA group (+20.0±9.2, $t(8)=3.2$ p=0.01) compared with placebo (-10.8±3.2). Similar changes were evident for corneal nerve fibre length (CNFL, mm/mm^2), which increased from baseline at day 90 in the omega-3 EFA group (+2.9±1.6, $t(8)=3.4$ p=0.01) compared with placebo (-2.7±0.5). There was a negative correlation between CTBD and tear osmolarity ($r(10)=-0.70$, p=0.01). No significant changes were observed for basal epithelial cell or corneal dendritic cell density.

Conclusion: These pilot study findings suggest that ω-3 EFA supplementation imparts neuroprotective effects in the corneal sub-basal plexus that correlate with the extent of tear osmolarity normalisation.

MAIN TEXT

Introduction

Dry eye disease (DED) is a major public health issue, owing to its high prevalence, negative impact upon quality of life and financial burden on healthcare systems.[1] Although the pathogenesis of DED is not fully understood, the condition is recognised to involve an immune-based inflammation of the anterior eye.[2] DED is characterised by tear hyperosmolarity,[3, 4] which may be triggered by lacrimal gland dysfunction and/or excessive tear evaporation. Tear hyperosmolarity promotes ocular surface inflammation,[5] leading to corneal and conjunctival epithelial cell apoptosis and inflammatory events that culminate in the loss of mucin-secreting goblet cells.[6] Together, these changes exacerbate tear film instability and perpetuate a pro-inflammatory cycle that promotes chronicity.

Given the role of inflammation in its pathogenesis, anti-inflammatory agents are often prescribed to manage the clinical signs and symptoms of DED. In addition to topical corticosteroids and cyclosporine-A, a novel immuno-modulatory agent (lifitegrast) that reduces T-lymphocyte mediated inflammation, has recently been approved by the United States of America Federal Drug Administration, for the therapeutic treatment of DED.[7] A further therapeutic option involves omega-3 (ω-3) essential fatty acid (EFA) supplementation. Omega-3 EFAs bias systemic prostaglandin metabolism towards the production of anti-inflammatory eicosanoids, which limit and resolve inflammation.[8] As summarised in a recent systematic review and meta-analysis,[9] several small clinical trials have investigated the use of oral ω-3 EFA supplements for treating DED. Together, these investigations report promising findings in terms of reducing dry eye symptoms and improving tear stability, however the mechanisms underlying the potential clinical benefits are not entirely understood.

There is increasing scientific interest in the role of neurosensory dysfunction in the aetiology of DED.[10] Patients with DED demonstrate changes to corneal sensation and a decreased number and density of sub-basal corneal nerves.[11, 12] Although yet to be demonstrated clinically, rationale for a link between corneal neuropathy and elevated tear osmolarity derives from recent experimental studies.[13, 14] Specifically, a negative correlation has been reported between corneal nerve fibre length and an acute hyperosmolar environment in a diabetic rat model.[13] Exposure to hyperosmolar solutions in another rodent model has been shown to reduce the physiologic sensitivity and morphologic integrity of the corneal nerves.[14]

In addition to notable structural and functional changes to corneal innervation in patients with DED, the effect of this inflammatory condition also extends, unsurprisingly, to the immune status and cellular integrity of the epithelium. Patients with DED, as diagnosed by standardised symptom scores and clinical signs, such as corneal sodium fluorescein staining, have increased numbers of epithelial dendritic cells (DCs)[15, 16] and increased numbers of basal epithelial cells; the latter effect is considered to be a hyperproliferative response that attempts to repair the ocular surface damage that occurs in DED.[16] Treatment with topical corticosteroids for 30 days can reduce the density of epithelial DCs (DCD), and this is associated with improved OSDI scores and other clinical signs.[17] However, the relationship between tear osmolarity and changes to DCD and/or epithelial cell density has not been directly examined.

In relation to the potential benefits of ω-3 EFA supplementation on altering corneal neuropathy, docosahexaenoic acid (DHA), has been previously shown to promote the regeneration of corneal nerves in an experimental rabbit model of corneal stromal nerve resection.[18] However, the potential effect of ω-3 EFA supplementation on corneal nerves and/or the corneal immune response in human DED remains unknown.

The major aim of this pilot study was to investigate whether supplementation with oral, long-chain ω-3 EFAs induces changes to the architecture of the central corneal sub-basal nerve plexus in people with DED and whether nerve alterations correlate with tear

osmolarity measures. The secondary objective was to assess the effect of ω-3 EFA supplementation on the corneal immune response (dendritic cell density/DCD) and corneal epithelial homeostasis (basal cell density).

