
Clinical applications of omega-3 fatty acids for corneal and peripheral nerve health

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“Knowledge is love and light and vision.”

~ Helen Adams Keller

Abstract

Peripheral neuropathy, a common complication of diabetes, can lead to debilitating functional impairment and adversely impact daily living. In diabetes, damage to small sensory nerves in the cornea, visible using *in vivo* confocal microscopy (IVCM), precedes large nerve fibre involvement. Quantitative corneal nerve parameters, derived from IVCM images, provide reliable markers for evaluating small fibre damage and repair in diabetic peripheral neuropathy. A current challenge in the management of diabetic peripheral neuropathy is a lack of effective treatments. Omega-3 polyunsaturated fatty acids (PUFAs) modulate systemic inflammation and impart neurotrophic effects and, thus, show promise as neuroprotective agents. Although omega-3 PUFAs have established utility in the management of a number of ocular conditions, including dry eye disease, their potential role for modulating corneal and peripheral nerve health in diabetes has not been thoroughly investigated.

This thesis focuses on evaluating the role of omega-3 PUFAs in improving peripheral nerve health using corneal nerve parameters as surrogate markers. First, a clinician survey was developed and administered to explore current practices relating to omega-3 fatty acids in eye care settings. The survey outcomes provide an overview of Australian and New Zealand optometrists' knowledge and practice patterns relating to omega-3 PUFAs and identify potential avenues for improving clinical implementation. To assist in providing a tailored clinical approach, a dietary questionnaire for quantifying an individual's omega-3 PUFAs intake was designed and validated.

In a cross-sectional study, the association between systemic omega-3 fatty acid levels and corneal nerve parameters was evaluated in healthy controls and individuals with diabetes. This study identified a relationship between corneal nerve structural parameters and the systemic Omega-3 Index, a metric combining erythrocyte docosahexaenoic acid (DHA) and eicosapentanoic acid (EPA) levels. Furthermore, an association between corneal nerve structure and DHA levels, but not EPA levels, was identified.

Using a systematic review methodology, randomised controlled trials (RCTs) evaluating the effects of oral omega-3 PUFA supplementation on peripheral nerve structure and function were identified, appraised, and synthesised. This review found, with low certainty, that omega-3 PUFAs attenuate sensory function deficits in chemotherapy-induced peripheral neuropathy. It also identified a paucity of RCTs evaluating the role of omega-3 PUFAs in treating diabetic peripheral neuropathy. These findings supported the rationale for conducting an RCT evaluating the effects of six-months of omega-3 PUFA supplementation in individuals with type 1 diabetes. This study found that, relative to placebo, oral omega-3 PUFA supplementation for six months significantly improved corneal nerve parameters, consistent with a corneal neuroregenerative effect. However, no significant differences were found for small or large nerve fibre function relative to placebo.

Overall, this body of work advances scientific understanding of the clinical practices relating to omega-3 PUFAs in eye care settings and provides a dietary assessment tool for improving clinical implementation. Using corneal nerve health as a marker, findings from the prospective clinical studies provide evidence for the role of omega-3 PUFAs in modulating peripheral nerve health.

Declaration

The candidate declares that

- I. This thesis comprises original work towards the Doctor of Philosophy degree, except where indicated in the preface.
- II. Due acknowledgment has been made in the text to all other material used.
- III. The thesis is less than 100,000 words in total length, exclusive of tables, bibliographies and appendices.

Signed:

Alexis Ceecee Zhang

Preface

This thesis contains the candidate's own work and no materials that have been submitted for any other qualifications. All the work included in this thesis was conducted following the candidate's enrolment in the PhD course. No third-party editorial assistance was provided in preparation of the thesis outside of the contribution supervisors and co-authors.

The PhD candidate was supported by an Australian Government Research Training Program Scholarship.



For the experimental work included in this thesis, the candidate was chiefly responsible for the following tasks:

- a. initial preparation of study protocols and documents for ethics approval;
- b. undertaking data collection, including participant recruitment and follow-up, and performing examination procedures for all study visits, except for the neurophysiology examination undertaken at St Vincent's Hospital Melbourne in the randomised controlled trial (**Chapter 7**; two study visits per participant, which were undertaken by Dr Jordan Kamel and Dr Leslie Roberts);
- c. data management and curation of collected data;
- d. formal analysis;
- e. manuscript preparation.

This thesis contains four published first-author papers (included in **Chapters 3, 4, and 6**) and one paper submitted for publication (**Chapter 7**). As first author, the candidate's contribution was greater than 50% for all of the publications included in this thesis. Other co-authors contributed towards conceiving the scientific ideas, revising study protocols and documentation, data collection, data analytical plans, interpretation of results, manuscript preparation, and generating funding to support. The publication status, contributions of each author and funding sources regarding each of these papers are described below.

Chapter 3:

This chapter contains a peer-review publication, published in *Nutrients* on April 22, 2020. Supplementary materials for this publication are provided in

Appendix A.

Citation details: Zhang, A. C., Singh, S., Craig, J. P., & Downie, L. E. (2020). Omega-3 Fatty Acids and Eye Health: Opinions and Self-Reported Practice Behaviors of Optometrists in Australia and New Zealand. *Nutrients*, 12(4), 1179.
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Chapter 5:

This chapter contains unpublished material, not yet submitted for publication. For the study described in this chapter, A/Prof Andrew Anderson assisted with developing the double-staircase algorithm for the Non-Contact Corneal Aesthesiometer (NCCA) methodology. **Appendices C** and **D** contain additional experiments undertaken to evaluate the analysis methods adopted in the study. For these experiments, Rajni Rajan (PhD candidate, University of Melbourne) assisted with: (i) data collection for the test-re-test sessions of corneal sensitivity thresholds measured using the NCCA (**Appendix C**); (ii) cell counting for the evaluation of intra- and inter-observer repeatability of dendritic cell quantification (**Appendix D**). PhD supervisors, A/Prof Laura Downie and A/Prof Jennifer Craig, provided assistance with experimental design, data analysis, and manuscript editing.

Chapter 6:

This chapter includes two publications; the systematic review protocol was published in *BMJ Open* on March 25, 2018, and the outcomes of the systematic review were published in *Nutrition Reviews* on September 17, 2019. Supplementary materials for these publications are provided in **Appendix E**.

Publication I: Systematic review protocol

Citation details: Zhang, A. C., MacIsaac, R. J., Roberts, L., Kamel, J., Craig, J. P., Busija, L., & Downie, L. E. (2018). Omega-3 polyunsaturated fatty acid supplementation for improving peripheral nerve health: protocol for a systematic review. *BMJ Open*, 8(3), e020804. <https://doi.org/10.1136/bmjopen-2017-020804>

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Publication II: Systematic review

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Chapter 7:

This chapter includes a paper that has been submitted for publication in *Lancet Diabetes and Endocrinology*. A/Prof Laura Downie was the Principal Investigator of the clinical trial described in this chapter. Supplementary materials for this publication include the clinical trial protocol (**Appendix F**), statistical analysis plan (**Appendix G**), and results of exploratory analyses (**Appendix H**).

Author contributions: L.E.D created the study concept. A.C.Z, L.E.D, R.J.M, L.J.R, and J.K developed the trial protocol. L.E.D, J.K, L.J.R, and R.J.M acquired the funding. A.C.Z, L.E.D, L.J.R and J.T.K implemented the trial procedures. R.J.M, J.K, and L.J.R provided administrative and technical support. A.C.Z, L.E.D, J.T.K, L.R acquired the data. A.C.Z, L.E.D and S.B developed the statistical analysis plan. A.C.Z and S.B performed the statistical analysis. L.E.D, J.P.C and R.J.M supervised the study. A.C.Z and L.E.D have verified the underlying data. A.C.Z and S.B have independently verified the data analysis. A.C.Z wrote the first draft of the report with input from L.E.D. All authors revised the article.

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All peer-reviewed publications, cited above, are reproduced in their entirety without modification, except for the following:

- I. Tables and figures are re-numbered within the thesis for consistency in style throughout.
- II. References have been formatted and incorporated into the main reference list for the thesis, with the exception of the systematic review publication (**Chapter 6**). For this publication (Zhang *et al.*, *Nutr Rev*, 2019), the reference style is retained as the preferred style of the publisher to improve readability. The reference list for this publication is listed at the end of the corresponding chapter.

Other works arising during candidature:

Conference abstracts

- I. **Zhang, A. C.**, Craig, J. P., & Downie, L. E. (2018). A systematic review of omega-3 polyunsaturated fatty acid (PUFA) supplementation for improving peripheral nerve health (poster). Presented at the 15th Scientific and 9th Educators' Meeting in Optometry (SEMO), Melbourne, Australia, April 2018.
- II. **Zhang, A. C.**, Craig, J. P., & Downie, L. E. (2019). Changes to corneal sensitivity precede symptoms of peripheral neuropathy in diabetes (poster). *Investigative Ophthalmology & Visual Science*, 60(9), 3241-3241. Presented at the 2019 Association for Research in Vision and Ophthalmology (ARVO) Annual Meeting, held in Vancouver, Canada, April 28 – May 2, 2019.
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Other publications

- I. De Silva, M. E. H., **Zhang, A. C.**, Karahalios, A., Chinnery, H. R., & Downie, L. E. (2017). Laser scanning in vivo confocal microscopy (IVCM) for evaluating human corneal sub-basal nerve plexus parameters: protocol for a systematic review. *BMJ Open*, 7(11), e018646. <https://doi.org/10.1136/bmjopen-2017-018646>
- II. Kamel, J. T., **Zhang, A. C.**, & Downie, L. E. (2019). Corneal epithelial dendritic cell response as a putative marker of neuro-inflammation in small fiber neuropathy. *Ocular immunology and inflammation*, 1-4. <https://doi.org/10.1080/09273948.2019.1643028>
- III. Stepp, M. A., Pal-Ghosh, S., Downie, L. E., **Zhang, A. C.**, Chinnery, H. R., Machet, J., & Di Girolamo, N. (2020). Corneal Epithelial "Neuromas": A Case of Mistaken Identity? *Cornea*, 39(7), 930-934. <https://doi.org/10.1097/ICO.0000000000002294>
- IV. Cote, S., **Zhang, A. C.**, Ahmadzai, V., Maleken, A., Li, C., Oppedisano, J., Nair, K., Busija, L., & Downie, L. E. (2020). Intense pulsed light (IPL) therapy for the treatment of meibomian gland dysfunction. *Cochrane Database of Systematic Reviews*, (3). <https://doi.org/10.1002/14651858.CD013559>
- V. **Zhang, A. C.**, Muntz, A., Wang, M. T. M., Craig, J. P., & Downie, L. E. (2020). Ocular Demodex: a systematic review of the clinical literature. *Ophthalmic and Physiological Optics*, 40(4), 389-432. <https://doi.org/10.1111/opo.12691>

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Abbreviations

AA	Arachidonic Acid
AE	Adverse Event
ALA	Alpha-Linolenic Acid
AGE	Advanced Glycation End-product
AMD	Age-Related Macular Degeneration
AREDS	Age-Related Eye Disease Study
BMI	Body Mass Index
CIPN	Chemotherapy-Induced Peripheral Neuropathy
CMAP	Compound Muscle Action Potential
CNBD	Corneal Nerve Branch Density
CNFD	Corneal Nerve Fibre Density
CNFL	Corneal Nerve Fibre Length
CODS	Clinical Omega-3 Dietary Survey
CSP	Cutaneous Silent Period
DBS	Dried Blood Spot
DC	Dendritic Cell
DCCT/EDIC	The Diabetes Control and Complications Trial (DCCT)/The Epidemiology of Diabetes Interventions and Complications (EDIC)
DED	Dry Eye Disease
DHA	Docosahexaenoic Acid
DPA	Docosapentaenoic Acid
DQES	Dietary Questionnaire for Epidemiology Studies
DSP	Diabetic Sensorimotor Polyneuropathy
EFA	Essential Fatty Acid
EPA	Eicosapentaenoic Acid
EQ-5D-5L	EuroQol Five-Dimensional Five-Level Descriptive System
ETDRS	The Early Treatment Diabetic Retinopathy Study
FFQ	Food-Frequency Questionnaire
GPCR	G-Protein Coupled Receptors

GRADE	Grading of Recommendations, Assessment, Development and Evaluation
HbA _{1c}	Haemoglobin A _{1c}
IENFD	Intraepidermal Nerve Fibre Density
imDC	Immature Dendritic Cell
ITT	Intention-To-Treat
IVCM	<i>In Vivo</i> Confocal Microscopy
LA	Linoleic Acid
mDC	Mature Dendritic Cell
MDNS	Michigan Diabetic Neuropathy Score
MHC	Major Histocompatibility Complex
MNSI	Michigan Neuropathy Screening Instrument
Na ⁺ /K ⁺ -ATPase	Sodium Potassium Adenosine Triphosphatase
NCCA	Non-Contact Corneal Aesthesiometry
NCS	Nerve Conduction Studies
Norfolk QoL-DN	Norfolk Quality of Life–Diabetic Neuropathy
NPD ₁	Neuroprotectin-D ₁
NFκB	Nuclear Factor Kappa-B
PEDF	Pigment Epithelial–Derived Growth Factor
PKC	Protein Kinase-C
PN	Peripheral Neuropathy
PNS	Peripheral Nervous System
PPAR	Peroxisome Proliferator-Activated Receptor
PUFA	Polyunsaturated Fatty Acids
QSART	Quantitative Sudomotor Axonal Reflex Test
QST	Quantitative Sensory Testing
RCT	Randomised Controlled Trial
ROS	Reactive Oxidation Species
rTNS	Reduced Total Neuropathy Score
SAE	Serious Adverse Event
SBNP	Sub-Basal Nerve Plexus
SNAP	Sural Nerve Action Potential
TEd	Depolarising Threshold Electrotonus
TEh	Hyperpolarising Threshold Electrotonus
TFOS DEWS II	Tear Film and Ocular Surface Society Dry Eye Workshop II
TRPM8	Transient Receptor Potential cation channel, subfamily M, member 8
TRPV ₁	Transient Receptor Potential cation channel, subfamily V, member 1
VAS	Visual Analogue Scale

1

Chapter 1. Introduction

Peripheral neuropathy describes a distressing and potentially disabling condition caused by damage to the nerves of the peripheral nervous system. Associated with symptoms such as numbness, pain, and loss of balance, such damage can lead to significant functional and psychosocial impairment and have a profound impact on quality of life.

In the developed world, the most common cause of chronic peripheral neuropathy is diabetes (Azhary et al., 2010; Hanewinckel et al., 2016). Optimal clinical care for diabetic peripheral neuropathy is challenged by a lack of disease-modifying treatments. There is a need to identify novel therapeutic agents that can improve outcomes for patients with peripheral neuropathy. This thesis aims to address this unmet need by evaluating the clinical utility of omega-3 polyunsaturated fatty acids (PUFAs) for improving peripheral nerve health, using corneal nerve parameters as surrogate markers.

Corneal nerve parameters are sensitive surrogate markers for evaluating peripheral nerve health

In diabetes, damage to small sensory nerve fibres, including those in the cornea, occurs prior to the involvement of large nerve fibres (Breiner et al., 2014). Currently, the only established method for attenuating progressive nerve damage in diabetes is optimising glycaemic control, which is effective only in reducing the incidence of neuropathy in type 1 diabetes (Callaghan et al.,

2012b). To date, few potential disease-modifying interventions have been found to improve peripheral nerve outcomes in clinical trials (Boulton et al., 2013).

A particular challenge is the selection of suitable clinical endpoints, as most standard tests for evaluating peripheral nerve function have a poor diagnostic yield for detecting changes in small nerve fibres. To address this challenge, this thesis uses corneal nerve parameters as surrogate markers for evaluating peripheral nerve health (Malik et al., 2003). Corneal sub-basal nerve parameters, quantified from *in vivo* confocal microscopy images, are valid morphological markers for both detecting peripheral neuropathy and quantifying change over time (Azmi et al., 2019). Corneal sensitivity, a functional marker, allows the exploration of the corneal structure-function relationship that has, to date, not been fully characterised in individuals with diabetes.

Omega-3 fatty acids show promise as a neuroprotective intervention in diabetes

There is scientific rationale that omega-3 PUFAs modulate peripheral nerve health. Omega-3 PUFAs are essential dietary components that cannot be synthesised *de novo* and must be derived from diet or supplementation. Once ingested, omega-3 PUFAs with their lipid-mediators impart a range of anti-inflammatory and neurotrophic effects (James et al., 2000; Robson et al., 2010). Preclinical studies have demonstrated that oral omega-3 PUFA supplementation can ameliorate corneal and peripheral neuropathy in experimental models of diabetes (Coppey et al., 2020; Gerbi et al., 1999). In a single-arm clinical study, omega-3 PUFAs promoted corneal nerve regeneration in individuals with diabetes (Lewis et al., 2017); however, this effect has not been thoroughly evaluated in randomised controlled clinical trials.

Omega-3 PUFAs have established applications in eye care practice

Although the clinical effects of omega-3 PUFAs for peripheral nerve outcomes in diabetes have not been thoroughly investigated, omega-3 PUFAs play a role in the management of several ocular conditions. In dry eye disease, dietary

intervention with omega-3 PUFAs is a strategy for reducing ocular surface inflammation by promoting the production of anti-inflammatory mediators.

The effects of oral omega-3 PUFA supplementation on symptoms and signs of dry eye disease have been widely examined (Downie et al., 2019); however, there is a lack of certainty in the current evidence concerning the optimal intervention protocol, particularly with regard to dosage and form. Furthermore, few clinical studies have quantified omega-3 intake at baseline, with consideration of specific patient populations that may preferentially benefit from dietary enhancement. Such factors potentially contribute to the divergent findings in clinical trials to date and have implications for the clinical implementation of dietary recommendations. Yet, little is known about current clinical practice behaviours and the existing knowledge of eye care practitioners relating to omega-3 PUFAs and eye health. This knowledge gap provides the rationale for the first two studies in this thesis, which examine current clinical practices relating to omega-3 PUFAs by Australian and New Zealand optometrists and describe a new dietary assessment tool suitable for implementation in this setting.

Thesis overview

The overall aim of this thesis is to investigate the role of omega-3 PUFAs in modulating corneal and peripheral nerve health. The thesis comprises eight Chapters. **Chapter 1** introduces the thesis, and **Chapter 2** presents a review of the relevant literature. **Chapter 3** describes optometric practice behaviours and clinician knowledge relating to omega-3 PUFAs for eye health. **Chapter 4** describes the development and validation of a short food frequency questionnaire for estimating the sufficiency of dietary omega-3 fatty acid intake.

The subsequent chapters describe studies that relate to the potential neuroprotective role of omega-3 PUFAs in systemic conditions associated with peripheral neuropathy, with a focus on diabetes. **Chapter 5** is a cross-sectional study that investigates the relationship between systemic omega-3 fatty acid

levels and corneal nerve structure and function in individuals with and without diabetes. To comprehensively examine the best-available clinical evidence for the efficacy and safety of oral omega-3 PUFA supplementation on peripheral nerve outcomes, a systematic review and meta-analysis in **Chapter 6** reports on the identification, appraisal and synthesis of this evidence from randomised controlled trials. This review identified a paucity of high-quality evidence evaluating omega-3 PUFA supplementation for modifying peripheral nerve health in individuals with diabetes. Finally, **Chapter 7** reports results from a randomised placebo-controlled trial evaluating the efficacy and safety of oral omega-3 PUFA supplementation for modulating peripheral nerve health in type 1 diabetes. An overall discussion of the findings, future directions, and concluding remarks is presented in **Chapter 8**.

2

Chapter 2. Review of the literature

2.1 Introduction

The first part of this chapter describes the epidemiology and management of diabetic neuropathy and current clinical evidence evaluating potential treatments. This is followed by a review of the effects of diabetes on the ocular surface. To understand the clinical utility of omega-3 fatty acids, their biological effects and current applications for improving ocular health are considered. Finally, a summary of the evidence informing a role for omega-3 fatty acids in modulating peripheral nerve health is presented.