METHODS

This project was conducted in accordance with the tenets of the Declaration of Helsinki and approved by the University of Melbourne Human Research Ethics Committee.

Participants

Participants were recruited from the University of Melbourne eye care clinic (Victoria, Australia), a community-based optometry clinic, and all provided written informed consent to participate in the study. Eligibility criteria, which have been previously reported in detail,[19] enabled the recruitment of adults with moderate DED, defined as an Ocular Surface Disease Index (OSDI) symptom score≥ 18 and < 65 and tear osmolarity ≥ 316 mOsmol/L in at least one eye. In addition, potential participants needed to have distance best-corrected visual acuity≥ 20/40 (Snellen equivalent) in each eye and intra-ocular pressure ≤ 21 mmHg in both eyes.

The study exclusion criteria involved: (i) general health conditions: the presence of uncontrolled systemic disease, diabetes, bipolar disorder, atrial fibrillation, implanted defibrillator, familial adenomatous polyposis, systemic immunocompromise, bleeding disorders or history of liver disease, (ii) a major change to diet or dietary supplement intake in the three months prior to enrolment, (iii) consumption of essential fatty acid oral supplements in the three months prior to enrolment, (iv) consumption of systemic anti-coagulants, (v) known allergy or sensitivity to the study supplements or any of their components, (vi) females of childbearing potential who were planning a pregnancy over the course of the study, or currently pregnant or breastfeeding, (vii) anticipated contact lens wear during the study or contact lens wear in the month prior to enrolment, (viii) scheduled or planned ocular or systemic surgery during the study, (ix) presence of severe dry eye at baseline (defined as either an OSDI score > 65, or, corneal or conjunctival sodium fluorescein staining of Grade 5 (Oxford scheme) in any zone of either eye), (x)

the start date of any systemic medication which may affect tear film or vision being less than three months prior to enrolment or when a change in dosage was anticipated during the study, (xi) presence of any of: active ocular infection or non-keratoconjunctivitis sicca ocular inflammation, active ocular allergy, history of recurrent herpes keratitis or active disease within six months of baseline, a corneal disorder or abnormality that affects corneal sensitivity or normal spreading of the tear film (except superficial punctate keratitis), severe blepharitis, which in the judgement of the investigator, may interfere with the interpretation of the study results, (xii) occlusion of the lacrimal puncta, with either punctal plugs or cauterisation, in the three months prior to enrolment, (xiii) history of ocular surgery/trauma, which could affect corneal sensitivity and/or tear distribution within six months of enrolment, or (xiv) the use of any of the following topical medications in the three months prior to baseline: corticosteroids, non-steroidal anti-inflammatories or cyclosporine.

Data were collected at days 1 (baseline) and 90±7 from 12 participants, being the final 12 enrolments from a larger randomised, double-masked, placebo-controlled clinical trial (Australian New Zealand Clinical Trials Registry, ACTRN12614001019695), involving 60 participants, who attended for monthly study visits over three months.[19] Corneal nerve data were unable to be captured for earlier enrolments as we only acquired the corneal confocal microscope to enable corneal nerve imaging during the later stages of participant recruitment for the larger study.

Participants were instructed to maintain their current dietary habits. As previously described,[19] the approximate dietary intake of omega-3 EFAs was determined by asking participants about their consumption of ω-3–rich foods over the preceding month. Participants were asked to quantify the approximate serving size (25g, 50g, 100g, 150g) and frequency of consumption of foods containing greater than 1g of combined EPA, DHA, docosapentaenoic acid and α-linolenic acid per 100g edible portion (Australia New Zealand Food Authority, 2011; Nutrient Data Laboratory and Beltsville Human Nutrition Research Centre, 2011).

Interventions

Participants were randomly assigned to either placebo (olive oil, 1500mg/day) or long-chain ω-3 EFA supplements (~1000mg/day eicosapentaenoic acid [EPA] + ~500mg/day docosahexaenoic acid [DHA] in either re-esterified triacylglyceride or phospholipid forms) for 90 days, as previously described.[19] In brief, an independent data manager generated a participant randomisation sequence using a random number generator in Microsoft Excel (Microsoft Corporation, Redmond, Washington, 2007). This randomisation schedule was provided to an independent compounding pharmacist (Dartnell's Pharmacy, Victoria, Australia) who repackaged the study supplements into identical, opaque containers using the randomisation schedule. Eligible participants were sequentially enrolled by a masked research optometrist, who dispensed the investigational product labelled with the appropriate randomisation code.

Four participants received placebo and eight participants received an ω-3 EFA intervention. All participants were instructed to consume five capsules each day. Participants and all study personnel were masked to treatment allocations throughout the study. Compliance was assessed by participants returning supplement containers, with unused capsules, at each follow-up visit for counting by an independent researcher.

Study assessments

Study assessments were performed at days one and 90. Participants underwent thorough anterior eye examinations, including the assessment of: best-corrected visual acuity, DED symptoms (OSDI; Allergan, USA), tear osmolarity (http://www.tearlab.com), comprehensive slit lamp examination and corneal imaging using *in vivo* confocal microscopy (HRT3 laser scanning confocal microscope with Rostock Corneal Module; https://business-lounge.heidelbergengineering.com/int/products/hrt/#Rostock-Cornea-Module). The tear osmometer was calibrated according to the manufacturer's instructions and room temperature was maintained between 20ºC and 24ºC to ensure measurement accuracy.[20] Participants using topical lubricant eye drops at baseline were permitted to continue using these during the study. At each visit, the investigator questioned participants with regard to how frequently they had used lubricant eye drops over the past

month. Participants using lubricating eye drops were instructed not to instil these for at least two hours prior to study appointments, which was confirmed prior to taking measurements. Right eyes were measured first.

Image analyses

Central corneal images were collected using the HRT3 laser scanning confocal microscope with Rostock Corneal Module as previously described,[21] from eyes having the highest tear osmolarity at baseline (day 1). Several sequence scans, focused at the level of the corneal sub-basal plexus, and one corneal volume scan of the anterior corneal layers were collected from the central cornea of each participant. Raw files from each sequence scan (100 frames, 384x384µm) were batch-processed using the automated Photomerge function in Photoshop (https://www.adobe.com/products/photoshop.html) to create large, montaged images. From these montages, at least eight non-overlapping images were extracted at each time point; the use of this number of non-overlapping images has been shown to provide adequate sampling accuracy for the quantification of corneal sub-basal nerve parameters.[22] As an additional safeguard to ensure that corneal regions were not analysed more than once, extracted images were processed using the Photomerge function. Failure of the image to successfully merge confirmed that the regions selected for analysis were non-overlapping. Individual images were de-identified for all analyses.

Corneal nerve parameters were analysed using the most recent version of the fully-automated and validated ACCmetrics program (Version 2.0, available at: http://www.click2go.umip.com/i/software/accmetrics_v2.html),[23] which enables automated quantification of the following parameters: corneal nerve fibre density (CNFD), corneal nerve fibre length (CNFL), corneal nerve branch density on the main fibre (CNBD), total corneal nerve branch density (CTBD), nerve fibre width (CNFW) and area (CNFA). The number of hyper-reflective DCs was manually quantified using the 'Cell Counter' tool function in FIJI/ImageJ (https://fiji.sc/) from the same images used for the corneal nerve analyses. Using the volume scans collected for each cornea at each study visit, one representative image of the basal corneal epithelium was extracted, using

established criteria for defining this layer,[24] and the number of basal epithelial cells was manually counted using the 'Cell Counter' tool in Fiji/ImageJ. The average image area used to quantify basal epithelial cell density in each image was 80,000µm^2.

Statistical analyses

Analyses were performed on the eye with highest tear osmolarity at day 1, as an established measure of disease severity.[25] Data were analysed using GraphPad Prism (version 6.01, https://www.graphpad.com/scientific-software/prism/). Descriptive statistics are expressed as mean ± SEM, F values for two-way ANOVA with repeated measures are expressed as $F_{a,b}$, where $_a$ represents DFn and $_b$ represents DFd. When t-tests are used, t values are expressed as t(df). The normality of data was tested using the Shapiro-Wilk test. Given a normal distribution of data, a repeated measures analysis of variance was used to assess for differences in the post-treatment means between groups for each outcome. Pairwise correlations were explored using Pearson's product-moment correlation coefficient (r). Data were expressed as r(df). For data expressed as proportions, the Chi-squared test was performed. An alpha value of 0.05 was adopted for statistical significance for all analyses.