2.2 Peripheral neuropathy

2.2.1 The peripheral nervous system

The nervous system is responsible for processing sensory data and transmitting motor information to coordinate body functions and movement. The brain and spinal cord constitute the central nervous system; nerves elsewhere, including in the cornea, are part of the peripheral nervous system (PNS). The PNS acts as a communication network between the central nervous system and the rest of the body, mediating sensory, motor or autonomic functions (von Holst, 1954).

Cranial nerves originate from the brain to supply the head and sensory organs; other nerves stem from the spinal cord, innervating the limbs and the rest of the

body. The optic nerve is the only cranial nerve that is not a part of the PNS. In conjunction with the ophthalmic division of the trigeminal nerve, it plays a role in vision and the sensory innervation of the eye. In the PNS, large nerve fibres convey pressure and balance information; small myelinated A δ -fibres detect sharp pain and cold sensation, and small unmyelinated C-fibres detect heat and pain perception (Muller et al., 1996; Pop-Busui et al., 2017).

2.2.2 Peripheral neuropathies

2.2.2.1 Subtypes and classifications

Peripheral neuropathy describes damage to the peripheral nervous system caused by a heterogenous group of disorders (England & Asbury, 2004; Martyn & Hughes, 1997). Such damage presents with a wide array of clinical symptoms and prognoses (England & Asbury, 2004). Peripheral nerve damage can be acute or chronic; it can be axonal, affecting neuronal axons, or demyelinating, affecting the myelin sheath; it can also be classified as a mononeuropathy, affecting a single nerve group, or a polyneuropathy, affecting multiple nerve groups (Hughes, 2002).

The presentation of neuropathy differs depending on the disease subtype and cause. The most common cause of peripheral neuropathy is diabetes mellitus (Dyck et al., 1993). Other causes include altered metabolism, hereditary factors, infectious or inflammatory conditions, neurotoxic substances, neoplasms, and trauma (Martyn & Hughes, 1997). Neuropathy without an identified aetiology, occurring in up to one-third of cases, is defined as idiopathic peripheral neuropathy (Gordon Smith & Robinson Singleton, 2006).

2.2.2.2 Diabetic neuropathy

Diabetes is a chronic condition affecting over 451 million people worldwide (Cho et al., 2018). This metabolic disorder is characterised by hyperglycaemia due to altered insulin signalling. There are two main types of diabetes; type 1, caused by absolute insulin deficiency due to autoimmune islet β -cell

destruction, and type 2, caused by insulin resistance due to progressive β -cell function loss (ADA, 2020a), although in type 2 diabetes, a proportion of individuals are also considered to have a primary β -cell defect (Ahlqvist et al., 2018). In diabetes, chronic hyperglycaemia can lead to vascular complications; the three main microvascular complications of diabetes are nephropathy, retinopathy, and neuropathy (ADA, 2020b).

2.2.2.2.1 *Classifications and symptoms*

Diabetic neuropathy can present in several forms. The most common form, diabetic sensorimotor polyneuropathy (DSP), predominantly affects sensory and motor nerves. The other main form, autonomic neuropathy, affects sympathetic and parasympathetic autonomic nerves (Pop-Busui et al., 2017; Tesfaye et al., 2010).

DSP can involve damage to small or large nerve fibres. It is hypothesised that sensory neurons, in particular small unmyelinated C-fibres, are the earliest affected in the disease process (Breiner et al., 2014; Malik et al., 2005). As the condition progresses, segmental demyelination is observed, followed by axonal degeneration of myelinated nerve fibres (Feldman et al., 2017).

DSP typically progresses in a length-dependent fashion, affecting first the feet and distal limbs, before spreading proximally to other body parts (Callaghan et al., 2012a). Symptoms vary depending on the class of fibres involved. Small nerve fibre damage alters sensation, causing symptoms such as reduced thermal discrimination, dysesthesias, and/or pain (Pop-Busui et al., 2017). Neuropathic pain affects about 30-40% of people with diabetes (Abbott et al., 2011; Harris et al., 1993). It is frequently described as burning, tingling, or shock-like sensations that are often worse at night (Peltier et al., 2014). Large nerve fibre damage presents as numbness, tingling without pain, and loss of balance (Pop-Busui et al., 2017). Up to 50% of individuals with diabetic neuropathy may be asymptomatic (Pop-Busui et al., 2017). This poses a problem, as in some individuals a diagnosis is not made until late-stage disease,

increasing the risk of end-stage complications, such as foot ulceration and amputation (Hicks & Selvin, 2019).

2.2.2.2.2 Epidemiology and risk factors

The prevalence of DSP reported in key epidemiology studies ranges from 8 to 54% (summarised in **Table 2-1**). Some of this variation may be due to differences in study population characteristics (for example: mean age, diabetes type and duration, glucose control, and comorbidities such as hypertension and hyperlipidaemia) and the DSP definition used. DSP diagnosis and classification depend on both the diagnostic criteria (i.e., whether it incorporates symptoms, signs, or a combination of both) and the tests used to define disease status. As demonstrated in the Anglo-Danish-Dutch Study of Intensive Treatment in People with Screen-Detected Diabetes in Primary Care, DSP prevalence in the study population ranged from 7 to 35% depending on the criteria used (Charles et al., 2011). This heterogeneity in DSP diagnostic criteria not only limits capacity for comparing prevalence across multiple studies, but also poses a problem for defining endpoints in interventional trials (discussed further in section **2.2.3**).

Table 2-1. Diabetic neuropathy prevalence and risk factors

Cross-sectional studies				
Study	Population	Diagnostic criteria and examination	Neuropathy prevalence	Identified risk factors
The Rochester diabetic neuropathy study (Dyck et al., 1993)	380 adults with diabetes (T1D n=102; T2D n=278)	2+ of: abnormalities in neuropathy symptoms score, neuropathy disability score, NCS, QST or autonomic examinations. At least one of NCS or autonomic function	T1D: 54%; T2D: 45%.	Not reported
San Luis Valley Study (Franklin et al., 1990; Franklin et al., 1994)	277 adults with T2D and 577 controls	Study-specific screening neurological examination including history and clinical examination (Franklin 1990)	T2D: 27.8%	Age, HbA1c, duration of disease, lower fasting C-peptide, diabetic retinopathy, diabetic nephropathy

EURO-Diab (Tesfaye et al., 1996)	3250 patients with T1D	Abnormalities in 2 or more of 1) symptoms; 2) absence of two or more ankle or knee reflexes; 3) vibration perception threshold; and 4) abnormal autonomic function	Overall: 28% (including DSP and autonomic neuropathy)	Age, diabetes duration, weight, HbA1c, smoking status, severe ketoacidosis, microalbuminuria, retinopathy, elevated BP, severe ketoacidosis, fasting triglyceride, microalbuminuria
Pittsburgh Epidemiology of Diabetes Complications (Maser et al., 1992)	400 adults with T1D	Abnormalities in 2 or more of: sensory or motor signs, symptoms consistent with neuropathy, or decreased tendon reflexes.	Overall: 34% (age 18-29 yrs: 18%; ≥30 yrs: 58%)	Diabetes duration, HbA1c, reduced HDL-C, smoking, macrovascular disease
SEARCH for Diabetes in Youth Study (Jaiswal et al., 2017)	1734 youths (mean age 15.8 years) with T1D and 258 with T2D	MNSI >2	T1D: 7% T2D: 22%	Age, diabetes duration, smoking, lower HDL-C. T1D additional risk factors: increased diastolic BP; obesity, increased LDL-C, and triglycerides,
(Young et al., 1993)	6487 adults with diabetes (T1D: 2414; T2D: 3949; unknown 124)	Moderate signs (assessed using Neuropathy disability score) with or without symptoms (assessed with Neuropathy symptom score), or mild signs with moderate symptoms	Overall: 28.5%	Age, diabetes duration
Longitudinal studies				
Study	Population	Diagnostic criteria for prevalence	DSP prevalence at baseline	Effect of Intensive glucose control
UKPDS (UKPDS, 1998)	3867 newly diagnosed T2D	- Biothesiometer reading of >25V. - Alternative method using loss of reflexes in both ankle or both knee.	Biothesiometry >25V: IGT: 11.4% CON 11.8%	RR not significant at 0, 3, 6, 9, or 12 years follow-up. At 15 years: RR 0.80 (0.39 to 0.94)

DCCT/ EDIC (Martin et al., 2014; Nathan, 2014)	1441 patients with T1D	- MNSI >2 - Clinical DN also assessed as abnormal neurologic examination plus either abnormal NCS in 2 peripheral nerves or abnormal autonomic nerve testing	Based on MNSI At baseline, IGT: 17.8%; CON: 28.0%. At ~14 years of follow-up: 33%.	DSP at 5 years: RR: 60% (38-74%) compared to standard treatment
ACCORD (Ismail-Beigi et al., 2010)	10251 patients with T2D	MNSI >2 (3 other neurological outcomes were assessed: vibratory sensation loss, ankle jerk loss and loss of light touch)	Based on MNSI At baseline: IGT: 43%; CON: 42%. At ~10 years: IGT: 45.4%; CN: 47.9%%	Based on MNSI: - annualized RR of 0.70% (95% CI 1.40 to -0.01) compared to standard treatment. - At 3.7 years: relative RR: 0.95, 95% CI: 0.89 to 1.00) compared to standard treatment
BARI 2D (Pop- Busui et al., 2013)	2368 patients with T2D and coronary artery disease	MNSI >2	Overall prevalence at baseline: 51%	Over 4 years, overall OR 0.99 (0.87 to 1.12) compared to standard treatment
ADDITION- Denmark study (Charles et al., 2011)	1533 patients with T2D	Various cut-offs, including MNSI ≥ 7 ; vibration detection threshold, light touch, ankle brachial index	Between 7.3% to 34.8% depending on the diagnostic method used	No significant intergroup difference in neuropathy prevalence across all measurement methods after 6 years follow-up.
Veteran study (Duckworth et al., 2009)	1791 military veterans with T2D	New neuropathy assessed using complete annual physical examination	Not reported	After mean of 5.6 years, HR: 0.88 (0.75 to 1.05) compared to standard treatment

Abbreviations: ACCORD, Action to control cardiovascular risk in diabetes; ADDITION, Anglo-Danish-Dutch Study of Intensive Treatment in People with Screen-Detected Diabetes in Primary Care; BARI-2D, Bypass Angioplasty Revascularization Investigation 2 Diabetes; CON, control group; DCCT/ EDIC, diabetes control and complications /epidemiology of diabetes interventions and complications; DN, diabetic neuropathy; DSP, diabetic sensorimotor polyneuropathy; HDL-C, high-density lipoprotein cholesterol; HR, hazard ratio; IGT, intensive glucose control; LDL-C, low-density lipoprotein cholesterol; MNSI, Michigan neuropathy screening instrument; OR, odds ratio; NCS, nerve conduction studies; QST, quantitative sensory testing; RR, risk reduction; T1D, type 1 diabetes; T2D; type 2 diabetes; UKPDS, UK Prospective Diabetes Study (UKPDS) Group; yr, years.

DSP prevention focusses on glucose control and, in type 2 diabetes, lifestyle modification to regulate metabolic risk factors (Pop-Busui et al., 2017). In type 1 diabetes, glycaemic control is the only known method for delaying DSP onset and slowing its progression (Pop-Busui et al., 2017). This evidence was established in a pivotal, large-scale observational study: The Diabetes Control and Complications Trial (DCCT) and its associated follow-up study, The Epidemiology of Diabetes Interventions and Complications (EDIC). Compared with conventional therapy, treatment with intensive glucose control imparted a 64% risk reduction (95%CI: 45-76%) in DSP development, measured using the Michigan Neuropathy Screening Instrument (MNSI), after 6.5 years of follow-up (Martin et al., 2014). Similar benefits have been reported in other large epidemiology studies (Klein et al., 1996; Tesfaye et al., 1996), and are further supported by Callaghan *et al.* in a Cochrane systematic review (Callaghan et al., 2012b). Combining data from two studies (including the DCCT/EDIC), this review concluded, with high certainty, that enhanced glucose significantly reduces the risk of developing clinical neuropathy in type 1 diabetes, with an annualised risk reduction of 1.84% (95%CI: 2.56 to 1.11%).

In type 2 diabetes, the evidence for whether intense glucose control can prevent DSP onset and/or reduce progression is less conclusive (Boussageon et al., 2011). The Action to Control Cardiovascular Risk in Diabetes (ACCORD) study did not find a significant risk reduction in DSP incidence after 3.7 years of intensive glucose treatment compared with standard treatment (RR 0.95; 95%CI: 0.89 to 1.00) (Ismail-Beigi et al., 2010). The UK Prospective Diabetes Study reported a significant risk reduction (RR 0.80; 95%CI: 0.39 to 0.94) in DSP measured using bioesthesiometry, but only after 15 years of follow-up (UKPDS, 1998). In the same systematic review, Callaghan *et al* (2012), from a meta-analysis of four RCTs (including the ACCORD study), found that compared with conventional treatment, intensive glucose control did not significantly reduce the incidence of diabetic neuropathy in type 2 diabetes; however, treatment significantly improved nerve conduction and vibration threshold abnormalities (Callaghan et al., 2012b).

Notably, in type 2 diabetes, most of these randomised controlled trials (RCTs) included clinical neuropathy measures only as a secondary outcome or in *post hoc* analyses, rather than as a primary outcome. Considering the differences in disease pathogenesis, the presence of comorbidities and metabolic risk factors, such as hypertension, dyslipidaemia, and obesity, may play a more prominent role in DSP pathogenesis in type 2 diabetes compared type 1 diabetes (Ang et al., 2014; Callaghan et al., 2016).

2.2.2.2.3 DSP pathogenesis and disease-modifying treatments

Current management for established DSP aims to reduce disease progression and treat painful symptoms (Boulton et al., 2005). Epalrestat, in Japan and India, and alpha-lipoic acid, in Germany, are the only agents approved to target the underlying pathogenesis of diabetic neuropathy (Alabdali et al., 2015; Ziegler et al., 1999b).

Many compounds effective in attenuating nerve damage in animal models have been unsuccessful in their translation to human clinical trials (RCT findings summarised in **Table 2-2**), for example, nerve growth factor and ruboxistaurin (Boulton et al., 2013). These failures could relate to the complex and multifactorial mechanisms that underlie DSP pathogenesis in humans. Current understanding of DSP pathogenesis is largely derived from rodent models, where disease progression shares neurological features with those of humans (e.g., slowing of axonal transport and nerve conduction) (Sullivan et al., 2008b). However, rodents have a shorter lifespan, altered PNS structure, and different metabolic needs; thus, may respond differently to treatment.

Table 2-2. Current treatments targeting DSP pathophysiology

Compound	Modes of action	Main RCT findings (primary outcome)
Aldose-reductase inhibitors	Reduces sorbitol accumulation via the polyol pathway	- In participants with DN (n=594) Epalrestat significantly reduced median motor nerve conduction velocity loss after 3 years, relative to placebo (Hotta et al., 2006) - In patients with mild-moderate DN (n=208), no significant differences between the Zenarestat and placebo treatment groups in change from baseline in summed sensory or motor nerve conduction velocity after 52 weeks (Greene et al., 1999). - Withdrawn due to adverse effects: Sorbinil, Tolrestat, Zenarestatm Lidorestat, Zopolrestat (Boulton et al., 2005). - Unclear efficacy: Ponalrestat, Fidarestat (Boulton et al., 2005).
Alpha-lipoic acid	Antioxidant; inhibits oxidation	- ALADIN-III: Using combined intravenous and oral treatment, no significant difference was found in change from baseline in total symptom score over 7 months compared to placebo (n=509) (Ziegler et al., 1999a) - NATHAN-1: In mild-moderate DN(n=460), no significant difference in change of the Neuropathy Symptoms Score (composite clinical score) over 4 years compared to placebo (Ziegler et al., 2011)
Actovegin	Increases cellular metabolism, glucose and oxygen uptake, and adenosine triphosphate turnover	In T2D (n=567), combined intravenous and oral treatment improved total symptom score of the lower limbs and vibration perception threshold after 160 relative to placebo days (Ziegler et al., 2009)
Vasodilators (e.g., angiotensin-converting enzyme inhibitors)	Increases nerve blood flow	In normotensive diabetes (n=41), Trandolapril significantly increased peroneal motor nerve conduction velocity after 12 months compared to placebo (n=41) (Malik et al., 1998)
C-peptide	Increases nerve blood flow	In T1D (n=139), no difference in change from baseline in sensory nerve conduction velocity at 6 months between treatment and placebo groups (Ekberg et al., 2007)

Benfotiamine	Inhibits advanced glycation end-products (AGE) formation	- BENDIP- In diabetic polyneuropathy (n=165), relative to placebo, treatment group had significantly higher neuropathy symptom score after 6 weeks compared to placebo, but similar total symptom scores (Stracke et al., 2008) - BEDIP – in diabetic polyneuropathy for <2 years (n=40) significantly improved neuropathy score after 3 weeks, relative to placebo (Haupt et al., 2005) - In T1D (n=64) no significant difference in change in peripheral nerve function after 24 months between treatment and placebo groups (Fraser et al., 2012)
Fenofibrate	Lowers lipids (via PPAR pathway)	DSP not investigated as primary outcome. In a prespecified analysis of the FIELD study, patients with T2D (n=9795) without macrovascular disease, treated with fenofibrate for 5 years experienced reduced risk of minor amputation relative to placebo (Rajamani et al., 2009)
Nerve growth factor	Neurotrophic factor; promotes nerve regeneration	- In symptomatic DSP (n=1019) treatment with nerve growth factor injections did not significantly improve neuropathy impairment score of the lower limbs after 48 weeks, relative to placebo (Apfel et al., 2000) - Treatment associated with painful adverse effects (hyperalgesia; pain at injection site)
ARA 290	Non-hematopoietic peptide	- In individuals with T2D and painful neuropathy, ARA 290 administered subcutaneously for 28 days improved neuropathic symptoms and increased corneal nerve fibre density compared with placebo (Brines 2014).

Abbreviations: ALADIN, Alpha-Lipoic Acid in Diabetic Neuropathy; BEDIP, Benfotiamine in the treatment of Diabetic Polyneuropathy; BENDIP, BENfotiamine in Diabetic Polyneuropathy; DN, diabetic neuropathy; DSP, diabetic sensorimotor polyneuropathy; FIELD, Fenofibrate Intervention and Event Lowering in Diabetes; NATHAN, Neurological Assessment of Thioctic Acid in Diabetic Neuropathy; PPAR, peroxisome proliferator-activated receptors, T1D, type 1 diabetes; T2D, type 2 diabetes.

The aetiology of DSP is not yet fully understood, but is hypothesised to involve multiple pathways, including accelerated metabolism in the polyol pathway, accumulation of advanced glycation end-products (AGEs), activation of protein kinase C (PKC) pathways, and oxidative and inflammatory stress (Brownlee, 2001; Feldman et al., 2017; Yagihashi et al., 2011). These key pathways (summarised in **Figure 2-1**) and associated therapeutic targets are discussed below.