A *post-hoc* power calculation was performed to determine the statistical power obtained for detecting the observed between-group mean difference in CNBD at day 90, with the nominated sample sizes in each group (n=4 placebo and n=8 omega-3). Assuming an alpha of 0.05 for the Type I error rate, we obtained 77% power to detect a between-group difference, using the observed standard deviations within each group (placebo: 23.0 and omega-3: 18.4).

RESULTS

Participants

All 12 participants completed the study and exceeded the pre-specified, minimum level of compliance to the study regimen (defined, as in other neutraceutical trials[26] as ≥75% capsule consumption), based upon capsule counts; no data were excluded from analysis

on these grounds. Participant baseline (day 1) data are summarised in Table 1; there were no significant inter-group differences for age, sex, DED severity or corneal nerve, DC or basal epithelial cell parameters at baseline ($p > 0.05$ for all comparisons).

At baseline, there were two (out of four) participants in the placebo group and five (out of eight) participants in the omega-3 group using topical lubricant eye drops. The lubricant products used by participants were Systane Ultra, Alcon Laboratories, (n=2 placebo group, n=3 omega-3 group), Systane Balance, Alcon Laboratories (n=1 omega-3 group) and Refresh minims, Allergan Pty Ltd (n=1 omega-3 group). There was no significant change to the frequency of eye drop utilisation, compared with baseline, in either intervention arm throughout the study (data not shown). Participants' combined daily dietary intake of EPA and DHA from food sources was similar in both intervention groups at baseline (placebo: 130.0±21.4 mg/day, fish oil: 140.1±27.9 mg/day). None of the participants reported major changes to their intake of foods containing high levels of ω-3 fatty acids throughout the study.

Table 1: Demographic and clinical characteristics of the study groups at baseline

	Placebo (n = 4)	**Omega-3** (n = 8)	***t*(df), p-value**
Demographics			
Age (years)	46 ± 10	42 ± 7	$t(10)=0.3$, $p = 0.75$
Sex (female/male)	3/1	6/2	$\chi^2(1, N = 12) = 0$, $p = 1$
Dry eye clinical parameters			
OSDI score (/100)	30.6 ± 3.5	32.6 ± 4.5	$t(10)=0.3$, $p = 0.78$
Tear osmolarity (mOsmol/L)	329.5 ± 6.2	321.9 ± 1.2	$t(3.2)=1.2$, $p = 0.31$
Corneal sub-basal nerve plexus parameters			

CNBD (branches on main fibre/mm^2)	34.6 ± 13.0	46.7 ± 6.8	$t(10)=0.8$, p = 0.38
CTBD (total branches/mm^2)	50.9 ± 12.4	72.9 ± 9.0	$t(10)=1.4$, p = 0.19
CNFD (fibres/mm^2)	20.9 ± 4.9	26.6 ± 3.1	$t(10)=1$, p = 0.33
CNFL (mm/mm^2)	16.5 ± 3.7	17.4 ± 2.0	$t(10)=0.4$, p = 0.71
CNFW (nerve fibre/mm^2)	0.022 ± 0.001	0.021 ± 0.0004	$t(10)=0.8$, p = 0.41
CNFA (length of nerve per unit area)	0.008 ± 0.001	0.008 ± 0.001	$t(10)=0.1$, p = 0.88
Other corneal parameters			
Basal epithelial cell density (cells/mm^2)	7394.7 ± 659.7	7796.6 ± 346.6	$t(10)=0.6$, p = 0.56
Dendritic cell density (cells/mm^2)	63.4 ± 36.1	118.1 ± 66.5	$t(10)=0.7$, p = 0.47

Data are expressed as mean ± SEM. Legend: corneal nerve branch density on the main fibre (CNBD); corneal nerve fibre area (CNFA); corneal nerve fibre density (CNFD); corneal nerve fibre length (CNFL); corneal nerve fibre width (CNFW); total corneal nerve branch density (CTBD); Ocular Surface Disease Index (OSDI).

At day 90, absolute levels of DED symptoms, quantified using OSDI score, were similar between groups (placebo: 27.8 ± 7.7, $t(10)= 1.4$, versus ω-3 EFA: 17.2 ± 3.9, p = 0.19), however relative to day 1 (baseline), OSDI score was significantly reduced at day 90 in the omega-3 EFA group (-15.4±2.8 units, $t(5)= 2.6$, p = 0.04) compared with placebo (-2.8±4.2 units). At day 90, the absolute level of tear osmolarity (placebo: 321.5 ± 3.6 versus ω-3 EFA: 299.3 ± 5.5 mOsmol/L, $t(10)= 2.7$, p = 0.02) was significantly lower in the ω-3 EFA supplementation group, compared with placebo. Relative to day 1, tear osmolarity was reduced at day 90 in the omega-3 EFA group (-22.6±5.7 mOsmol/L, $t(9)=2.3$, p=0.04) compared with placebo (-8.0±2.8 mOsmol/L).