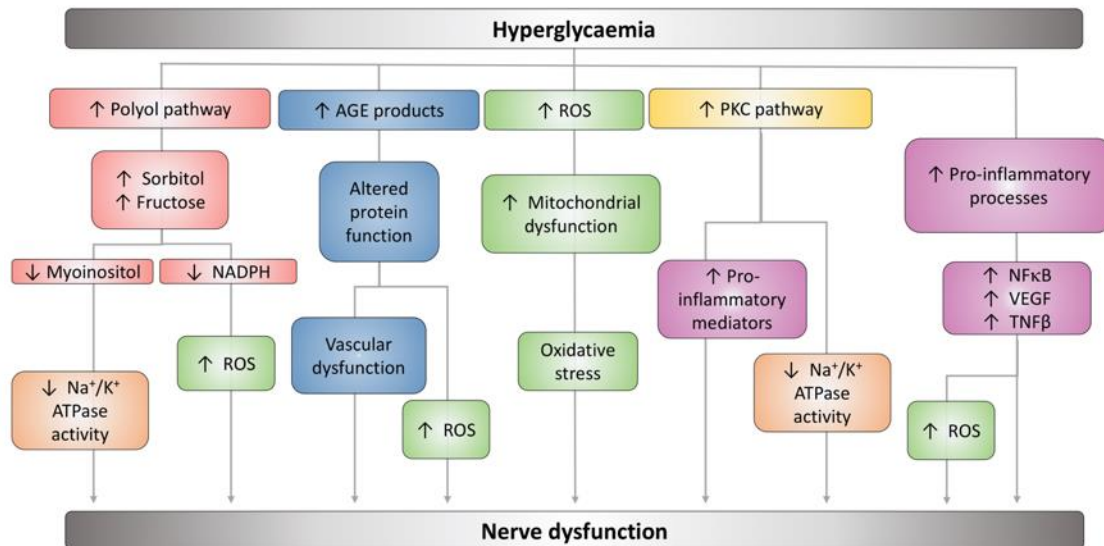


Figure 2-1. DSP pathogenesis. Adapted from Markoulli *et al.* (2018). **Abbreviations:** AGE, advanced glycation end-products; Na^+/K^+ ATPase, sodium potassium adenosine triphosphatase; NADPH, Nicotinamide adenine dinucleotide phosphate; $\text{NF}\kappa\text{B}$, nuclear factor kappa-B, ROS, reactive oxidation species; PKC, protein kinase-C; $\text{TNF-}\beta$, transforming growth factor- β ; VEGF, vascular endothelial growth factor.

2.2.2.2.3.1 Polyol pathway

As hyperglycaemia saturates the glycolytic pathway, excess intracellular glucose is diverted into the polyol pathway and converted into sorbitol by the enzyme aldose reductase (Brownlee, 2001). Accumulation of sorbitol and its metabolite, fructose, reduces membrane myoinositol levels and sodium potassium adenosine triphosphatase (Na^+/K^+ -ATPase) activity, slowing nerve conduction velocity (Greene *et al.*, 1984). Increased polyol pathway activity also depletes intracellular nicotinamide adenine dinucleotide phosphate, a primary antioxidant, which exacerbates the accumulation of reactive oxidation species (ROS) (Cashman & Hoke, 2015).

Aldose-reductase inhibitors aim to suppress sorbitol accumulation. Several compounds that showed promise in animal models have been unsuccessful in their clinical translation, due to limited efficacy and/or severe dose-limiting adverse effects in humans (Greene *et al.*, 1999; Santiago *et al.*, 1993). Epalrestat was approved in Japan in 1992, after a large, multicentre RCT showed that it is effective for improving motor nerve conduction and DSP symptoms compared to placebo (Hotta *et al.*, 2006). However, this trial included

only patients with stable glycaemic control (HbA1c $\leq 9\%$). The analysis also excluded participants who withdrew from the study. To date, all RCTs investigating the long-term effects of Epalrestat have been open-label studies, risking bias from a lack of masking. Furthermore, a Cochrane systematic review concluded, from combining 32 RCTs, that compared with placebo, there was no significant benefits of aldose-reductase inhibitors for DSP on neurological examination findings in individuals with diabetes (Chalk et al., 2007).

2.2.2.2.3.2 Advanced glycation end-products

The glycation of glucose and glucose-derived precursors with intracellular proteins forms advanced glycation end-products (AGEs), altering protein function (Brownlee, 2001; Sugimoto et al., 2008). In the sciatic nerve of rats, glycation of extracellular matrix and basement membrane proteins reduces sensory neurite outgrowth and impairs axonal regeneration (Duran-Jimenez et al., 2009). In diabetes, increased AGE accumulation has been detected in axonal cytoskeletal and myelin proteins, suggesting glycation plays a role in axonal degeneration and segmental demyelination (Ryle & Donaghy, 1995). Interactions of AGEs with their receptors further elicit oxidative stress by upregulating nuclear factor kappa-B (NF κ B) transcription factor and activating pro-inflammatory pathways (Cameron & Cotter, 2008; Sugimoto et al., 2008).

Several RCTs have investigated the efficacy of Benfotiamine, a thiamine derivative that inhibits AGE accumulation, on diabetic neuropathy (**Table 2-2**). Although treatment for a short duration (<6 weeks) showed promising results by improving DSP symptoms and clinical scores (Haupt et al., 2005; Stracke et al., 2008), a large RCT conducted over 24 months did not find a difference in peripheral nerve function with Benfotiamine relative to placebo (Fraser et al., 2012).

2.2.2.2.3.3 Protein kinase C activity

Hyperglycaemia increases the formation of protein kinase C (PKC) isoforms. PKC activity upregulates the expression of angiogenic and pro-inflammatory

mediators, including vascular endothelial growth factor (VEGF), transforming growth factor- β (TGF- β) expression, and NF κ B; this process induces vascular abnormalities and ROS formation (Brownlee, 2001; Geraldles & King, 2010). Hyperglycaemia-induced PKC activation also inhibits NA⁺/K⁺-ATPase activity, disrupting nerve perfusion and reducing nerve conduction velocity (Cameron et al., 1999). In a double-masked RCT, ruboxistaurin, a PKC- β inhibitor, did not improve vibration detection thresholds (primary trial outcome) after 12 months, relative to placebo (Vinik et al., 2005a).

2.2.2.2.3.4 *Oxidative stress*

Excess ROS accumulation precipitates oxidative stress, which causes mitochondrial dysfunction and neuronal apoptosis (Babizhayev et al., 2015; Vincent et al., 2005). In DSP, in addition to the pathways previously described, ROS accumulates from elevated glucose oxidative metabolism in the mitochondria (Babizhayev et al., 2015). PNS neurons and Schwann cells, containing larger volumes of mitochondria than other organelles, are more susceptible to oxidation-induced mitochondrial injury, resulting in nerve conduction loss and axonal demyelination (Hamid et al., 2014; Markoulli et al., 2018).

Alpha-lipoic acid, an antioxidant, is proposed to improve symptomatic DSP by reducing oxidative stress (Ziegler et al., 1999a). While earlier studies showed intravenous alpha-lipoic acid to be effective for reducing symptomatic neuropathy and neuropathic deficits over three to five weeks (Mijnhout et al., 2012; Ziegler et al., 2004), longer-term treatment with combined intravenous and oral therapies failed to demonstrate similar efficacy (Ziegler et al., 1999a). Furthermore, a double-masked RCT showed oral alpha-lipoic acid treatment for four years did not significantly improve neuropathy impairments relative to placebo (**Table 2-2**) (Ziegler et al., 2011).

2.2.2.2.3.5 Intracellular and inflammatory signalling

In addition to the metabolic, neurochemical and vascular irregularities described, inflammatory signalling also plays a critical role in the pathogenesis of neuropathy, with mechanisms occurring in parallel pathological pathways (Pop-Busui et al., 2016).

Diabetic neuropathy features low-grade, chronic sub-clinical inflammation (Pop-Busui et al., 2016; Zhou & Zhou, 2014). NF κ B activation is hypothesised to play a central role in mediating inflammation-related nerve damage (Pop-Busui et al., 2016). NF κ B is a key transcription factor involved in the activation of inflammatory responses of immune cells triggered by exogenous stimuli. NF κ B release up-regulates the expressions of a number of pro-inflammatory mediators, including tumour necrosis factor- α (TNF- α), endothelin-1, cyclooxygenase-2, lipoxygenase, and inducible nitric oxide synthase (Allam-Ndoul et al., 2016). This process further exacerbates oxidative stress in a feedback loop (Cameron & Cotter, 2008). In rats, inhibition of NF κ B and its downstream mediators improved motor nerve conduction velocity and restored nerve blood flow (Kellogg et al., 2007; Kumar et al., 2011).

Fenofibrate, a peroxisome proliferator-activated receptor (PPAR)- α agonist, aims to reduce inflammation through inhibiting the NF κ B pathway. Fenofibrate reduced diabetic retinopathy progression in type 2 diabetes and was recently approved in Australia for this indication (Keech et al., 2007). In mice with DSP, Fenofibrate restored neuropathic impairments in the limbs and the cornea (Matlock et al., 2020). However, PPAR- α agonists have not been specifically investigated for the clinical treatment of DSP.

In diabetes, a lack of neurotrophic support is hypothesised to contribute towards the pathogenesis of DSP (Andreassen et al., 2009). Neurotrophic signalling plays a critical role in neuron differentiation and survival; reduced nerve growth factor and brain-derived neurotrophic factor is found in both animals and humans with diabetes (Andreassen et al., 2009). Insulin and C-peptide

deficiencies can also impair neurotrophic signalling (Sima et al., 2000). However, various treatments aiming to deliver neurotrophic support have not convincingly demonstrated efficacy (**Table 2-2**).

2.2.2.2.4 Limitations of current clinical trial evidence

In addition to the complexities associated with finding an effective therapeutic target, other factors to consider in DSP trials are study design, participant selection, and the precision of the outcome(s) selected as trial endpoints (Alabdali et al., 2015; Boulton et al., 2013). Ascertaining clinically significant improvement is difficult in populations without neuropathy; however, in patients with advanced neuropathy and undetectable nerve potential signals at baseline, there is also limited ability to quantify change in these variables (Apfel et al., 2000). Ziegler and Luff suggested that RCTs should focus preferentially on mild or moderate DSP, with an aim to prevent, rather than to reverse, the condition would be more likely to yield significant results (Ziegler & Luff, 2002). However, given the progressive nature of DSP, these trials must be undertaken over extended durations to allow adequate time to observe any treatment-related effects, and this would require significantly more resources.

With regard to study methodology, per-protocol analyses do not provide an unbiased comparison of study treatment groups, on account of excluding participants that are lost to follow-up (Hotta et al., 2006; Stracke et al., 2008; Ziegler et al., 1999a). Masking is a critical consideration as a significant placebo effect has been observed in DSP, particularly for outcomes related to symptoms (Tesfaye et al., 2007; Ziegler et al., 2009). For studies involving compounds that have significant side effects, the ability to undertake adequate masking may be unintentionally compromised in the treatment group (Apfel et al., 2000).

Another important consideration is the selection of outcome measures as endpoints. As demonstrated in epidemiological studies (**Table 2-1**), a range of clinical and neurophysiological outcomes have been adopted for assessing DSP. The absence of a universally-accepted outcome measure set for

quantifying nerve damage in peripheral neuropathies limits the ability to compare outcomes across multiple trials (Merkies & Lauria, 2006). Furthermore, clinical endpoints should be sensitive enough to detect both deterioration in the placebo group and improvement in the treatment group to clearly ascertain treatment efficacy (Boulton et al., 2013).

2.2.3 Clinical evaluations for peripheral neuropathy

Several clinical methods are available for assessing peripheral nerve structure and function (**Table 2-3**).

Table 2-3. Clinical examination of peripheral nerve structure and function

Test	Nerve fibres assessed	Assessment method for diabetic neuropathy
Nerve conduction studies (Mallik & Weir, 2005)	Fastest, large-diameter nerve fibres	Stimulation of a sensory or motor nerve (in the limbs) with electrical pulses and measuring the responses of the nerve obtained through a muscle (motor nerves) or a different point along the same nerve (sensory nerves)
Nerve excitability (Kiernan et al., 2020)	Axonal ion channel function	Stimulation of a sensory or motor nerve (in the limbs) with submaximal stimuli and measuring the properties of the axonal membrane
Quantitative sensory testing (QST) (ADA, 1992)	Small A δ - and C-fibres	Subjective response to thermal, vibration and pain stimuli presented at different thresholds; usually evaluated in the limbs.
Cutaneous silent period (CSP) (Floeter, 2003)	Thinly myelinated A δ -fibres	Measures the brief suppression of voluntary muscle contraction after strong stimulation of the cutaneous nerve in the limbs
Quantitative sudomotor axonal reflex testing (QSART) (Sletten et al., 2010)	Unmyelinated postganglionic sudomotor C-fibres	Measures axon reflex from the stimulation of the eccrine sweat glands through iontophoresis of acetylcholine
Intraepidermal nerve fibre density (Lauria & Lombardi, 2007)	Somatic small nerve fibres	<i>Ex vivo</i> examination of the density of intraepidermal small nerve fibres taken using skin punch biopsy, usually from the limbs
<i>In vivo</i> corneal confocal microscopy (Perkins et al., 2018)	Small A δ - and C-fibres	<i>In vivo</i> visualisation and quantification of corneal sensory nerve fibre parameters from the sub-basal nerve plexus

Neuropathy impairment is often assessed using validated composite scores or neuropathy scales, quantifying symptoms (e.g., the MNSI), signs (e.g., the Neuropathy Disability Score), or a combination of both (e.g., the Toronto Clinical Neuropathy Score). Although Merkies and Lauria, in the 131st Europe Neuromuscular Centre international consensus document, stated that neuropathy impairment should be assessed as an endpoint in intervention studies, no recommendation was made with regard to a universal scoring system for RCTs (Merkies & Lauria, 2006). For DSP diagnosis, the American Academy of Neurology has stated that composite scores provide higher diagnostic accuracy, and the incorporation of electrophysiology results further increases specificity (England & Asbury, 2004).

Electrophysiology studies evaluate the characteristics of an electrical signal that travels through a nerve. Nerve conduction studies (NCS), which are non-invasive, objective, reproducible, and employ standardised methodology, form an integral component of DSP diagnosis (England et al., 2005; Mallik & Weir, 2005). However, NCS provide information only on the number and characteristics of the fastest conducting nerve fibres and no information on small nerve fibre function (Mallik & Weir, 2005). Nerve excitability testing assesses axonal membrane potential and provides information on ion channel function; in particular, on sodium and potassium channel function and Na⁺/K⁺-ATPase activity (Nodera & Kaji, 2006). In contrast to NCS, nerve excitability testing can detect axonal dysfunction in early and subclinical DSP, prior to irreversible nerve loss (Arnold et al., 2013; Sung et al., 2012). Hence, along with small nerve fibre evaluations, nerve excitability tests are considered superior clinical markers of axonal dysfunction earlier in the course of DSP. The application of nerve excitability testing has widened since the development of the TROND protocol (Kiernan et al., 2000), which addressed earlier issues pertaining to its time-consuming and technically-challenging nature (Nodera & Kaji, 2006). However, nerve excitability testing has still not been widely adopted as an endpoint in DSP clinical trials.

Quantitative sensory testing measures an individual's response to a thermal, vibrating and/or painful stimulus delivered to the skin. However, sensory function tests rely on subjective responses and are, as such, prone to greater variability. Its reproducibility can also vary depending on the examination technique, examiners' skill level, and testing conditions (e.g., temperature, humidity) (Shy et al., 2003). Cutaneous silent period (CSP) and quantitative sudomotor axonal reflex testing (QSART) respectively evaluate dysfunction of thinly myelinated A δ - and unmyelinated C-fibres. It was stipulated that the two tests used in combination provide better diagnostic accuracy (diagnosing small fibre neuropathy with 77% sensitivity) than individually, as small fibres show inter-subject variation in structural and functional properties (Kamel et al., 2015).

Nerve biopsy is currently the gold standard procedure for examining the structural integrity of small nerve fibres. Intraepidermal nerve fibre density (IENFD) can accurately diagnose and classify the extent of small fibre neuropathy in the absence of large fibre nerve damage (Lauria & Lombardi, 2007). However, biopsy procedures are invasive, expensive and not easily repeatable; therefore, they are infrequently utilised in longitudinal studies (Sommer et al., 2010).

Historically, clinical examinations assessing large fibre function have been reserved for DSP diagnosis rather than for assessing nerve regeneration and repair, owing to limitations in sensitivity (Dyck et al., 1985). The accurate assessment of small fibre integrity is important not only for diagnosing and monitoring disease but is also critical in evaluating the efficacy of new therapies. For this reason, outcome measures selected in the RCT described in **Chapter 7** include NCS and nerve excitability studies, for large nerve fibre and axonal membrane function, and CSP and QSART, for A δ - and C- small nerve fibre function. In addition, corneal nerve structural parameters, quantified from *in vivo* confocal microscopy (IVCM) images, are adopted as a surrogate marker to evaluate peripheral nerve structure.

In vivo confocal microscopy

The transparent and richly-innovated cornea is the only place in the body where peripheral sensory nerves can be non-invasively imaged. *En face* visualisation of corneal anatomical structures can be evaluated using IVCM. Corneal IVCM imaging was first described using the tandem-scanning microscope, followed shortly by the slit-scanning microscope (Cruzat et al., 2017). Both instruments capture images of the corneal layers, but have poorer resolution than modern laser-scanning IVCM, which is currently considered the gold-standard (De Silva et al., 2017).

Currently, the only commercially-available laser-scanning IVCM device is the Heidelberg Retinal Tomograph (HRT)-3 with Rostock Corneal Module (RCM) (Heidelberg Engineering, GmbH, Dossenheim, Germany). Images captured using laser-scanning IVCM have high contrast and signal-to-noise ratio and can be analysed to provide a range of quantitative clinical measures, such as the density and morphology of corneal nerves, epithelial cells, and resident immune cells.

The next section describes the impact of diabetes on the ocular surface and the utility of corneal sensory nerve parameters as surrogate markers for longitudinally monitoring DSP.

2.3 The ocular surface

2.3.1 Cornea

2.3.1.1 Gross anatomy

The cornea is a highly organised, transparent tissue that forms the most anterior portion of the eye. It comprises five specialised layers: epithelium, Bowman's layer (anterior limiting lamina), stroma, Descemet's membrane (posterior limiting lamina), and endothelium (**Figure 2-2**). The cornea provides most of the eye's refractive power and acts as barrier to protect internal ocular structures. As an avascular tissue, it obtains essential nutrients via adjacent fluid reservoirs; anteriorly from the tear film and limbal capillaries, and posteriorly from the aqueous humour. Significant disturbances to the tear film, such as in dry eye disease (DED) and diabetes, can disrupt corneal metabolism and affect corneal transparency.

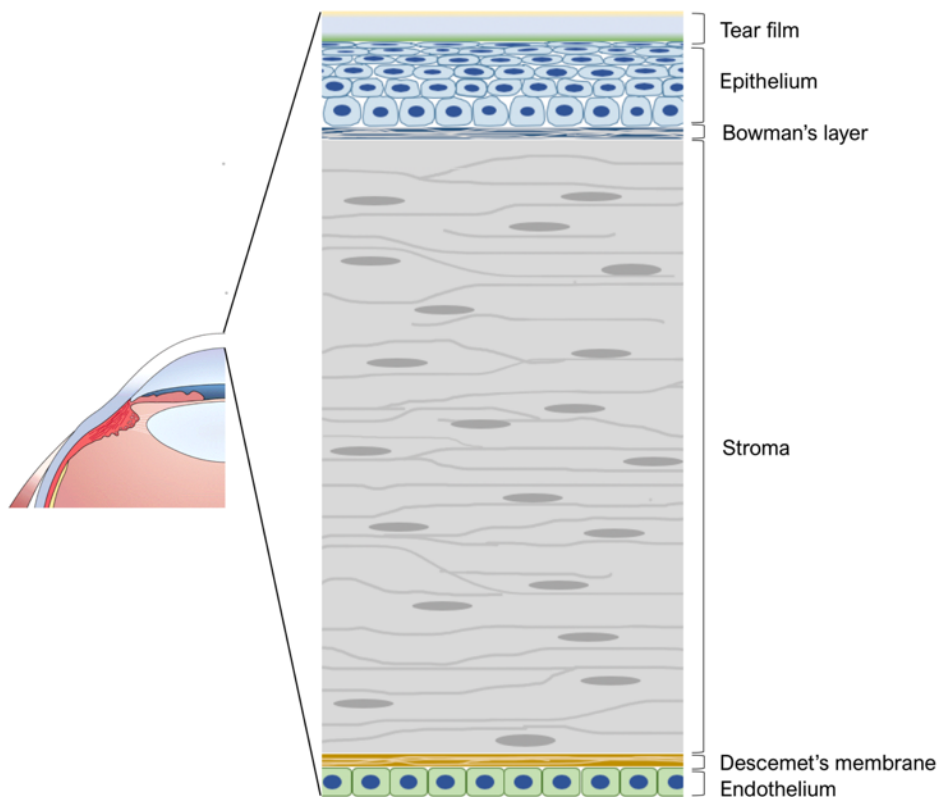


Figure 2-2. The gross structure of the cornea. Adaptation based on figure by Rowsey & Karamichos (Rowsey & Karamichos, 2017)

The epithelium, the most anterior corneal layer, comprises non-keratinised, stratified epithelium in three distinct layers: superficial squamous cells, elongated wing cells, and deeper cuboidal basal cells (Beuerman & Pedroza, 1996). Superficial squamous cells are held together by tight junctions that provide barrier function; the cells project, on their externally facing surface, microvilli that secrete glycocalyx, supporting tear film adhesion and stability (Gipson & Argüeso, 2003).

The human corneal epithelium is replaced completely approximately every seven days (Lemp & Mathers, 1989). Basal epithelial cells proliferate and migrate anteriorly, flattening to first form the wing cell layer, and eventually the superficial squamous cell layers, before sloughing into the tear film (Dua et al., 1994). The corneal sub-basal nerve plexus (SBNP) runs beneath and parallel to the basal epithelium (**Figure 2-3**). Dendritic cells (DCs), situated in the basal epithelium, assist in maintaining corneal homeostasis and wound repair (see section **2.3.1.3**) (Gao et al., 2011). Basal epithelial cells are firmly anchored to the basement membrane by hemidesmosomes and sit atop Bowman's layer.

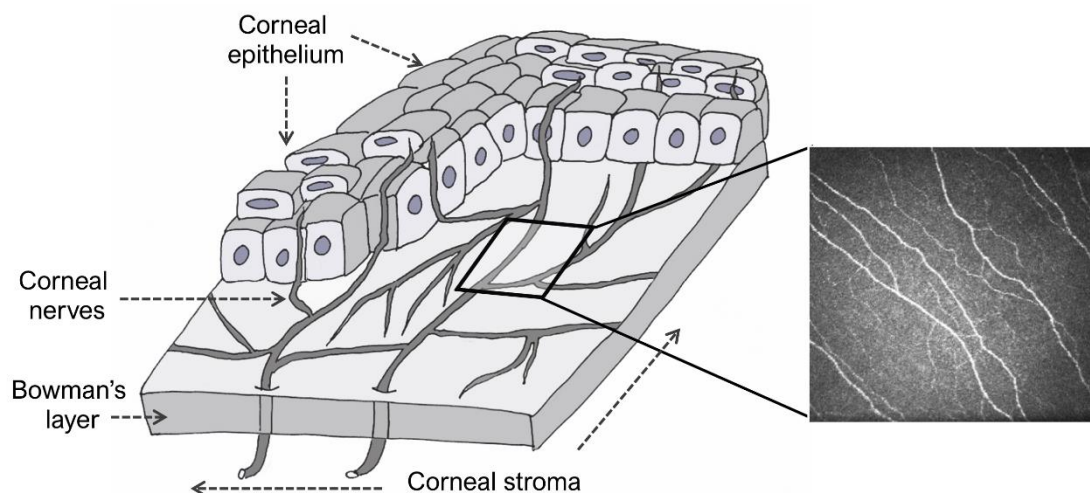


Figure 2-3. Corneal sub-basal nerve plexus (SBNP). Adapted based on figure by Erie et al. (Erie et al., 2005). Inset: *En face* image of the Corneal SBNP, captured using *in vivo* confocal microscopy.

Bowman's layer is an acellular layer composed of randomly distributed, but closely arranged, collagen fibrils, proteoglycans and amorphous matrix (Marshall et al., 1991). Bowman's layer does not possess regenerative properties.

The stroma is the thickest corneal layer, making up approximately 90% of the total corneal thickness. It comprises lamellae of tightly-packed, parallel collagen fibrils, keratocytes, nerves, and extracellular matrix (Marshall et al., 1991). The regular collagen arrangement and extracellular matrix provide transparency, regulate hydration, and uphold corneal rigidity. Stromal keratocytes play a crucial role in synthesising collagen, glycosaminoglycans and proteoglycans to maintain the extracellular matrix environment (Meek & Knupp, 2015).

Descemet's membrane is a second acellular layer located between the corneal stroma and endothelium. It is secreted by, and acts as the basement membrane to, the endothelium and serves as an inner protective barrier. It comprises widely-spaced collagen fibrils and, unlike Bowman's layer, can regenerate (Obata & Tsuru, 2007).

The endothelium is the most posterior of the corneal layers, comprising a monolayer of tightly-packed, flattened, hexagonally-shaped cells held together by modified desmosomes (DeMonte & Kim, 2011). Its main function is to regulate corneal hydration. Na^+/K^+ -ATPase pumps, located in the basolateral endothelial membrane, actively transport ions from the stroma to the aqueous humour, maintaining the osmotic gradient and corneal transparency (Huang et al., 2003). Correspondingly, nutrients, such as glucose, are transported from the aqueous humour to provide metabolic support to the cornea.

2.3.1.2 Corneal nerves

2.3.1.2.1 Structure

2.3.1.2.1.1 Gross structure

The cornea is the most densely innervated tissue in the human body (Rózsa & Beuerman, 1982). Its rich sensory innervation is crucial for providing neurotrophic support to the ocular surface (Muller et al., 2003). Corneal nerves arise from the ophthalmic branch of the trigeminal nerve and are supplied by small myelinated A δ -fibres and unmyelinated C-fibres (Muller et al., 1997). Corneal nerves become demyelinated at approximately one millimetre from the limbus to aid corneal transparency (Al-Aqaba et al., 2010).

The architecture of the corneal nerve network has been mapped using both light and electron microscopy (Muller et al., 1996; Muller et al., 1997), as well as IVCM (Misra et al., 2012; Patel & McGhee, 2005). Nerves enter from the limbus multi-directionally through the mid-stroma and traverse centrally and anteriorly. In the mid-peripheral stroma, some corneal nerves turn 90 degrees to perforate Bowman's layer, forming the SBNP (Muller et al., 1996). These nerves terminate into bulb-like endings in the most superficial aspect of the corneal epithelium (Marfurt et al., 2010).

Corneal nerves in the SBNP are arranged centripetally in a whorl-like pattern, which is centred infra-nasally around 1-3 mm below the corneal apex (Al-Aqaba et al., 2010; Patel & McGhee, 2005). As a consequence, nerves in the central cornea are arranged predominantly vertically, and in the peripheral cornea course in various directions and can change direction abruptly to reorientate towards the corneal apex (Marfurt et al., 2010). Central SBNP nerves are also dense and homogenous, whereas in the peripheral cornea they are thinner and more sparse (Marfurt et al., 2010).

2.3.1.2.2 *Function*

2.3.1.2.2.1 *Types of sensory nerves*

The cornea contains three functional receptor subtypes that differ by their response to stimuli: polymodal nociceptors that respond to thermal, chemical and mechanical stimuli; cold thermoreceptors that respond to cooling sensations and increases in tear osmolarity; and mechano-nociceptors that respond only to mechanical stimulation (Belmonte et al., 2004) (**Table 2-4**).

Table 2-4. Corneal receptor subtypes

Receptor subtype	Main fibre type	Receptor subtype	Stimuli to activate	% in cornea
Polymodal receptors	C-fibres	TRPV1	- Heat ($> \sim 37^{\circ}\text{C}$) - Chemical (exogenous irritants; pH ~ 5-6.5 or CO_2; endogenous chemical mediators) - Mechanical (threshold lower than that of mechano-receptors)	70%
Cold thermo-receptors	A δ - and C- fibres	TRPM8	- Thermal ($> \sim 0.5^{\circ}\text{C}$ from the ocular surface temperature) - Osmolarity (from 10 mOsmol/L; significant increase from ~ 310 to 340 mOsmol/L)	10-15%
Mechano-receptors	A δ -fibres	Piezoz2	Mechanical (~ 0.6 mN)	20-30%

Based on information reported by Belmonte *et al.*(2004) and Gallar *et al.*(1993) (Belmonte et al., 2004; Gallar et al., 1993) (Belmonte et al., 2004; Gallar et al., 1993) (Belmonte et al., 2004; Gallar et al., 1993) (Belmonte et al., 2004; Gallar et al., 1993) (Belmonte et al., 2004; Gallar et al., 1993) (Belmonte et al., 2004; Gallar et al., 1993) (Belmonte et al., 2004; Gallar et al., 1993) (Belmonte et al., 2004; Gallar et al., 1993) (Belmonte et al., 2004; Gallar et al., 1993) (Belmonte et al., 2004; Gallar et al., 1993) (Belmonte et al., 2004; Gallar et al., 1993) (Belmonte et al., 2004; Gallar et al., 1993) (Belmonte et al., 2004; Gallar et al., 1993) (Belmonte et al., 2004; Gallar et al., 1993) (Belmonte et al., 2004; Gallar et al., 1993) (Belmonte et al., 2004; Gallar et al., 1993) (Belmonte et al., 2004; Gallar et al., 1993) (Belmonte et al., 2004; Gallar et al., 1993). **Abbreviations:** Piezo₂, Piezo-type mechanosensitive ion channel component 2; TRP, transient receptor potential

Polymodal nociceptors terminate in the epithelium in either simple endings without branching or ramifying endings that run parallel to the corneal surface (Ivanusic et al., 2013). Polymodal receptors express the transient receptor potential (TRP) cation channel, subfamily V, member 1 (TRPV1). It has been

hypothesised that different molecular phenotypes of TRPV1-expressing polymodal nociceptors exist, but the functional significance of this molecular heterogeneity has not been elucidated (Belmonte et al., 2017). TRPV1-expressing nerve terminals release neuropeptides, substance P and calcitonin gene-related peptide, which mediate neurogenic inflammation and promote corneal epithelial cell proliferation (Belmonte et al., 1997).

Cold thermoreceptors comprise a mixture of A δ - and C-fibres (Gallar et al., 1993). Axons of cold thermoreceptors express TRP subfamily M, member 8 (TRPM8) channels that form complex endings in the epithelial wing and squamous cell layers (Ivanusic et al., 2013). TRPM8-containing axons are more abundant and structurally-complex in the peripheral cornea of mouse and guinea pig (Ivanusic et al., 2013). Whether this neural structural complexity is evident in humans is not yet known.

Mechano-nociceptors of the A δ -fibre type are phasic sensory receptors that convey sharp pain (MacIver & Tanelian, 1993). They can signal the presence of a mechanical stimulus, but only poorly differentiate the stimulus duration and/or intensity (Belmonte et al., 2017). Compared to mechano-nociceptors in the skin, the threshold to activate corneal mechanoreceptors is relatively low, but higher than that required to activate polymodal nociceptors (Belmonte et al., 1997).

2.3.1.2.2.2 Functional assessment

Corneal sensation can be measured using both contact and non-contact methods. The Cochet-Bonnet contact aesthesiometer is the most widely used clinical instrument. Similar to von Frey hairs for skin assessment, Cochet-Bonnet aesthesiometry uses pressure from a nylon filament that is applied to the corneal surface with increasing force, by incrementally shortening the filament length, until sensation is detected. The method is rapid and portable, but invasive, and has discrete and limited intensity ranges (Murphy et al., 1998). The Cochet-Bonnet aesthesiometer was unable to accurately measure corneal

sensitivity in up to 60% of normal individuals due to values being below threshold (Golebiowski et al., 2011; Murphy et al., 1998).

Non-contact corneal aesthesiometry (NCCA) uses a controlled air pulse to measure corneal sensation. Unlike contact aesthesiometry, which provides a mechanical stimulus, NCCA measures sensation to a composite stimulus comprising the effects of air pressure, tear film disruption, and cooling (Murphy et al., 1996). Comparing the two techniques, both Golebiowski *et al.*, and Murphy *et al.*, reported poor correlation in the corneal sensitivity thresholds of healthy individuals measured using the NCCA and the Cochet-Bonnet aesthesiometer (Golebiowski et al., 2011; Murphy et al., 1998). The poor correlation may be due to the different stimulus modalities provided by the instruments and the limited intensity range of the Cochet-Bonnet aesthesiometer. In that regard, the NCCA holds key advantages over contact methods as the technique is non-invasive, has superior repeatability, and is more sensitive (Golebiowski et al., 2011; Murphy et al., 1998).

2.3.1.3 Immune cells

In the cornea, significant interactions between the immune and nervous systems play a role in maintaining ocular surface homeostasis (Gao et al., 2016).

Resident immune cells of the cornea comprise macrophages, located predominantly in the posterior stroma, and presumed DCs in the epithelium and anterior stroma (Chinnery et al., 2008).

DCs serve as a bridge between the innate and adaptive immune systems, acting as professional antigen-presenting cells that stimulate T-lymphocyte-dependent immune responses. Immature DCs, with a high capacity for antigen binding and low capability for T-cell activation, undergo maturation in response to local and systemic inflammation. This process downregulates their endocytic activity and upregulates the expression of major histocompatibility complex (MHC) class II and costimulatory molecules (Hamrah et al., 2003).

In the cornea, epithelial immune cells have been identified as DCs based on the expression of cell-surface receptors using immunohistochemistry (Hattori et al., 2011). Historically, DCs in the uninflamed cornea were thought to be MHC class II-negative cells that undergo maturation and migrate centrally in response to corneal inflammation (Hamrah et al., 2002). However, this view has been challenged by several studies that found that corneal DCs universally express MHC class II receptors, suggesting that resident DCs are mature cells (Leppin et al., 2014; Mayer et al., 2007).

Using IVCM, cellular structures identified within the corneal sub-basal nerve plexus are presumed to be DCs. These cells have also been referred to as Langerhans cells in some studies (Hamrah et al., 2002; Hattori et al., 2011). However, their expression of cell-surface markers has not been characterised using molecular studies. Using IVCM, DC numbers are approximately three-fold higher in the peripheral cornea than in the central region.

DC classification has been proposed by several studies based on observed cellular morphological features via IVCM (Lagali et al., 2018; Mastropasqua et al., 2006). Cells with long, slender bodies and dendrite-like processes were considered to be mature cells; cells with larger bodies and shorter processes were classified as immature DCs (**Figure 2-4**). However, these classifications have not been confirmed by molecular phenotyping.

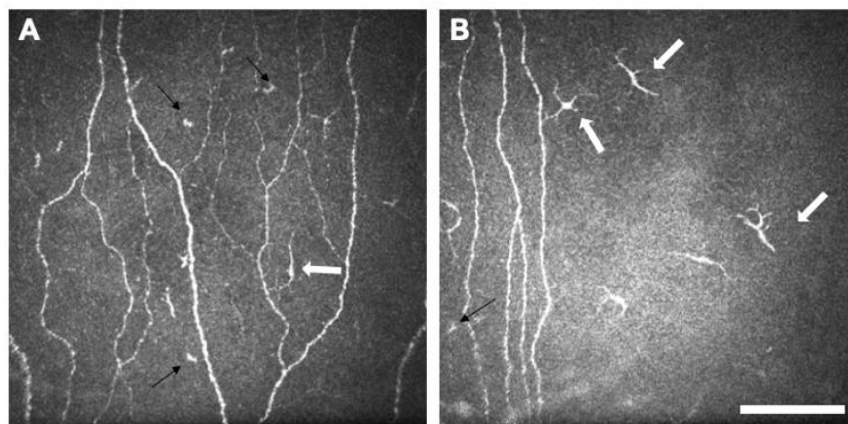


Figure 2-4. Corneal dendritic cells (DCs) visualised using *in vivo* confocal microscopy in the A) central and B) peripheral cornea. White arrows indicate putative mature DCs with long, slender bodies and branching dendrites; black arrows indicate putative immature DCs with larger bodies and short processes. Scale bar=100 μ m.

With respect to corneal DC density, Tavakoli *et al.* reported higher DC density in individuals with DSP compared with healthy controls (Tavakoli *et al.*, 2011a). In this study, central corneal DC density also correlated with DSP severity. In contrast, Lagali *et al.* did not find a difference in DC density, but found a higher proportion of cells to be mature DCs, in recent onset type 2 diabetes (<10 years) relative to healthy, age-matched controls (Lagali *et al.*, 2018). No longitudinal studies have examined how epithelial DC density or morphology changes throughout the course of DSP. It is also not clear whether this change occurs differently in the central versus peripheral cornea, potentially reflecting DC maturation and/or migration.

2.3.2 Tear film

The tear film is the primary refractive surface of the eye and provides the underlying cornea with nutrition and metabolic support and protection (Willcox *et al.*, 2017). As reported by the recent Tear Film And Ocular Surface Society Dry Eye Workshop II (TFOS DEWS II), the tear film is composed of an outermost lipid layer overlying a mix of aqueous and mucin components that constitutes a mucoaqueous gel (Willcox *et al.*, 2017) (**Figure 2-5**).

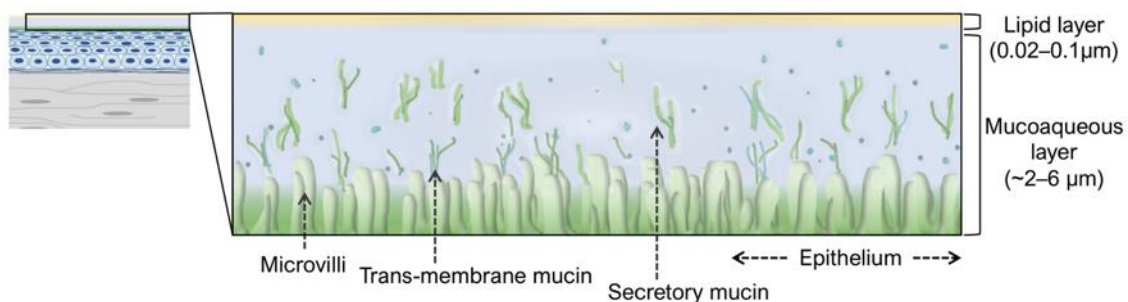


Figure 2-5. The pre-corneal tear film. Corneal structure of **Figure 2-2** with inset showing structures of the tear film.

The lipid layer contains meibum secreted from meibomian glands, modified sebaceous glands located in the eyelids. The lipid layer comprises both non-polar and amphiphilic lipids, the majority of which are thought to be wax and cholesterol esters, and it functions to maintain tear stability and prevent tear film evaporation (Wojtowicz *et al.*, 2009).

The aqueous component is secreted by the lacrimal gland, located superotemporally to the globe, and by accessory glands. It contains water, to provide lubrication; metabolites and electrolytes, to maintain nutrition and osmolarity; and proteins, to provide immune protection (Bron et al., 2017). Tear film osmolarity is a colligative property, determined by the concentration of electrolytes in the tears. After a blink, the tear film thins over the cornea, as a result of tear evaporation, and induces a hyperosmotic shift. In humans, physiological tear osmolarity ranges from approximately 270 to 315 mOsm/L (Jacobi et al., 2011; Tomlinson et al., 2006). Although elevated tear osmolarity is positively associated with ocular surface disease (Suzuki et al., 2010), there is overlap in the values in normal and diseased states (Sullivan et al., 2012).