For corneal sub-basal nerve plexus parameters, both CTBD (Figure 1A) and CNBD (Figure 1B) were increased after 90 days of ω-3 EFA supplementation, compared with placebo (Figure 1D-F; CTBD, ω-3 EFA: 91.1 ± 8.6 versus placebo: 45.1 ± 13.4, $F_{1,10}$ = 14, p = 0.004; CNBD, ω-3 EFA: 63.4 ± 6.4 versus placebo: 27.8 ± 11.5, $F_{1,10}$ = 6, p = 0.03). There was also a significant crossover interaction for CNFL between groups at day 90 (Figure 1C, ω-3 EFA: 20.3 ± 1.1 versus placebo: 13.8 ± 2.6, $F_{1,10}$ = 5.9, p = 0.03). There were no significant inter-group differences (p > 0.05 for all comparisons) for the other corneal nerve parameters at day 90: CNFA (placebo: 0.007 ± 0.002 versus ω-3 EFA: 0.009 ± 0.001 length of nerve per unit area, $F_{1,10}$ = 0.9, p = 0.37), CNFD (placebo: 21.9 ± 5.5 versus ω-3 EFA: 31.5 ± 2.5 fibres/mm^2 $F_{1,10}$ = 1.8, p = 0.20) or CNFW (placebo: 0.022 ± 0.001 versus ω-3 EFA: 0.022 ± 0.0001 nerve fibre/mm^2 $F_{1,10}$ = 0.3, p = 0.61).

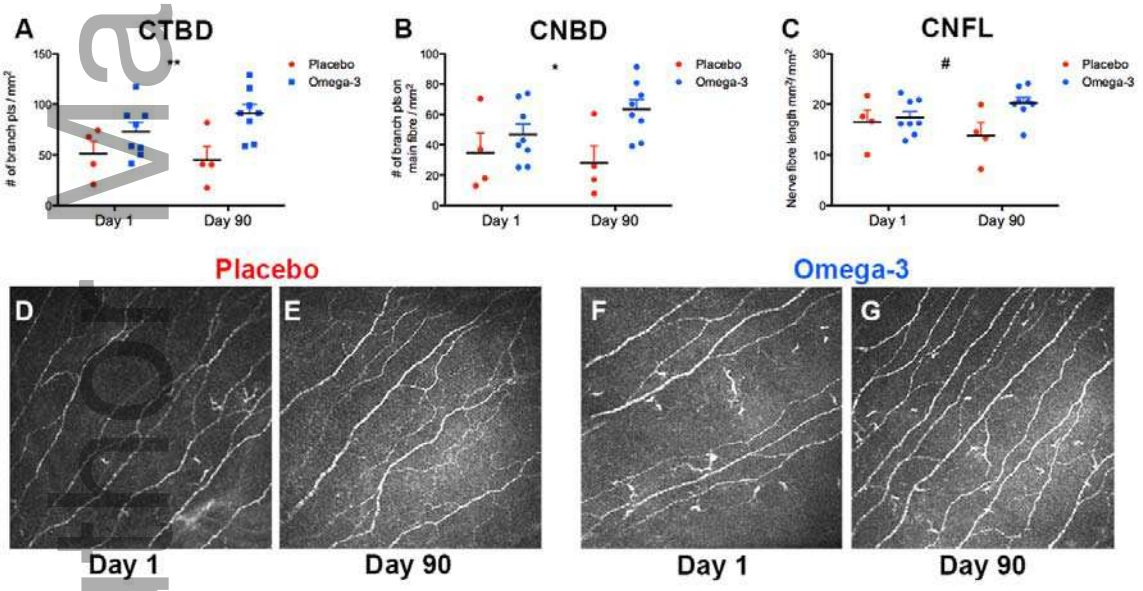

Figure 1. The effect of omega-3 essential fatty acid supplementation on central corneal nerve sub-basal plexus parameters, quantified using *in vivo* laser scanning confocal microscopy. A. Total corneal nerve branch density (CTBD, number of branches/mm^2) was significantly greater in the omega-3 EFA group, compared with placebo at day 90. **B.** Corneal nerve branch density on the main fibre (CNBD, number of branches on main fibre/mm^2) was also significantly higher in the omega-3 EFA group,