Mucins are large, hydrophilic glycoproteins that are bound to the apical surface of the corneal epithelium (transmembrane mucins) or secreted by conjunctival goblet cells (secretory mucins) (Willcox et al., 2017). The two types of secretory mucins are large, gel-forming mucins and small, soluble mucins (Corfield, 2015). In the tear film, mucins function to enhance the spreading of the tear layers and support tear film structure. In conjunction with the lipid layer and aqueous proteins, mucins may also play a role in preventing tear film evaporation (Gipson & Argüeso, 2003).

2.3.3 The impact of diabetes on the ocular surface

2.3.3.1.1 Corneal nerves

2.3.3.1.1.1 Structure

Corneal SBNP structural parameters, quantified from IVCM images, have been extensively studied as a surrogate marker for predicting, diagnosing and monitoring DSP (Dehghani et al., 2014b; Malik et al., 2003; Misra et al., 2015; Pritchard et al., 2015).

Cross-sectional studies have demonstrated the diagnostic utility of both corneal nerve fibre length (CNFL) and corneal nerve fibre density (CNFD) relative to

NCS and IENFD (Ahmed et al., 2012; Chen et al., 2015). Both corneal nerve parameters can be used to stratify DSP severity (Jiang et al., 2016; Tavakoli et al., 2010; Ziegler et al., 2014). CNFD loss has also demonstrated to reflect motor axonal ion channel dysfunction quantified using nerve excitability testing (Tummanapalli et al., 2020). Comparing assessments of small nerve fibre structure in the cornea with the skin, Quattrini *et al.* found that nerve density quantified using IENFD correlated with CNFD, but not CNFL, in individuals with diabetes (Quattrini et al., 2007). However, Ziegler identified no correlation between IENFD and CNFD in new-onset type 2 diabetes (Ziegler et al., 2014). In this study, both assessments detected nerve fibre loss, but 20.5% of individuals with abnormal CNFD had normal IENFD, and 11% with normal CNFD had abnormal IENFD. These results suggest that early manifestations of small nerve fibre neuropathy may be patchy, and supports the earlier argument that a combination of tests may have better diagnostic utility than a single test (Ziegler et al., 2014).

Comparing corneal SBNP parameters, Hertz *et al.* found CNFL to be most reproducible metric; CNFL also correlated with sural amplitude potential, which is hypothesised to be the earliest NCS parameter to show change in DSP (Hertz et al., 2011). In contrast, Ziegler *et al.* reported that CNFD was most sensitive for detecting corneal nerve pathology (Ziegler et al., 2014). Nonetheless, CNFL remains the more widely adopted parameter for assessing DSP severity in longitudinal studies (Perkins et al., 2018).

Table 2-5 provides a summary of studies that have reported both CNFL (using IVCM) and corneal sensitivity to air stimuli (using NCCA) in individuals with diabetes.

Table 2-5. Corneal structure and function in diabetes

Author (year), study population	Structure	Function	Structure-function relationship
	CNFL (mm/mm ²) [instrument]	Corneal sensitivity threshold assessed using NCCA (mbar)	
Cross-sectional studies			
Tavakoli <i>et al.</i> (2010)*, n=101 diabetes; n=17 controls	Significantly lower in mild (5.48 ± 4.52), moderate (3.01 ± 3.92) and severe DSP (2.99 ± 3.42) than controls (11.21 ± 3.63) [SSCM]	Significantly higher in severe (2.23 ± 5.13), but not mild (1.38 ± 1.11) or moderate (1.83 ± 4.32), DSP than controls (0.72 ± 1.48)	Not reported
Tavakoli <i>et al.</i> (2011)*, n=128 diabetes n=26 controls	Significantly lower in mild (5.31 ± 3.04), moderate (4.44 ± 2.55), and severe (3.49 ± 1.75) DSP than controls (8.76 ± 2.91), but not in diabetes without DSP (7.6 ± 3.82) [SSCM]	Significantly higher in severe (2.43 ± 2.2), but not in no (1.15 ± 0.45), mild (1.27 ± 0.61) or moderate (24.43 ± 15.28), DSP than controls (0.72 ± 0.02)	Not reported
Pritchard <i>et al.</i> (2014), n=74 T1D with DSP; n=168 T1D without DSP; n=154 controls	Significantly lower in T1D with DSP (14.0 ± 6.4) than T1D without DSP (19.1 ± 5.8); both lower than controls (23.2 ± 6.3) [LSCM]	Significantly higher in T1D with DSP (1.0 ± 1.1) than both T1D without DSP (0.7 ± 0.7) and controls (0.6 ± 0.4)	Not reported
Misra <i>et al.</i> (2015), n=53 T1D; n=40 controls	Significantly lower in T1D (11.04 ± 3.8) compared to controls (21.17 ± 4.2) [LSCM]	Significantly higher in T1D (1.3 ± 1.3) compared to controls (0.2 ± 1.3)	Inverse correlation between CNFL and corneal sensitivity ($r=-0.36$; $p=0.008$)
Longitudinal studies			
Mehra <i>et al.</i> (2007)*, n= 20 T1D (n=15 undergoing SPK); n=15 controls	Significantly lower in T1D (2.23 ± 1.25) compared to controls (9.69 ± 2.71) at baseline [LSCM]	Significantly higher in T1D (1.54 ± 1.25) compared to controls (0.77 ± 0.08) at baseline	CNFL increased to 3.6 ± 1.28 six months after SPK (from 1.81 ± 1.28 ; n=15), but corneal sensitivity (NCCA) was unchanged
Tavakoli <i>et al.</i> (2013)*, n=15 T1D; n=10 controls.	Significantly higher in T1D (11.35 ± 4.03) compared to controls (27.93 ± 3.98) at baseline [LSCM]	Significantly higher in T1D (1.78 ± 1.63) compared to controls (0.56 ± 0.32) at baseline	CNFL improved (to 15.63 ± 6.04) 12 months after SPK (n=15), but corneal sensitivity (NCCA) was unchanged

All data are reported in mean $\pm$ SD. *Data converted from mean $\pm$ SEM. **Abbreviations:** CNFL, corneal nerve fibre length; DSP; diabetic sensorimotor polyneuropathy; LSCM, laser-scanning confocal microscope; NCCA, non-contact corneal aesthesiometry; SPK, simultaneous pancreas and kidney transplant; SSM, slit-scanning confocal microscope; T1D, type 1 diabetes.

Several longitudinal studies have demonstrated that baseline CNFL can predict DSP incidence. Lovblom *et al.* found that a CNFL cut-off of 14.9 mm/mm² predicted DSP onset with 82% Sensitivity and 69% specificity over 3.5 years (Lovblom *et al.*, 2015). Similarly, Pritchard *et al.* found that a CNFL of 14.1 mm/mm² predicted DSP incidence with 63% sensitivity and 74% specificity over 4 years (Pritchard *et al.*, 2015). Lewis *et al.*, combining data from a consortium of five cohort studies, estimated a CNFL loss of 6% or more per year, which occurs in 17% of individuals with diabetes, to be associated with an increased risk of DSP progression (Lewis *et al.*, 2020).

In the context of corneal nerve regeneration, in 25 individuals with diabetes and mild-to-moderate DSP, improvements in HbA1c over 24 months resulted in improved CNFD, but not CNFL (Tavakoli *et al.*, 2011b). However, in 15 individuals with type 1 diabetes, simultaneous pancreas and kidney transplant significantly improved all corneal SBNP parameters from six months following treatment (Tavakoli *et al.*, 2013). In the same study, no significant differences in neurophysiology, quantitative sensory testing, corneal sensitivity, and IENFD were detected after 12 months, relative to baseline. This finding demonstrates that the evaluation of CNFL can not only identify individuals who are at risk of developing DSP, but also inform the therapeutic efficacy of DSP treatments.

2.3.3.1.1.2 Structure-function relationship

It is established that corneal sensitivity is reduced in severe DSP (**Table 2-5**). Pritchard *et al.* showed that corneal sensitivity thresholds of >0.66 mbar, measured using NCCA, can detect DSP with a sensitivity of 70% and specificity of 75% (against an NDS score of ≥ 6) (Pritchard 2010). However, the reported correlation coefficients comparing NCCA to NDS ranges from 0.20 to 0.35 (Pritchard *et al.*, 2012; Tavakoli *et al.*, 2007), and, to quantitative sensory testing thresholds, from -0.32 to 0.29 (Pritchard *et al.*, 2012). The strength of these relationships suggests that although corneal sensitivity may have some clinical application for DSP diagnosis, it does not have adequate diagnostic utility alone, and is currently indicated only as an adjunct method to existing clinical markers.

Although corneal nerve structural losses have been reported in individuals with subclinical DSP, potential corneal sensitivity changes have not been well characterised in this population. Rosenberg *et al.* reported reduced mechanical sensitivity only in severe DSP (Rosenberg *et al.*, 2000), but Tavakoli *et al.* found reduced mechanical sensitivity in all DSP severities (Tavakoli *et al.*, 2007). Reduced corneal sensitivity to NCCA has been consistently reported only in established neuropathy, and not in earlier stages of disease (Tavakoli *et al.*, 2007; Tavakoli *et al.*, 2010). In terms of corneal nerve regeneration, corneal sensitivity did not change in individuals with type 1 diabetes at 12 months after kidney and pancreas transplantation, despite an observed improvement in corneal nerve density (Tavakoli *et al.*, 2013).

The corneal structure-function relationship in individuals with DSP is also ambiguous (Patel *et al.*, 2009). Both Rosenberg *et al.* and Misra *et al.* reported an inverse correlation between corneal SBNP density and corneal sensitivity, using mechanical and non-contact aesthesiometers, respectively (Misra *et al.*, 2015; Rosenberg *et al.*, 2000). However, Pritchard found no relationship between corneal structure and sensation (using an NCCA) in large cohort of participants with DSP (n=300; unpublished data cited by author) (Pritchard *et al.*, 2012).

Characterisation of the structure-function relationship in individuals with diabetes is examined in **Chapter 5**. This relationship could provide insight into how corneal SBNP losses relate to the function of different receptor subtypes. In mice with corneal SBNP losses due to experimental diabetes, TRPM8-containing cold thermoreceptors, which more densely populate the peripheral cornea, are more markedly reduced than TRPV1-containing polymodal receptors (Alamri *et al.*, 2019). If these findings are replicated in humans, it would be consistent with the hypothesis that different subpopulations of corneal nerve fibres may be differentially affected in diabetes.

2.3.3.1.2 Tear film

In addition to the neurosensory deficits discussed above, tear film parameters are altered in diabetes. Contributing factors include loss of trophic support to the lacrimal tissue, microvascular damage to the lacrimal gland caused by hyperglycaemia, reduced lacrimal innervation caused by autonomic neuropathy, and reduced reflex tearing as a result of reduced corneal sensitivity (Bron et al., 2017; Shih et al., 2017).

The loss of corneal sensory innervation reduces the neurotrophic support for epithelial cell proliferation; consequently, there is a loss of neurotrophic factors that promote neurite growth, secreted by epithelial cells. This cycle further exacerbates corneal nerve loss (Markoulli et al., 2018; Muller et al., 2003).

The combination of a loss of tear film integrity and neurosensory deficits causes delays in corneal wound healing and can also precipitate ocular surface damage and symptoms of DED (Shih et al., 2017). Dry eye symptoms affect around 50% of individuals with diabetes, and are thought to mirror the severity of peripheral neuropathy (Lv et al., 2014).

2.3.4 Dry eye disease

Diabetes is only one of many disorders that can cause ocular surface disease and affect tear function. DED poses a significant burden on quality of life (McDonald et al., 2016), owing to the potential effects of ocular surface damage on vision and to symptoms that vary from mild intermittent ocular discomfort to debilitating chronic pain (Belmonte et al., 2017).

The 2017 TFOS DEWS II report defines DED as “a multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film, and accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles” (Craig et al., 2017b). In DED, disruptions to tear film homeostasis, observed clinically as tear film instability and

hyperosmolarity, perpetuate a 'vicious cycle' of ocular surface inflammation and ocular surface damage (Baudouin et al., 2016; Bron et al., 2017).

There are two main aetiological subtypes of DED. Evaporative DED is characterised by an abnormal tear-lipid layer due to hyper-evaporative tear loss from conditions affecting the eyelids (e.g., meibomian gland dysfunction) or the ocular surface. Aqueous deficient DED primarily arises from conditions affecting the lacrimal gland, including Sjögren's syndrome and diabetes (Bron et al., 2017). Most individuals with symptomatic ocular surface disease have a mixed aetiology. DED treatment is guided by targeting the primary aetiologies taking into account the presenting clinical signs (Jones et al., 2017).

2.3.5 The influence of diet on ocular surface disease

There is growing evidence that diet plays a role in modulating ocular surface inflammation. In particular, dietary enhancement with omega-3 polyunsaturated fatty acids (PUFAs) can improve tear-lipid dysfunction in evaporative DED and reduce the immune-mediated inflammatory responses associated with aqueous-deficient DED. Omega-3 PUFAs have a broad range of systemic anti-inflammatory effects. In adults with DED, dietary omega-3 PUFAs influenced polar lipid profile in meibomian gland secretions (Sullivan et al., 2002), and reduced tear inflammatory mediators (Deinema et al., 2017). The omega-3-derived mediator, resolvin E1, has also been shown to reduce the concentration of pro-inflammatory cytokines in corneal epithelial cells (Erdinest et al., 2014), and, in mice, to increase tear production and corneal epithelial integrity (Li et al., 2010). Furthermore, dietary omega-3 PUFAs and their mediators have been hypothesised to play a role in modulating corneal nerve regeneration (Chinnery et al., 2017; He et al., 2015).

The following sections describe the biological effects of omega-3 PUFAs (section 2.4), current applications of dietary omega-3 PUFAs in eye health (section 2.5), and the role of omega-3 PUFAs in modulating corneal and peripheral nerve integrity (section 2.6).

2.4 Polyunsaturated fatty acids (PUFAs)

2.4.1 Structure and nomenclature

Fatty acids consist of long, straight hydrocarbon chains with a carboxyl group at one end and a methyl group at the other (Surette, 2008). PUFAs are fatty acids with multiple double bonds, generally in a *cis*-configuration, each separated by a methylene group.

The two major classes of PUFAs important for human health and nutrition are omega-3 and omega-6 PUFAs. Their nomenclature is dictated by the position of the first bond from the terminal end of the molecule, designated omega (n) to represent the last letter of the Greek alphabet. Short-hand notations of PUFAs, for example 18:3 n -3, are constructed to indicate the number of carbon atoms in the chain, followed by a colon, then the total number of double bonds; the final number indicates the position of the first double-bond relative to the terminal end (Calder, 2012).

The short chain omega-3 PUFA, alpha-linolenic acid (ALA; 18:3 n -3), and short-chain omega-6 linoleic acid (LA; 18:2 n -6), are termed essential fatty acids. They are required by mammals for normal biological function and growth, but cannot be synthesised *de novo* and, thus, must be obtained from dietary sources, such as foods or supplementation (FAO, 2010). ALA is a precursor to long-chain omega-3 PUFAs, docosahexaenoic acid (DHA; 22:6 n -3) and eicosapentaenoic acid (EPA; 20:5 n -3) (Bradberry & Hilleman, 2013); LA is a precursor for long-chain omega-6 PUFA, arachidonic acid (AA; 20:4 n -6).

Long-chain omega-6 and omega-6 PUFAs can be obtained via conversion of their short-chain precursors, or directly from diet. This section will discuss the influence of circulating PUFAs and their balance on biological processes, and hypothesise why increasing dietary intake of long-chain omega-3s, EPA and DHA, is likely to provide a range of physiological benefits (Simopoulos, 2002a).

2.4.2 Conversion of short-chain to long-chain PUFAs

The biological conversion of short-chain to long-chain omega-3 and omega-6 PUFAs occurs through two independent pathways that share, and compete for, the same series of enzymes (**Figure 2-6**). Although it has been well-established that dietary ALA depletion causes symptoms of omega-3 fatty acid deficiency (Lamprey & Walker, 1976), there is much controversy surrounding whether dietary ALA obtained from the usual adult diet (approximately 2 g/day) can yield adequate tissue levels of long-chain omega-3 PUFAs, in particular DHA, for development and function (Emken et al., 1994; Fokkema et al., 2000). This is largely due to the various efficiencies of ALA conversion that have been reported, which range from 4 to 21% conversion to EPA (Burdge & Wootton, 2002; Emken et al., 1994) and between 0.2 and 5% conversion to DHA (Bourre et al., 1989; Emken et al., 1994).

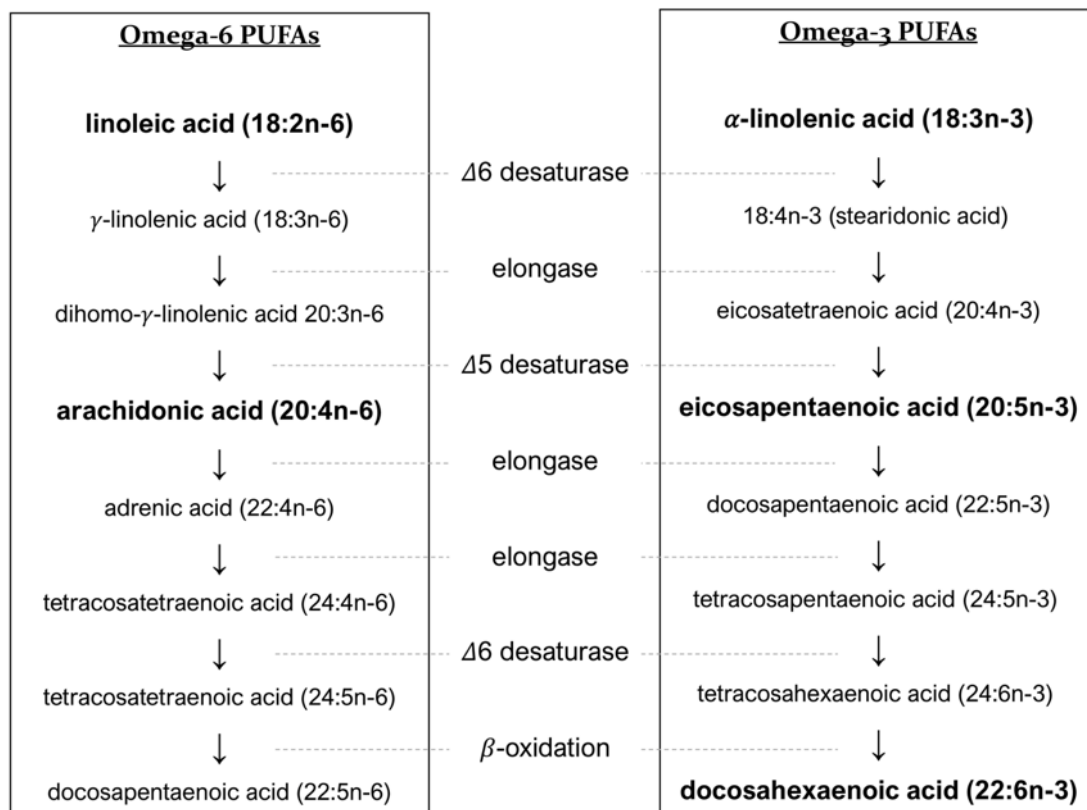


Figure 2-6. Conversion of short-chain to long-chain PUFAs. Main omega-3 and omega-6 PUFAs described in this thesis are indicated in **bold**.

Although human studies using stable isotope tracings have demonstrated negligible changes in plasma EPA and DHA following ALA supplementation (Hussein et al., 2005; Pawlosky et al., 2001), such biosynthesis is thought to occur, but in a tissue-dependent manner (Barcelo-Coblijn & Murphy, 2009; Sprecher et al., 1999). Fatty acid sampling in humans is limited to the extraction of accessible tissues, such as plasma. Although analysis of blood-derived cells can provide a good approximation of ALA conversion in the heart and liver, it may not accurately reflect the conversion of ALA to DHA in the brain, which may occur at a higher rate (Barcelo-Coblijn et al., 2005).