compared with placebo, at day 90. **C.** Corneal nerve fibre length (CNFL, mm/mm^2) showed a significant crossover interaction effect at day 90. **D-F**. Representative laser scanning confocal microscopy images, acquired from the central cornea, showing no major changes to sub-basal plexus nerve density in a participant allocated to the placebo intervention at day 1 **(D)** and day 90 **(E)**. Similar images acquired from a participant allocated to the omega-3 supplement group show an increase in the branching of the corneal sub-basal nerve plexus from day 1 **(F)** to day 90 **(G).** * indicates statistically significant inter-group difference (*p < 0.05; ** p < 0.01); # indicates statistically significant interaction effect (p < 0.05).

Relative to day 1 (baseline), CNBD (branches/mm^2) was increased at day 90 in the omega-3 EFA group (+20.0±9.2, $t(8) = 3.2$, p = 0.02) compared with placebo (-10.9±3.2). Similar changes were evident for corneal nerve fibre length (CNFL, mm/mm^2), which increased from baseline at day 90 in the omega-3 EFA group (+2.9±1.6, $t(8) = 3.4$ p = 0.01) compared with placebo (-2.7±0.5). Relative to day 1, there were no significant inter-group differences (p > 0.05 for all comparisons) for the other corneal nerve parameters.

There was no significant inter-group difference for dendritic cell density at day 90, placebo: 74 ± 27.5 versus ω-3 EFA: 86 ± 40.8 cells/mm^2 $F_{1,10} = 0.4$, p = 0.55) or basal epithelial cell density (day 90, placebo: 7104.1 ± 439.2 versus ω-3 EFA: 7923 ± 363.9 cells/mm^2 $F_{1,10} = 1.7$, p = 0.22).

Pearson's correlation analysis showed a significant negative correlation ($r(10)=-0.70$, p = 0.01) between tear osmolarity and CTBD at day 90 (Figure 2). There were no significant correlations between tear osmolarity and OSDI with any other anatomical features, including nerve fibre measurements, basal epithelial cell and dendritic cell density (p > 0.05 for all comparisons).

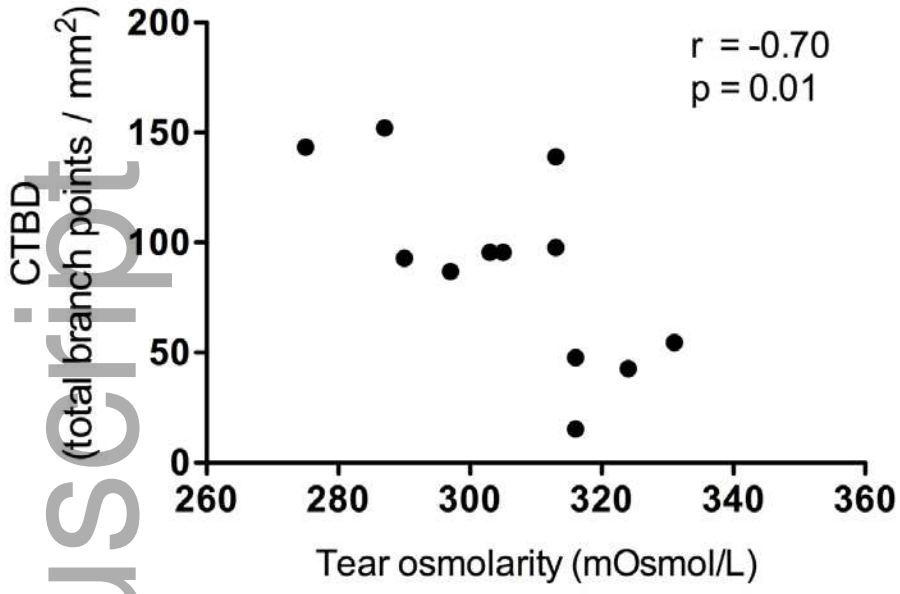

Figure 2. Relationship between tear osmolarity (mOsmol/L) and total corneal nerve branch density (branch points/mm^2) in the sub-basal plexus at the study endpoint. Pearson's correlation analysis shows a significant negative correlation between tear osmolarity and CTBD at day 90.