The efficiency of long-chain omega-3 biosynthesis from ALA is also affected by other dietary PUFAs (Pawlosky et al., 2001). In adults, increasing dietary LA intake from 15 to 30 g/day suppressed EPA and DHA synthesis by up to 50% (Emken et al., 1994). Increasing dietary DHA from <0.1 to 6.5 g/day also suppressed this synthesis, by up to 88%, via negative inhibitory feedback processes (Emken et al., 1999). A prospective cohort study found an 80% difference in estimated long-chain omega-3 intake between fish-eaters and non-fish eaters, indicating that a definitive method for obtaining biologically adequate concentrations of EPA and DHA is through direct dietary consumption (Welch et al., 2010). However, the percentage differences in total plasma PUFAs were smaller than those estimated on the basis of dietary intake, suggesting long-chain PUFA biosynthesis may be preferentially elevated in individuals who obtain inadequate concentrations from their diet. Consistent with previous studies (Burdge & Wootton, 2002), women had higher ALA conversion efficiencies than men, which could be the influence of oestrogen and driven by the need for an adequate foetal supply of DHA during pregnancy to support brain development (Welch et al., 2010).

2.4.3 Dietary sources

2.4.3.1 Food sources, the omega-6 to omega-3 ratio, and dietary recommendations

Omega-6 fatty acids are found in many food sources in the mammalian diet. LA is abundant in grains and plant oils made from safflower, sunflower, and corn; AA is found in most animal products, including meat, eggs and dairy products (Meyer et al., 2003). With respect to omega-3 PUFAs, ALA is present in relatively high amounts in flaxseed and chia seeds, and in smaller amounts in walnuts, plant oils from canola and soybean, and leafy green vegetables (Calder, 2013). EPA and DHA, synthesised by marine algae, are abundant in fish oils and marine-based food sources (Nettleton, 1991). Oily fish, such as salmon, mackerel and tuna, contain higher concentrations of EPA and DHA than lean fish, such as cod and snapper (Calder, 2013).

Western diets are characterised by food sources rich in omega-6 PUFAs, such as cereals and grains, and are relatively low in omega-3 PUFAs (Simopoulos, 2006). In the modern Western diet, the omega-6 to omega-3 ratio is estimated to range between 8:1 (Meyer et al., 2003) and 15:1 (Sanders, 2000). The optimal dietary omega-6 to omega-3 ratio for preventing chronic cardiovascular and inflammatory disease has often been quoted as 4:1 (de Lorgeril et al., 1994; Simopoulos, 2002a). However, the use of this ratio as a criterion for informing disease risk has been criticised, as it does not specify which of the dietary PUFAs are included (i.e., long-chain or short-chain), and exaggerates the negative impact of omega-6 PUFAs on general health. Not all omega-6 PUFAs are proinflammatory; prospective cohort studies have also associated higher LA intakes as being cardioprotective (Ascherio et al., 1996; Hu et al., 1997). Furthermore, a ratio cannot be used for providing dietary advice in a clinical setting (Harris, 2018).

Rather than the omega-6 to omega-3 ratio, Harris proposed quantifying the sufficiency of EPA and DHA intake (see section **2.4.4**) to be preferable for evaluating risk factors in chronic disease (Harris, 2018). The joint Food and

Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) Expert Consultation on Fats and Fatty Acids in Human Nutrition guidelines also specify that there is no rationale for recommending a specific omega-6 to omega-3 ratio if the omega-3 intake lies within established recommendations (FAO, 2010). For non-pregnant adults, this is suggested to be 250 mg/day of combined EPA and DHA. The American Diabetes Association and the American Heart Association recommend 500 mg of long-chain omega-3 PUFAs per day for general health (Kris-Etherton et al., 2002), which equates to approximately two standard servings of oily fish per week. In those with hypertriglyceridemia, supplementation with 4000 mg of EPA and DHA per day is recommended (Colquhoun et al., 2008). Similar recommendations exist in Europe (EFSA, 2012) and Australia (NHMRC, 2006).

With regard to the adequacy of dietary omega-3 intake, a systematic review, found low levels of long-chain omega-3 PUFAs (omega-3 index <4 %) in most regions with Westernised food habits, including North America and Europe (Stark et al., 2016). In Australia, up to 80% of the population may be failing to meet the recommended intake of long-chain omega-3 PUFAs (Meyer, 2016).

2.4.3.2 Supplements forms and bioavailability

Long-chain omega-3 PUFA supplements contain fish oils that are generally obtained from fish livers or their flesh (Calder, 2013). Whereas omega-3 PUFAs from natural fish oils exist in triglyceride forms, omega-3 supplements can be prepared in either ethyl ester forms, or re-esterified to a glycerol (e.g., in fish oil) or onto a phospholipid backbone (e.g., in krill oil). The chemical form and preparation of the supplement can affect its bioavailability (Schuchardt et al., 2011).

The bioavailability of phospholipids and re-esterified triglycerides was found to be comparable; both are superior to that of natural triglycerides (Schuchardt et al., 2011). Ethyl esters have the lowest bioavailability (Dyerberg et al., 2010;

Schuchardt et al., 2011; Ulven et al., 2011), but absorption may be enhanced in the presence of high dietary fats (Lawson & Hughes, 1988).

2.4.4 Systemic fatty acid analysis

Several methods can be used to evaluate systemic PUFA levels; their advantages and disadvantages are summarised in **Table 2-6**.

Table 2-6. Methods for assessing systemic fatty acid levels

Method	Advantages	Disadvantages
Biological markers	- Objective marker - Reflects other metabolic factors that influence EPA+DHA absorption (e.g., age, BMI)	- Invasive - Requires additional analysis time and laboratory expertise - Can be relatively costly - Reference values vary depending on the laboratory methods and biological tissue assessed
Food frequency questionnaires	- Non-invasive - Suitable for clinical settings	- Prone to recall bias - Limited to availability and accessibility of food compositional data in regional nutritional databases
Short-term dietary recalls (usually 24-hour)	- Non-invasive - Suitable for clinical settings	- Fail to account for day-to-day variations in habitual diet - Limited to availability and accessibility of food compositional data in regional nutritional databases
Food diaries (usually over 7-10 days)	- Precise record of food intake over a short period; not prone to recall bias	- Dietary behaviours may be influenced by the recording process over the capture period - Limited to availability and accessibility of food compositional data in regional nutritional databases

Abbreviations: BMI, body mass index; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid

Biological PUFA concentrations are typically assessed from erythrocytes using gas chromatography methods. Venous blood is the conventional source from which PUFAs are extracted, as samples show less degradation over time compared to capillary blood (Otto et al., 1997). However, venous blood collection is time-consuming and invasive. As such, both Harris & Polreis and Liu *et al.* have developed and validated analytical methods for longer-term

stabilisation of capillary dried blood spots that are commercially available (Harris & Polreis, 2016; Liu et al., 2014).

Erythrocyte PUFA levels directly correspond to dietary PUFA intake. However, the relative PUFA concentration measured in blood is also affected by biological and metabolic factors (for example, omega-3 absorption decreases with higher age and BMI) (Sands et al., 2005), and the analytical method used. A recent systematic review reported considerable variation in erythrocyte PUFA assessment methods across 298 studies (Stark et al., 2016). In these studies, omega-3 PUFA levels were estimated from different blood fractions, which have different total fatty acid components. In an effort to standardise the reporting of erythrocyte omega-3 PUFAs, Harris & Von Schacky proposed the Omega-3 Index, which describes the sum of the percentage weight of EPA and DHA relative to total fatty acids in erythrocytes (Harris & Von Schacky, 2004). The Omega-3 Index has since been validated as a marker for cardiovascular disease; an omega-3 index of <4% is considered high risk, whereas shifting from 4 to 8% has been estimated to reduce fatal cardiovascular disease risk by around 30% (Harris et al., 2017).

Although erythrocyte analysis provides a direct and objective measure of systemic PUFA levels, the test is invasive, time-consuming, and relatively costly, and, thus, not routinely performed in clinical settings. Furthermore, biological markers cannot differentiate PUFAs consumed from food sources and those from nutritional supplements, which, in the context of eye health, is particularly relevant (discussed in section **2.5**).

Dietary questionnaires offer an alternative, non-invasive method for assessing PUFA intake adequacy. Although various food frequency questionnaires (FFQs) have been developed and validated (Bassett et al., 2016; da Silva et al., 2018; Dahl et al., 2011; Dickinson et al., 2015; Lucas et al., 2009; Meyer et al., 2013; Sullivan et al., 2008a), many designed for use in epidemiology studies are lengthy, time consuming, and lack transparency with respect to how data are

derived. Their reliability further differs depending on the nutritional composition of food sources, which varies across different global regions (Stark et al., 2016).

2.4.5 Biological effects of dietary PUFAs

Once ingested, dietary PUFAs become incorporated into cellular phospholipid membranes and affect cellular mechanisms through multiple pathways. The anti-inflammatory effects of omega-3 derived eicosanoids have been widely studied, but lipid signalling and mediator production mechanisms that can provide neuroprotection still require greater understanding (Gordon & Bazan, 2013). Membrane omega-3 PUFAs alter membrane biophysical properties and regulate cellular signalling and gene expression (Deckelbaum et al., 2006). Through these mechanisms, omega-3 PUFAs can influence neurotransmission, delay neuronal apoptosis, and promote neuron survival (Bazinet & Laye, 2014).

2.4.5.1 Metabolite production

Eicosanoids are oxygenated metabolites, synthesised from 20-carbon PUFAs, that influence immune cell and cytokine production and, subsequently, the intensity and duration of inflammatory responses (Schmitz & Ecker, 2008). Circulating long-chain omega-3 and omega-6 PUFAs compete for the same oxidation enzymes that are required for eicosanoid synthesis (Calder & Grimble, 2002). Prostaglandins and thromboxane are synthesised via the cyclooxygenase pathway, and leukotrienes and lipoxins are synthesised from the lipoxygenase pathway (**Table 2-7**).

Table 2-7. Metabolites of long-chain omega-6 and omega-3 PUFAs and their opposing actions

AA derived		EPA/DHA derived	
Mediators	Cellular effects	Mediators	Cellular effects
Prostaglandins (PG)			
PGD ₂ PGE ₂ PGF ₂ PGI ₂	Vasodilator Pro-arrhythmic Pro-arrhythmic	PGD ₃ PGE ₃ PGF ₃ PGI ₃	Anti-arrhythmic
Thromboxanes (TX)			
TXA ₂ TXB ₂	Platelet activator Vasoconstriction	TXA ₃ TXB ₃	Platelet inhibitor Vasodilation
Leukotrienes (LT)			
LTA ⁴ LTB ⁴ LTC ⁴ LTD ⁴ LTE ⁴	Chemoattractant	LTA ⁵ LTB ⁵ LTC ⁵ LTD ⁵ LTE ⁵	Anti-inflammatory
Other messengers			
Lipoxins (LX) LXA ₄ Epoxyeicosatrienoic (EET) derivatives 5,6-EET 8,9-EET 11,12-EET 14,15-EET Hydroxyleicosatetraenoic (HETE) derivatives 5-HETE 12-HETE 15-HETE	Stimulates macrophage efferocytosis	Resolvins (RV) RVE ₁ RVD Protectins (P)/ Neuro-protectins (NP) PD ₁ /NPD ₁	Anti-inflammatory

Table adapted from Schmitz & Ecker (2008)

Omega-3 PUFAs are also precursors for pro-resolving lipid mediators, resolvins and protectins, which are synthesised via the cyclooxygenase-2 pathway. Resolvins are synthesised from EPA (E-series resolvins; RVE) and DHA (D-series resolvins; RVD). Furthermore, DHA is a precursor to the family of docosanoids called protectins, or neuroprotectins when found in neural tissues (Bradbury 2011). Resolvins and protectins promote the resolution of inflammation by reducing polymorphonuclear leukocyte recruitment, enhancing leukocyte phagocytosis of microbial agents, and protecting tissues from neutrophil-mediated injury (Chiang et al., 2015; Schwab et al., 2007; Spite et al., 2009).

2.4.5.2 Membrane dynamics and fluidity

Phospholipid omega-3 PUFAs provide biological membranes with a high degree of conformational fluidity (Rajamoorthi et al., 2005). Membrane fluidity plays a critical role in modulating membrane protein functions and signal transduction processes (Yehuda et al., 2002). Membrane-bound PUFAs also improve neurotransmission by displacing saturated fatty acids and its associated proteins from lipid rafts, which are membrane microdomains that facilitate intracellular signal transduction (Allen et al., 2007; Simons & Toomre, 2000). Through the regulation of membrane fluidity, dietary omega-3 PUFAs are hypothesised to influence Na^+/K^+ -ATPase activity and nerve conduction activity (Gerbi et al., 1998).

DHA is the main phospholipid constituent of neuronal membranes (Bazan, 2006). In the neural retina, DHA is sequestered in photoreceptor outer segments and plays an important role in visual function (Birch et al., 1998; Yee et al., 2010). DHA increases the hippocampal transcription of brain-derived neurotrophic factor (Rao et al., 2007; Redivo et al., 2016) and, through this mechanism, facilitates synaptic transmission, enhances synaptic plasticity, and improves cognitive function (Wu et al., 2004). Following traumatic brain injury in rodents, DHA supplementation normalised brain-derived neurotrophic factor

levels, reduced oxidative damage and improved neuronal function (Rao et al., 2007).

2.4.5.3 Cellular protein interactions and gene expression

Membrane-bound omega-3 PUFAs act as endogenous ligands for G-protein-coupled receptors (GPCR), influencing their intracellular signalling pathways (Guixà-González et al., 2016). Through the ligand binding with GPCR120, omega-3 PUFAs repressed macrophage-mediated inflammation and increased insulin sensitivity (Oh et al., 2010). The binding of DHA and retinal GPCR, rhodopsin, enhanced photoreceptor function and improved retinal phototransduction (Gawrisch et al., 2008). Furthermore, fatty acid-bound GPCRs are implicated in nociceptive signal processing, such that activation of fatty acid signalling has been proposed to reduce neuropathic pain (see **2.6.1**) (Karki et al., 2015).

Membrane omega-3 PUFAs regulate gene expression; of particular interest are their role in upregulating PPAR expression by acting as endogenous ligands for PPAR isomers (Kliwer et al., 1997; Xu et al., 1999). As discussed in section **2.2.2.2.3**, PPAR α agonists prevented corneal nerve degeneration in mice with diabetic neuropathy (Matlock et al., 2020). Following neurological injury, PPAR γ agonists enhanced neuroprotective chaperone expression and reduced neuronal apoptosis (Michael-Titus & Priestley, 2014; Yi et al., 2008).

Omega-3 PUFAs also downregulate NF κ B activity. As discussed in section **2.2.2.2.3**, NF κ B upregulation plays a major role in mediating proinflammatory pathways in DSP pathogenesis. Long-chain PUFAs inhibit NF κ B activation both directly, by inhibiting the GPCR signalling process that leads to its activation (Allam-Ndoul et al., 2016; Novak et al., 2003), and indirectly, through PPAR activation, as ligand-bound PPARs reduce NF κ B translocation (Mishra et al., 2004). In mouse Schwann cells, DHA also reduced oxidative stress-induced NF κ B activation by increasing intracellular glutathione (Tatsumi et al., 2019).

2.5 Omega-3 PUFAs for eye health: current clinical applications

2.5.1 Dry eye disease (DED)

Increasing dietary omega-3 PUFAs to maintain an optimal omega-6 to omega-3 systemic ratio yields anti-inflammatory effects and is associated with a reduced risk of DED (Miljanovic et al., 2005). The US Women's health study, involving 39,876 female participants, found an omega-6 to omega-3 dietary ratio of 15:1 to be associated with a 2.5-times higher incident risk of DED relative to a ratio of 4:1 (Miljanovic et al., 2005). However, clinical trials assessing omega-3 supplementation for treating DED have presented conflicting findings (Downie et al., 2019) (**Table 2-8**). Potential factors that could have contributed towards the observed heterogeneity in reported effect estimates include differences in intervention protocols with respect to dosage (ranging from 135 to 3000 mg/day), treatment duration (from 1 to 12 months), and supplement form (triglyceride, phospholipid or ethyl ester); study design; population selection; and the choice of outcome measures.

In a recent Cochrane systematic review of relevant RCTs, Downie *et al.* found, with moderate certainty, a probable benefit of EPA and DHA in increasing aqueous tear production and, with low certainty, a possible reduction in tear osmolarity in DED populations (Downie et al., 2019). However, a synthesis of four RCTs found, with low certainty, that omega-3 supplements had little to no effect in reducing DED symptoms relative to placebo. Thus, in the context of DED, there is current uncertainty with regard to the optimal intervention type and dose, and to the population most likely to benefit from dietary omega-3 PUFA intervention.

Table 2-8. Key randomised controlled trials comparing omega-3 PUFAs to placebo for the treatment of dry eye disease

Author (year)	Population characteristics		Intervention characteristics		
	Clinical population (number randomised)	Baseline omega-3 information	EPA/DHA dosage (form)	Comparator dosage (type)	Treatment duration
Asbell (2018)	Moderate to Severe DED (n=535)	Baseline fatty acid level in RBCs	2000/1000 (triglycerides)	5000 mg (olive oil)	12 mo
Deinema (2017)	Mild to moderate DED (n=60)	Approximate dietary intake of omega-3 over the preceding month	1000/500 (triglycerides) 945/510 (phospho-lipids)	1500mg (olive oil)	3 mo
Goyal (2017)	LASIK-induced DED (n=60)	Not reported	720/480 (not reported)	400mg (vitamin E)	13 wk
Kawashima (2016)	DED, probable DED and no DED (n=40)	Not reported	81/54 (not reported)	Placebo details not reported	6 mo
Bhargava (2016a)	DED in rosacea patients (n=130)	Not reported	720/480 (not reported)	Not reported (olive oil)	6 mo
Bhargava (2016b)	DED in VDU users (n=522)	Not reported	1440/960 (not reported)	Not reported (olive oil)	45 d
Bhargava (2015)	DED with computer vision syndrome (n=478)	Not reported	720/480 (not reported)	Not reported (olive oil)	3 mo
Kangari (2013)	DED (n=73)	Not reported	360/240 (not reported)	2000mg capsule (medium-chain triglycerides)	1 mo
Kawakita (2013)	DED (severity not reported) (n=37)	Not reported	1245/540 (not reported)	Placebo details not reported	3 mo
Pinazo-Duran (2013)	Mild to moderate DED (n=30)	Not reported	85/700 and 60mg DPA (not reported)	No treatment	3 mo

Adapted table using data extracted from Downie *et al.* (2019). **Abbreviations:** d, days; DED, dry eye disease; LASIK, Laser in situ keratomileusis; mo, months; VDU, visual display unit; wk, weeks

2.5.2 Age-related macular degeneration (AMD)

In addition to anti-inflammatory effects on the ocular surface, dietary omega-3 PUFAs have benefits in age-related macular degeneration (AMD). AMD is characterised by progressive degenerative changes to the macula, the part of the posterior eye that is responsible for central vision. The late-stage form of the disease can lead to vision loss due to atrophic degeneration of the outer retina and/or choroidal neovascularisation (Ferris et al., 2013). Omega-3 PUFAs are believed to impart clinical benefit in AMD via lipid mediators that target ischaemic, oxidative and inflammatory mechanisms, as well as providing retino-protective benefits through DHA-mediated effects (Prokopiou et al., 2019; Rezende et al., 2014).