DISCUSSION

To our knowledge, this is the first study to report the potential corneal neuroprotective effects of long-chain, ω-3 EFA supplementation in a clinical population. Compared with placebo, a moderate daily dose of ω-3 EFAs (~1000mg/day EPA + ~500mg/day DHA) for three months, resulted in enhanced nerve branch density in the corneal sub-basal plexus in people with mild-to-moderate DED. Notably, a significant correlation was evident between tear osmolarity and corneal nerve branch density at the study endpoint. Reduced tear osmolarity (i.e., the amelioration of tear hyperosmolarity) was associated with an increased total number of nerve branches, suggesting a specific link between tear hyperosmolarity and corneal neuropathy. Indeed, patients with diabetes mellitus having severe diabetic peripheral neuropathy have been shown to have abnormally elevated tear osmolarity.[27] Furthermore, using *in vivo* electrophysiological recordings from rat

trigeminal ganglion neurons, the application of hyperosmolar solutions has been found to reduce the physiologic sensitivity and the morphologic integrity of corneal nerves.[14]

Given the role of inflammation in the pathogenesis of DED, there has been increasing clinical and scientific interest in novel anti-inflammatory treatments.[28] Corticosteroids, the current mainstay anti-inflammatory therapy for DED, target inflammation by attenuating corneal dendritic cell responses, but do not appear to alter the structure of the corneal sub-basal nerve plexus.[17] Interestingly, we report the opposite effect with ω-3 EFA supplementation, at least at the three-month intervention time-point, whereby some corneal nerve parameters are improved without significant alterations to the corneal dendritic cell response or epithelial cell density relative to baseline. This finding is noteworthy given that ω-3 EFAs modulate systemic inflammation[8] and suggests that the time-course of immune-mediated changes in the cornea with ω-3 EFAs differs from the timing of the observed corneal neuroprotective effects.

That we observed significant changes to some corneal nerve parameters after three months of oral omega-3 supplementation is reasonable in the context of earlier studies. Scientific understanding of the timing of corneal nerve regeneration in the human cornea largely derives from studies that have longitudinally examined participants following surgical disruption to nerve integrity. As recently reviewed, following photorefractive keratectomy, in which photoablation results in severe damage to the sub-basal and anterior stromal corneal nerves, nerve regeneration within the sub-basal plexus begins at four weeks postoperatively, and is largely complete six months after surgery.[29] In a rabbit model of lamellar keratectomy, topical treatment with neuroprotectin D1 (a lipid-based derivative of DHA), in combination with DHA for six weeks, was found to induce significant anti-inflammatory and nerve regenerative effects.[30] In this study, anatomical nerve recovery was also accompanied by an increase in corneal sensitivity and tear secretion.

The mechanism underlying the observed improvements in corneal nerve branch density with systemic omega-3 supplementation may relate to the effects of anti-inflammatory

lipid mediators, in particular resolvins and protectins, formed by the oxidation of DHA and EPA within the body. Omega-3 long-chain EFAs have been shown to enhance neurite outgrowth in cell cultures of primary sensory neurons[31] and to improve functional recovery in a murine model of peripheral nerve injury.[32] In the body, metabolism of DHA results in the production of neuroprotectins and the D-series resolvins, which promote neuroprotection, the resolution of inflammation and tissue repair.[33] DHA has been shown to have therapeutic potential for a broad range of acute and chronic neurodegenerative conditions.[34] In this respect, our data corroborate findings reported in an experimental model of gross corneal nerve injury, involving nerve resection, where the administration of DHA was found to promote corneal nerve regeneration.[18] In this study, it was shown that nerve recovery was mediated by the DHA-derived lipid mediator neuroprotectin D1 (NPD_1). In animal models, NPD_1 has been implicated in several important physiological processes relevant to anterior eye health, including influencing the survival of epithelial cells,[35] modulation of wound healing[36] and corneal nerve regeneration.[36] Furthermore, the EPA-derived resolvins D1 (RvD_1) and E1 (RvE_1) have been found to be involved in the regulation of tear homeostasis under conditions of desiccating stress.[37, 38] Similar to our data, autologous serum tears, which are reserved for treating severe DED, have also been recently shown to promote corneal sub-basal nerve recovery in patients with several neuropathy, without altering dendritic cell numbers, suggesting a potential overlap in mechanism of action.[39]