Prospective cohort studies have associated diets containing high intakes of omega-3 rich foods with both a reduced lower risk of developing early AMD (Tan et al., 2009) and reduced AMD progression (SanGiovanni et al., 2008; Wu et al., 2017). However, a number of RCTs investigating oral omega-3 supplements, either as a standalone treatment or in conjunction with other vitamins, have found no benefit associated with omega-3 PUFA supplementation on AMD progression (**Table 2-9**). In a Cochrane systematic review, Lawrenson and Evans concluded that, based on the synthesis of data from the age-related eye disease 2 (AREDS 2) and the nutritional AMD treatment 2 (NAT2) studies, omega-3 PUFA supplements for a period of five years does not reduce the risk of progression to late AMD in those with existing disease (HR: 0.96; 95%CI: 0.84 to 1.10) (Lawrenson & Evans, 2015).

In AMD, distinction should be made between the potential clinical benefits of omega-3 PUFAs derived from food sources and the lack of benefit derived from omega-3 supplementation; this distinction should be reflected in the clinical guidance given to AMD patients relating to omega-3 PUFAs for AMD management.

Table 2-9. Key randomised controlled trials on omega-3 supplementation, with/without co-interventions, for the treatment of age-related macular degeneration

Study name (year)	Clinical population	Baseline omega-3 information	Treatment information	Follow-up duration
AREDS ₂ (2013)	Existing AMD with high risk of progressing to advanced AMD, and advanced AMD in fellow eye	Not reported	2x2 factorial design: AREDS formulation* plus one of 1. lutein (10 mg) + zeaxanthin (2 mg) 2. DHA (350 mg) + EPA (650 mg), form not stated 3. lutein + zeaxanthin and DHA + EPA 4. placebo	5 yrs
NAT ₂	Neovascular AMD in one eye	Omega-3 intake at baseline quantified	1. Treatment: Reti-Nat supplements containing 840 mg DHA and 270 mg EPA, 6 mg vitamin E (triglycerides) 2. Placebo: 602mg olive oil	3 yrs
VITAL (2016)*	Ancillary study: subpopulation of AMD in primary VITAL trial in CVD and cancer prevention in middle-aged men and women	Fish serving quantified at baseline	2x2 factorial design: 1. Active omega-3 (Omacor containing 465 EPA 385 DHA ethyl esters) and vitamin D (cholecalciferol; 2000 IU/d) 2. Active omega-3 and placebo vitamin D 3. Placebo omega-3 and active vitamin D 4. Placebo omega-3 and placebo Vitamin D	5 yrs

*ARED formulation was randomised to: original AREDS formulation (vitamin C [500 mg], vitamin E [400 IU], beta carotene [15 mg], zinc [80 mg, as zinc oxide], and copper [2 mg, as cupric oxide]), AREDS formulation without beta carotene, AREDS formulation with lower zinc dose (25 mg), or AREDS formulation with no beta carotene and lower zinc dose. **Abbreviations:** AMD, age-related macular degeneration; AREDS, Age-Related Eye Disease Study; NAT₂: The Nutritional AMD Treatment 2 study; VITAL: the Vitamin-D and Omega-3 Trial; yrs, years.

2.5.3 The role of optometrists in making dietary omega-3 recommendations

Optometrists, as primary eye care providers, are ideally placed to inform the public about the impact of diet and nutrition on eye health. Clinical advice should consider the potential benefits and risks of omega-3 PUFAs, optimal food sources and intake frequency, and supplement types and dosage. Furthermore, eye care professionals should seek to provide tailored advice to individuals who are most likely to benefit from increased dietary omega-3 fatty acid intake.

Two studies have reported on optometrists' nutritional recommendations in eye care practice. Lawrenson and Evans found that UK optometrists are actively engaged in providing nutritional advice relevant to AMD, but highlighted a need for increased awareness of the relevant research evidence (Lawrenson & Evans, 2013). Downie and Keller found 60% of Australian optometrists report routinely asking their patients about diet, and 50% ask about nutritional supplement intake (Downie & Keller, 2015). No studies have specifically investigated optometrists' practice patterns relating to the use of omega-3 PUFAs, which is important for understanding whether current research evidence is being appropriately applied in clinical practice. A survey investigating optometrists' opinions and clinical practices relating to omega-3 PUFAs in eye care practice is reported in **Chapter 3**.

2.6 Omega-3 PUFAs for peripheral nerve health: current evidence

As discussed in section **2.4.5**, membrane omega-3 PUFAs can reduce oxidative stress, ameliorate neuro-inflammation, and improve neurotransmission (Janssen & Kiliaan, 2014; Yorek, 2017). Via these mechanism, dietary omega-3 PUFAs can attenuate axonal degeneration and improving neurological function (Jolivald et al., 2012). This section examines the preclinical and clinical evidence on omega-3 PUFAs and their mediators for improving corneal and peripheral nerve health.

2.6.1 Peripheral nerve injury

Mechanical injury

Several preclinical studies have found that omega-3 PUFAs modified the response of the PNS to acute injury. Robson *et al.*, in an *in vitro* study, found that omega-3 PUFA incubation increased neurite outgrowth in dorsal root ganglia neurons of both developing and aged rats (Robson et al., 2010). Extending these findings, Gladman *et al.* demonstrated that dorsal root ganglia neurons from mice expressing the fat-1 gene, which allows for the endogenous conversion of omega-6 to omega-3 PUFAs, were more resistant to mechanical and hypoxic injury, and exhibited more abundant and complex neurite growth, compared to wild-type mice (Gladman et al., 2012). Moreover, the same study, in an *in vivo* model, found that fat-1 encoded mice had improved structural and motor functional recovery after sciatic nerve crush injury than wild-type mice. However, it was recognised in these studies that the distances over which regeneration would need to occur in humans is much greater than those in the mouse, thus collateral sprouting may be limited (Gladman et al., 2012).

Dietary omega-3 PUFAs may alleviate neuropathic pain. Mice administered concentrated fish oil supplements after sciatic nerve injury were found to be protected from mechanical and thermal sensitisation, and had reduced neuroinflammation, compared to mice on vehicle treatment (Silva et al., 2017). Other animal models have reported similar improvements in neuropathic pain following peripheral nerve injury using treatment with dietary omega-3 PUFAs (Unda et al., 2019) and their mediators, RvE1 (Xu et al., 2013a) and NPD1 (Xu et al., 2013b). Both fish oil and DHA supplements have demonstrated to attenuate diabetes-associated neuropathic pain in rats (Heng et al., 2015; Li et al., 2015).

In humans, few studies have evaluated whether omega-3 PUFA supplements might alleviate neuropathic pain. A case-series reported high-dose oral omega-3 supplements (2400-7200 mg/day) for up to 19 months significantly reduced associated symptoms in five patients with chronic neuropathic pain (Ko

et al., 2010); however, high-level clinical evidence is required to rigorously examine this reported effect.

Trigeminal nerve injury

Following corneal dissection in rabbits, treatment with topical DHA and pigment epithelial-derived growth factor (PEDF), a neurotrophin that stimulates NPD1 biosynthesis, produced corneal nerve regeneration after 2 weeks (Cortina et al., 2010). Corneal sensation and tear secretion also improved after treatment (Cortina et al., 2013; He et al., 2015). Subsequent studies by the same research group also showed similar results in rabbits infected with herpes simplex virus infection (He et al., 2017a) and in mice with diabetic keratopathy (He et al., 2017b). However, the effects of topical DHA or NPD1 for corneal neuropathy have not been examined in humans.

In a clinical population with moderate DED, a placebo-controlled, double-masked RCT found that 1500 mg/day combined EPA and DHA oral supplements for three months induced corneal nerve regeneration. (Chinnery et al., 2017). DED, as described in section 2.3.5, is characterised by mild corneal neuropathy. This study found a significant increase in CNFL in the omega-3 group relative to placebo (+2.9 mm/mm² versus -2.7 mm/mm²). However, this small pilot study involved only 12 participants. Future trials with larger clinical populations and longer follow-up durations are required to confirm these effects.

2.6.2 Diabetic neuropathy

In animal studies of DSP, dietary omega-3 PUFA supplementation has not only slowed the progression of diabetic neuropathy and its associated functional impairments, but also showed potential in stimulating nerve regeneration (Yorek, 2017). Earlier studies in streptozotocin-induced diabetic rats showed that fish oil supplementation prevented Na⁺/K⁺-ATPase loss, restored nerve function, and improved sciatic nerve conduction velocity (Gerbi et al., 1999; Gerbi et al., 1998). Coste *et al.* later suggested that treatment effects are

mediated through DHA, as DHA supplementation in rats prevented the 30% reduction in nerve conduction velocity observed in non-supplemented animals (Coste et al., 2003). However, this study did not find an intergroup difference in sciatic nerve Na⁺/K⁺-ATPase activity (Coste et al., 2003).

Another research group investigated the effects of dietary omega-3 PUFAs using interventions of dietary menhaden oil, composed of ~85% omega-3 PUFAs (ALA, EPA, and DHA) and ~15% omega-6 PUFAs (LA and AA) (Shireman, 2003). In experimental models of type 1 diabetes, rats supplemented with menhaden oil had improved nerve conduction and tactile responses compared to control animals (Coppey et al., 2015). Furthermore, menhaden oil prevented corneal nerve fibre loss and improved corneal sensitivity in treated animals. Similar findings were reported in rodent models of type 2 diabetes. Dietary modification with menhaden oil reduced motor and sensory nerve conduction losses, improved IENFD profiles (Coppey et al., 2012), and mitigated corneal nerve loss and vascular dysfunction (Coppey et al., 2020). This intervention not only prevented diabetic neuropathy, but when administered later in the disease course, was able to reverse corneal nerve loss and promote regeneration (Yorek et al., 2017). Even though menhaden oil contains both omega-6 and omega-3 PUFAs, the authors reported a seven-fold difference in the omega-6 to omega-3 ratio between treated and non-treated groups, which suggested that the increase in omega-3 PUFAs was the primary mechanism for the observed treatment effects.

These preclinical data highlight the promise for omega-3 PUFAs as a neuroprotective agent in DSP; however, few clinical studies have explored this potential benefit. In a cohort with type 2 diabetes and peripheral neuropathy, a non-randomised intervention study demonstrated that oral EPA (1800 mg/day; ethyl esters) for eight weeks improved clinical neuropathic symptoms and vibration perception thresholds from baseline (Okuda et al., 1996). However, this study involved only a single group of participants (n=21) with no comparator group.

In ten participants with type 1 diabetes, Stiefel *et al.* demonstrated that 960 mg/day of oral EPA and DHA for 90 days significantly increased nerve conduction velocity relative to a non-supplemented group (n=8). Even though this was a parallel-group RCT, masking was not performed. Furthermore, a functional improvement was only observed in the median motor nerve and not in the sensory nerves (Stiefel *et al.*, 1999). More recently, an open-label, single-arm clinical trial demonstrated, in 40 people with type 1 diabetes, that treatment with oral omega-3 PUFAs (2330 mg/day EPA + DHA + docosapentaenoic acid) for 12 months increased CNFL by 29% relative to baseline. No change was found in quantitative sensory testing thresholds or in nerve conduction studies (Lewis *et al.*, 2017). The lack of a comparator group and the absence of masking limit confidence in the reported treatment effect; however, this evidence posits corneal nerve quantification as a suitable endpoint for DSP intervention trials. Furthermore, these findings support the conduct of a well-designed, parallel-group RCT to confirm the potential role of dietary omega-3 PUFAs in promoting peripheral nerve health in diabetes.

In summary, omega-3 PUFAs have anti-inflammatory and neuroprotective effects. There is strong scientific rationale that dietary modification with omega-3 PUFAs can improve corneal and peripheral nerve health.

Although RCTs have demonstrated a probable benefit of omega-3 PUFAs supplementation in treating ocular surface disease, there remains much uncertainty surrounding the optimal dosage and formulation, as well as how to identify individuals who might derive the greatest benefit from this form of dietary enhancement. It is unclear how such uncertainty influences current practices relating to omega-3 PUFA recommendations.

There is a need for high-level, prospective clinical studies to investigate whether oral omega-3 PUFAs can improve peripheral nerve outcomes in individuals with diabetes. Corneal nerves morphology, evaluated using IVCM, is a sensitive marker for demonstrating peripheral nerve repair. Evidence supporting a role

for omega-3 PUFAs in modulating peripheral nerve health could potentially be translated into practice and lead to improved clinical care for the millions of individuals with diabetes.

2.7 Aims and hypotheses

Based upon this consideration of the literature, the aims and hypotheses to be considered in this thesis are:

Chapter 3

Aim: To assess the attitudes and self-reported practice behaviours of optometrists towards omega-3 PUFA recommendations for eye health, and their understanding of the potential benefits and risks associated with oral omega-3 PUFA intake.

Hypothesis: Optometrists will recommend both omega-3 rich food sources and omega-3 PUFA supplements to their patients for improving dry eye disease, but will fail to demonstrate a thorough understanding of the differences in biological efficacies between different forms (i.e., marine-based vs plant-based) of omega-3 fatty acids.

Chapter 4

Aim: To undertake preliminary validation of a novel clinically-applicable food-frequency questionnaire for assessing dietary long-chain omega-3 PUFA intake, relative to an objective systemic biomarker and a previously-validated full dietary profile survey.

Hypothesis: The clinically-applicable food-frequency questionnaire will provide a reliable estimate of an individual's dietary omega-3 PUFA intake, relative to both the objective systemic biomarker and the validated dietary profile survey.

Chapter 5

Aim: (i) To investigate central and peripheral corneal sensitivity and the associated corneal structure-function relationship in individuals with diabetes without neuropathy symptoms; and (ii) to investigate the association of systemic fatty acid levels and corneal morphological and functional parameters, in both healthy individuals and those with diabetes without symptomatic neuropathy.

Hypothesis: A similar corneal structure-function relationship will be evident in controls and in individuals with diabetes without symptomatic neuropathy. Systemic Omega-3 Index will be associated with corneal sub-basal nerve structure and corneal nerve function.

Chapter 6

Aim: To identify, appraise, and synthesise the current, best-available evidence on the efficacy and safety of oral omega-3 supplementation for improving peripheral nerve health, in a systematic review, in order to inform clinical practice and future research in the field.

Hypothesis: Oral omega-3 PUFA supplementation can be demonstrated to improve both peripheral nerve structure and function in populations with acute and chronic systemic conditions.

Chapter 7

Aim: To evaluate the efficacy and safety of a six-month supplementation period with oral omega-3 PUFA supplements on peripheral nerve parameters in individuals with type 1 diabetes, in a randomised, double-masked, placebo-controlled clinical trial.

Hypothesis: Treatment with oral omega-3 PUFAs for six months will increase corneal nerve fibre length, relative to placebo, in individuals with type 1 diabetes.

3

Chapter 3. Omega-3 fatty acids for eye health: knowledge, opinions and practice patterns of optometrists in Australia and New Zealand

3.1 Introduction

The publication presented in this chapter examines the current clinical use of omega-3 polyunsaturated fatty acids (PUFAs) in eye care practice by evaluating the recommendations made by optometrists to their patients in relation to omega-3 PUFAs.

Results from randomised controlled trials indicate a possible role for omega-3 supplements in the clinical management of dry eye disease (DED) (Downie et al., 2019). Understanding practitioners' opinions and practices relating to omega-3 PUFAs is useful for identifying gaps in current knowledge and barriers to evidence-based practice. This information can also inform whether a simple dietary assessment questionnaire (**Chapter 4**) could be a useful clinical tool for eye care clinicians. To date, no studies have specifically evaluated eye care clinicians' practices relating to omega-3 PUFAs.

This survey examines optometrists' clinical recommendations relating to both omega-3 rich foods and omega-3 PUFA supplements in the clinical management of DED and age-related macular degeneration (AMD).

Although the management of AMD with omega-3 PUFAs is not the central focus of this thesis, the concept of there being differences in the clinical efficacy of food sources and supplements for modifying AMD risks is important for clinical practice. Whether this distinction is reflected in current optometric practice is, therefore, also examined in this chapter.

The citation for this peer-reviewed publication is provided below. **Section 3.2** is the verbatim content of the published paper formatted in thesis style, with the exception that tables, figures, and appendices have been re-numbered (indicated in bold) and references have been formatted to combine with the full reference list for the thesis.

Zhang, A. C., Singh, S., Craig, J. P., Downie, L.E. (2020). Omega-3 Fatty Acids and Eye Health: Opinions and Self-Reported Practice Behaviors of Optometrists in Australia and New Zealand. *Nutrients*, 12(4), 1179.
<https://doi.org/10.3390/nu12041179>

The supplementary material for this publication is included in **Appendix A** of the thesis.

3.2 Publication: Omega-3 Fatty Acids and Eye Health: Opinions and Self-Reported Practice Behaviors of Optometrists in Australia and New Zealand

3.2.1 Abstract

This study investigated optometrists' attitudes and self-reported practice behaviors towards omega-3 fatty acids for eye health, and knowledge and understanding of their potential risks and benefits. An anonymous online survey was distributed to optometrists in Australia and New Zealand. Questions included practitioner demographics and practice modality; self-reported practices and recommendations relating to diet, nutritional supplements, and omega-3 fatty acids for age-related macular degeneration (AMD) and dry eye disease (DED); and practitioner knowledge about omega-3 fatty acids. Of 206 included surveys, most respondents (79%) indicated recommending for their patients to consume omega-3 fatty acids to improve their eye health. Sixty-eight percent of respondents indicated recommending omega-3-rich foods for AMD management, while 62% indicated recommending omega-3 supplements. Most respondents (78%) indicated recommending omega-3-rich foods or supplements for DED. For DED, recommended omega-3 supplement dosages were (median [inter-quartile range, IQR]) 2000 mg [1000–2750 mg] per day. The main sources of information reported by respondents to guide their clinical decision making were continuing education articles and conferences. In conclusion, optometrists routinely make clinical recommendations about diet and omega-3 fatty acids. Future education could target improving optometrists' knowledge of differences in the evidence for whole-food versus supplement sources of omega-3 fatty acids in AMD. Further research is needed to address uncertainties in the evidence regarding optimal omega-3 dosage and formulation composition in DED.

3.2.2 Introduction

Diet is a major lifestyle factor that can influence eye health (Lawrenson & Downie, 2019). There is evidence to suggest that diets rich in omega-3 essential fatty acids, obtained from food sources or supplementation, may have ocular benefits. Omega-3 fatty acids are termed ‘essential’ as they cannot be synthesized in the body and, thus, must be obtained from the diet. The other major essential fatty acid family is the omega-6 fatty acids. A person’s dietary intake and balance of omega-6 and omega-3 fatty acids has been reported to be important for regulating vascular activity, mediating immune and nervous system function, and influencing the balance of systemic lipid-derived mediators (Calder & Grimble, 2002).

Omega-3 fatty acids can be obtained from several sources. Short-chain omega-3, alpha-linoleic acid (ALA), is found mostly in plant-based foods (e.g., flaxseed, chia seeds). Long-chain omega-3 fatty acids, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), are predominantly obtained from marine-based foods (e.g., oily fish). As the biological conversion of ALA to EPA and DHA is incomplete *in vivo* (estimated in the range of 5 to 20% conversion) (Barcelo-Coblijn et al., 2005; Emken et al., 1994; Gerster, 1998), direct intake of long-chain omega-3 fatty acids is considered preferable (Nettleton, 1991). Once ingested, long-chain omega-3 fatty acids compete with the long-chain omega-6 fatty acid, arachidonic acid (AA), for incorporation into cellular membranes (Janssen & Kiliaan, 2014). Countering pro-inflammatory AA-mediated pathways, increasing omega-3 fatty acid intake modulates systemic inflammation through the production of anti-inflammatory metabolites. The optimal ratio of dietary omega-6 to omega-3 is considered to be approximately 4 to 1 (Simopoulos, 2002a). Excessive consumption of foods rich in omega-6 fatty acids, a recognized feature of modern Western diets, can shift the omega-6:omega-3 ratio closer to 15 to 1 (Simopoulos, 2002a). Moreover, it has been reported that up to 80% of adults in developed countries may not obtain the daily, recommended dietary intake of long-chain omega-3 fatty acids for optimal

health (Meyer, 2016). Diets rich in long-chain omega-3 fatty acids have been suggested to provide long-term benefits for several chronic ocular conditions, including dry eye disease (DED) and age-related macular degenerations (AMD) (Rand & Asbell, 2011; SanGiovanni & Chew, 2005).