The corneal neural recovery that we report with ω-3 EFA supplementation occurred in association with a significant clinical improvement in DED status, as indicated by a profound normalisation of tear osmolarity (mean post-treatment tear osmolarity: 299 mOsmol/L) and an average 57% reduction in OSDI score from baseline. Similar clinical improvements, as quantified by a relative decrease in tear osmolarity and an attenuation of dry eye symptoms, has recently been reported in another ω-3 EFA intervention trial, involving a higher daily dose of ω-3 EFAs (1680mg EPA + 560mg DHA) for 12 weeks.[40] Our findings suggest that providing neurotrophic support to corneal nerves is linked to an alleviation of DED symptoms with ω-3 EFA supplementation.[9] Indeed, the restoration of

corneal nerve topography, using autologous serum tears, has recently been shown to attenuate patient-reported photoallodynia in severe corneal neuropathy.[39]

We acknowledge the limitations of this proof-of-principle study, in particular the small sample size. Nevertheless, the study was undertaken in a robust manner, with double masking and a placebo-control group, thereby minimising major potential sources of bias. Analysis of corneal nerve morphology was performed in a double-masked manner, using validated, automated image analysis software (ACCMetrics),[41, 42] further reducing measurement bias. Future work will be directed towards understanding how changes to sub-basal nerve parameters correlate with alterations to corneal sensitivity in larger clinical populations. In addition, longer follow-up durations are required to ascertain the period over which the corneal neuroprotective effects are retained.

In conclusion, this pilot study suggests that oral ω-3 EFA supplementation imparts neuroprotective effects in the corneal sub-basal plexus in DED patients that correlate with the extent of normalisation of tear osmolarity. This study provides novel insight into the potential mechanism of action of ω-3 EFAs in treating corneal neuropathy.

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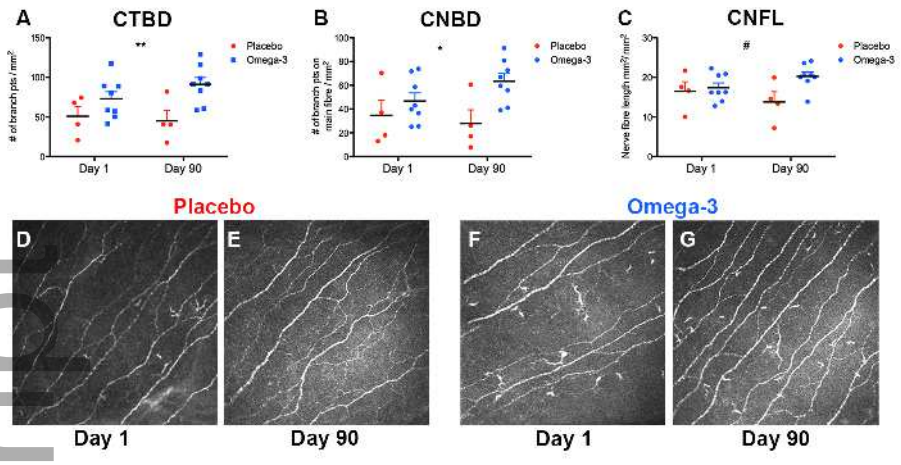

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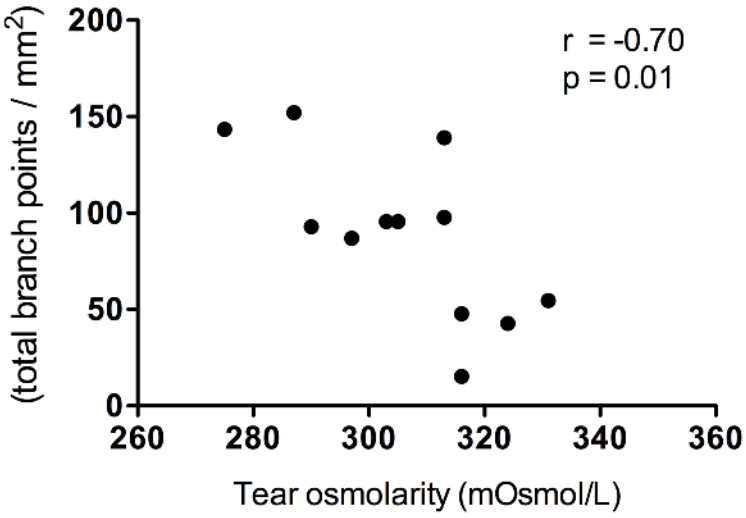

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