DED is one of the most common reasons for patients to seek ophthalmic care (Stapleton et al., 2017). DED can impose a significant burden on quality of life and is associated with substantial direct and indirect health costs (McDonald et al., 2016; Patel et al., 2011). The condition is characterized by a loss of tear film homeostasis and potential disruption to ocular surface integrity (Craig et al., 2017b). DED is commonly associated with ocular surface inflammation (Bron et al., 2017). Dietary modification with omega-3 fatty acids is thought to reduce DED symptoms and signs by modulating ocular surface inflammation and improving tear-lipid profiles (Sullivan et al., 2002; Walter et al., 2016). The United States (US) Women's Health Study showed a higher incidence of DED in women with low dietary omega-3 fatty acid intake (Miljanovic et al., 2005). However, clinical trials assessing the potential effects of omega-3 supplementation on the clinical expression of DED have presented apparently contradictory findings (Asbell et al., 2018; Deinema et al., 2017; Epitropoulos et al., 2016; Kangari et al., 2013; Wojtowicz et al., 2011). A recent Cochrane systematic review concluded that while a possible role exists for long-chain omega-3 supplements in managing DED, the best available evidence is currently uncertain and inconsistent (Downie et al., 2019). The role of omega-3 fatty acids in the management of DED is, thus, of continued interest and debate.

In addition to its anti-inflammatory effects, DHA is implicated in maintaining the structural and functional properties of the retina (van Leeuwen et al., 2018). Diets rich in polyunsaturated fatty acids improve the retinal cellular response to ischemic, oxidative, and inflammatory damage in animal models of AMD (Reme et al., 1994; Simón et al., 2016; Tuo et al., 2009). Epidemiology studies have associated dietary long-chain omega-3 intake with a lower risk of developing early-stage AMD (Tan et al., 2009) and progression to late-stage, sight-

threatening forms of disease (Wu et al., 2017). However, omega-3 nutritional supplements have been shown not to confer the same benefit of reducing the risk of developing AMD as whole foods (Lawrenson & Evans, 2015). The reason for this difference in efficacy may be potential synergistic interactions of fatty acids with other nutrients and vitamins that are present in whole foods but not supplements (Merle et al., 2018); these differences should be recognized when clinical recommendations relating to omega-3 fatty acids are made for AMD.

Eye care clinicians have an important role in informing their patients and the public about modifiable risk factors for ocular disease, including diet.

Optometrists, as major providers of primary eye care in Australia and New Zealand, are ideally positioned to counsel patients about the benefits and risks of nutritional supplements and their potential impact on eye health. Two studies have investigated the self-reported recommendations made by optometrists relating to nutrition. In the UK, Lawrenson and Evans (2013) reported active engagement by optometrists in providing nutritional advice relating to AMD, but highlighted a need for increased awareness of the relevant research evidence (Lawrenson & Evans, 2013). In an Australian study of self-reported optometric practice patterns, Downie and Keller (2015) found 60% of optometrists indicated routinely asking their patients about diet, and approximately half indicated asking about nutritional supplement intake (Downie & Keller, 2015). To date, no studies have specifically investigated eye care practitioner recommendations relating to omega-3 fatty acids.

In both Australia and New Zealand, the professional scope of optometry includes the diagnosis, assessment, and management of ocular disorders, including the prescription of topical and, in New Zealand, oral medications. In both countries, optometrists are assessed to equivalent competency standards (Kiely & Slater, 2015). Research has highlighted that most of the general public who seek optometric care expect to be counselled about their diet, as it relates to their eye health (Downie et al., 2017). It is, thus, critical that the information delivered by eye care providers is accurate and up to date.

The aim of this study was to investigate the current attitudes and self-reported practice behaviors of optometrists towards omega-3 fatty acid recommendations for eye health, and to assess their opinions and understanding of the potential benefits and risks associated with oral omega-3 fatty acid intake.

3.2.3 Materials and Methods

3.2.3.1 Participants

An anonymous, web-based survey was distributed to optometrists in Australia and New Zealand from June to October 2019. The survey was circulated electronically through professional organizations (Optometry Australia and associated early career networks, Cornea and Contact Lens Society of Australia, New Zealand Association of Optometrists, and Cornea and Contact Lens Society, New Zealand) and at a major national professional education conference, held in Melbourne Australia (July 2019).

The project was approved by both the University of Melbourne Human Research Ethics Committee (HREC #1853287.1) and University of Auckland Human Participants Ethics Committee on 12 March 2019 for three years (ref. 022730).

3.2.3.2 Survey Design

This survey was administered using Qualtrics (Qualtrics, Provo, UT, USA) and comprised 39 questions, divided into four sections. Questions within each section sought responses that included: Select yes/no, select all that apply, select one option only, and optional free-text boxes. Reviewing or altering prior responses was not permitted. The survey was piloted to optimize the clarity of questions and the flow. Respondents were informed at the beginning of Section 2 that, for the purpose of this survey, diet was defined as “the food(s) and drink(s) that are regularly consumed”.

Figure 3-1 illustrates the survey flow and question logic. As summarized in **Table 3-1**, the survey's four sections comprised: (1) Participant demographics, (2) self-reported clinical practices relating to diet and nutritional supplementation, (3a) clinical approach to recommending omega-3 fatty acids for AMD and DED, or (3b) reason(s) for not making omega-3 recommendations or for recommending that patients not consume omega-3 fatty acids, (4) knowledge relating to omega-3 fatty acids.

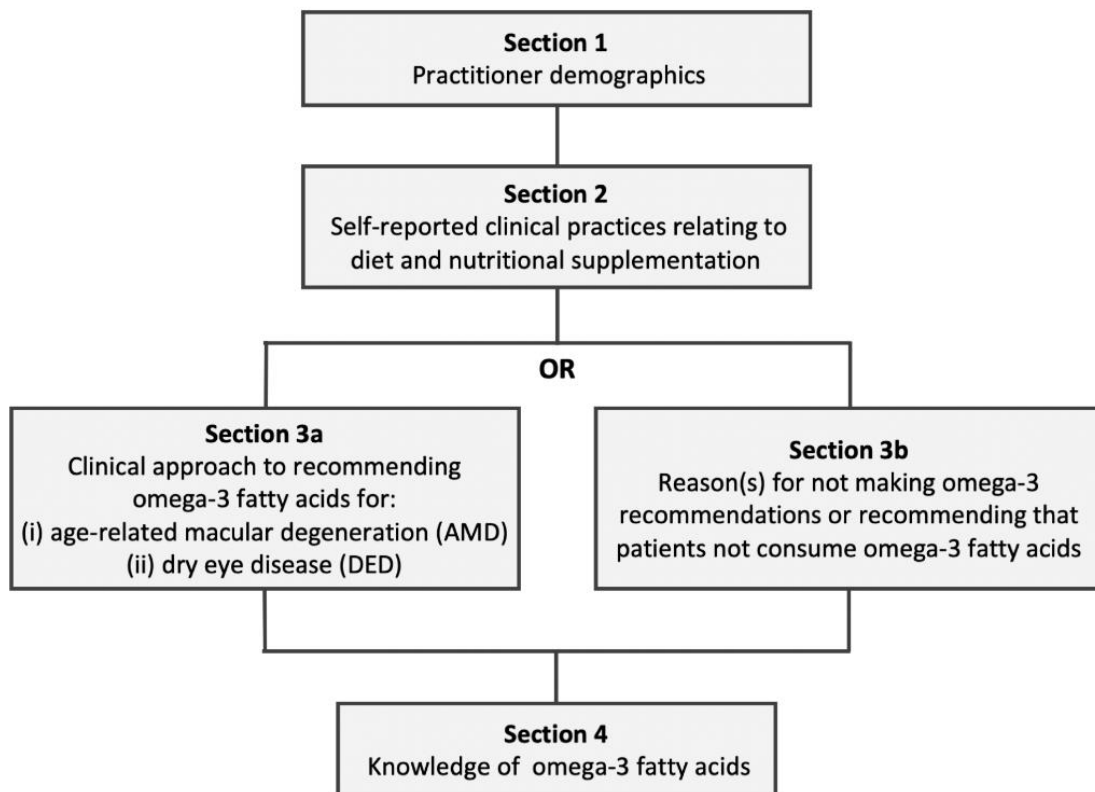


Figure 3-1. Survey structure.

After completing Section 2, branch logic was used to direct respondents to either Section 3a or Section 3b depending on their current general clinical approach to omega-3 fatty acids, according to their response to the following three options:

- (1) I make recommendations for my patients to consume omega-3 fatty acids (in either oral supplementation form or via dietary modification) to improve their ocular health;
- (2) I do **not** make recommendations for my patients to consume omega-3 fatty acids (in either oral supplementation form or via dietary modification) to improve their ocular health;
- (3) I recommend for my patients **not** to consume omega-3 fatty acids to improve their ocular health.

Participants who selected option (1) were directed to complete Section 3a (to obtain data about their clinical recommendations relating to omega-3 fatty acids for AMD and DED). Participants who selected options (2) or (3) were directed to complete Section 3b (to explore reasons for not making clinical recommendations relating to omega-3, or recommending that patients not consume omega-3 fatty acids).

Table 3-1. Summary of survey questions.

Section	Question Surveyed
1. Practitioner demographics	▪ Gender ▪ Age ▪ Location of optometry degree completion ▪ Year of graduation from optometry degree ▪ Location of principal practice ▪ Postcode of principal practice ▪ Therapeutic endorsement status ▪ Mode of optometry practice (academic, corporate, hospital clinic or public health clinic, independent, refractive surgery clinic, other) ▪ Average hours per week providing patient care ▪ Approximate age distribution of patients (percentage for each of: under 18 years, 18–40 years, 41–60 years, over 60 years, so that percentages summed to 100%) ▪ Areas of self-declared clinical interest (contact lenses, diabetes, dry eye or ocular surface disease, gerontology or aged care, glaucoma, myopia, paediatrics or binocular vision, retinal disease, other)

2. Self-reported clinical practices relating to diet and nutritional supplementation

- Frequency of enquiry about patients' diet (never, rarely, sometimes, often or always)
 - Whether quantitative tools were used to survey dietary habits *
- Frequency of enquiry about patients' usual intake of nutritional/vitamin supplements (never, rarely, sometimes, often or always)
- Frequency of providing dietary advice to patients (never, rarely, sometimes, often or always)
- Statement that best describes respondents' current general clinical approach to omega-3 fatty acid recommendations for ocular health ^
- Sources of information used to guide clinical decision-making regarding omega-3 fatty acids
- Percentage of patients seen in practice estimated to have AMD
- Percentage of patients seen in practice estimated to have DED

Section 3a #

3a. Clinical approach to recommending omega-3 fatty acids for AMD and DED

Age-related macular degeneration (AMD)

- Frequency of recommending foods rich in omega-3 fatty acids
 - Specific food sources recommended (fish, non-fish seafood [e.g., prawns, oysters etc.], cooking oils or margarines, vegetables or fruits, nuts and seeds, other) *
 - If fish and/or seafood was selected above: frequency of intake recommended
- Frequency of recommending plant-based omega-3 supplements
- Frequency of recommending marine-based omega-3 supplements
- Open text box to record specific recommendations relating to omega-3 supplements for AMD

Dry eye disease (DED)

- Frequency of recommending foods rich in omega-3 fatty acids
 - Specific food sources recommended (fish, non-fish seafood [e.g., prawns, oysters etc.], cooking oils or margarines, vegetables or fruits, nuts and seeds, other) *
 - If fish and/or seafood was selected above: frequency of intake recommended
 - Frequency of recommending any omega-3 supplements
 - Specific recommendations relating to the type of supplement(s) *
-

	• Open text box to record specific recommendations relating to omega-3 supplements for DED • Selection of DED severity considered for recommending omega-3 fatty acids via dietary modification or supplementation • Selection of DED disease subtypes considered for recommending omega-3 fatty acids via dietary modification or supplementation
3b. Reason(s) for not making omega-3 recommendations or for recommending that patients not consume omega-3 fatty acids	Section 3b [#] • Reason(s) for not currently recommending omega-3 fatty acids (from either the diet or via supplementation) ▪ If previously recommended omega-3 fatty acids, reason(s) for no longer making this recommendation • Other dietary supplements recommended to patients
4. Knowledge of omega-3 fatty acids	• Awareness of differences in the biological effects of plant-based and marine-based omega-3 fatty acids • Perceived anti-/pro-inflammatory effects of omega-3 fatty acids • Perceived anti-/pro-inflammatory effects of omega-6 fatty acids • Perceived ideal ratio of omega-6:omega-3 in dietary intake • Knowledge of the approximate recommended adult daily dietary target of long-chain omega-3 fatty acids • Yes/no choice about whether omega-3 fatty acids are considered to have any potential side effects ▪ If yes, perceived side effects associated with omega-3 fatty acids (options offered: nose bleeds, bloating, constipation, gastric reflux [fishy after-taste], hypercholesterolaemia [high systemic levels of cholesterol], excess lacrimation, other) • Perceived daily safety limit for omega-3 fatty acid intake • Perceived contraindications to omega-3 fatty acid supplementation

[^] Stratification question, depending on the response selected, participants were directed to either Section 3a or Section 3b. [#] Only one of these sections was displayed to the respondent.

* Only displayed if any answers other than "Never" were selected in the previous question. AMD, age-related macular degeneration; DED, dry eye disease.

3.2.3.3 Data Analysis

Data contributed by optometrists practicing outside of Australia or New Zealand, or from participants who did not complete the survey up to the end of Section 3, were not included in the analysis.

Statistical analysis was performed using IBM SPSS statistics software version 21.0 (<https://www.ibm.com/analytics/spss-statistics-software>). Graphs were plotted in GraphPad PRISM (version 6.01 for Windows, GraphPad Software, La Jolla, CA, USA, www.graphpad.com). Descriptive statistics were used to analyze participant demographics, clinical practices relating to diet and nutrition, clinical practice relating to omega-3 fatty acids, and practitioner knowledge of omega-3 fatty acids. Fisher's exact test was used to compare data relating to the proportions of respondents across different groups. Specific recommendations relating to omega-3 supplementation, derived from responses in the free-text boxes, were represented using a 'word cloud' (Wordle™; <http://www.wordle.net/>). The size of the lettering in the 'word cloud' reflects the frequency with which respondents reported the individual words.

Univariate and multivariate binocular logistic regression analyses were performed to assess factors influencing a clinician's self-reported practices relating to omega-3 fatty acid recommendation for ocular health, and knowledge relating to the biological effects and dietary targets of omega-3 fatty acids. A p-value of less than 0.05 was used to define statistical significance. To quantify participant knowledge about omega-3 fatty acids, the accuracy of respondents' answers was scored out of five (i.e., one point for each correctly answered question). The five knowledge questions surveyed and the answers that were considered as 'correct' are provided in Table A1 (**Appendix A**). A score of three or more (i.e., 60%) was considered a 'pass' for the 'omega-3 knowledge score'.

3.2.4 Results

3.2.4.1 Participant Demographics

Of 234 total responses received, 206 responses were included in the analysis. Twenty-eight surveys were excluded because they were either not completed up to the end of Section 3 or were completed by optometrists practicing outside of Australia and New Zealand. **Table 3-2** summarizes the demographics of participating optometrists. Approximately two-thirds of responses were from optometrists practicing in Australia (64%) and one-third practicing in New Zealand (36%). The majority of respondents were endorsed to prescribe ocular therapeutic medications (Australia, 86%; New Zealand, 91%).

Most respondents completed their optometry training in Australia or New Zealand (94%); the remainder completed their degree in the UK, South Africa, or US (and additional competency examinations to practice optometry in Australia or New Zealand). Australian respondents practiced in all 6 states; most were practicing in Victoria (48%), New South Wales (25%), and Queensland (12%). From New Zealand, responses were received from optometrists in all 16 regions except Gisborne, Tasman, Marlborough, West Coast, and Southland. Most New Zealand optometrists practiced in Auckland (35%), Waikato (14%), Wellington (13%), and Hawkes Bay (13%).

Approximately half of the respondents worked in independent optometric practice (49%), 30% worked principally in corporate practice, 11% in a hospital or public health clinic, and 8% within academia. The vast majority of respondents (92%) selected one or more areas of clinical interest, with 50% self-declaring interest in DED or ocular surface disease, 26% in retinal disease, and 8% in gerontology or aged care.

Table 3-2. Summary of participant demographics.

Characteristic (n = 206 for All Categories)	Number of Responses (%)
Gender	
Male	80 (38.8)
Female	125 (60.7)
Other/gender diverse	1 (0.5)
Age (years)	
<30	91 (44.2)
31-45	67 (32.5)
46-60	39 (18.9)
>60	9 (4.4)
Completed optometry training in Australia or New Zealand?	
Yes	194 (94.2)
No	12 (5.8)
Country of principal practice	
Australia	131 (63.6)
New Zealand	75 (36.4)
Therapeutically endorsed	
Yes	180 (87.4)
No	26 (12.6)
Principal type of optometric practice	
Academic	16 (7.8)
Corporate	61 (29.6)
Hospital or public health clinic	22 (10.7)
Independent	101 (49.0)
Refractive surgery clinic	2 (1.0)
Other	4 (1.9)
Average hours spent providing patient care as an optometrist per week	
0	2 (1.0)
1-10	20 (9.7)
11-20	23 (11.2)
>20	161 (78.2)
Areas of specific clinical interest	
Binocular vision	58 (28.2)
Contact lenses	96 (46.6)
Diabetes	65 (31.6)
Dry eye or ocular surface disease	103 (50.0)
Gerontology or aged care	17 (8.3)
Glaucoma	72 (35.0)
Myopia	120 (58.3)
Paediatrics	58 (28.2)
Retinal disease	54 (26.2)
Other	12 (5.8)

3.2.4.1.1 Clinical Recommendations Relating to Diet

As responses from Australian and New Zealand practitioners were similar, pooled data across both locations are presented. **Figure 3-2** self-reported clinical practice behaviors relating to diet and nutritional supplements.

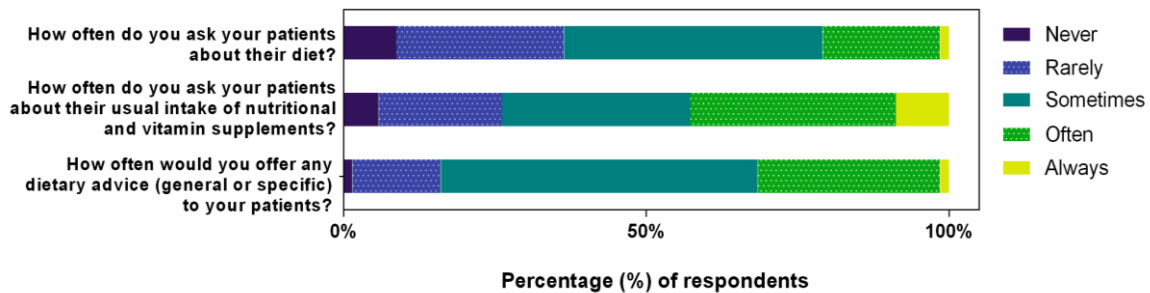


Figure 3-2. Optometrists' self-reported clinical practices as related to diet and nutritional supplementation. Percentage (%) of respondents, from $n = 206$, who selected each frequency of practice, on a five-step Likert scale.

Of the 91% of respondents who asked their patients about their diet, 98% did not use any quantitative tools to survey dietary habits. Three respondents indicated that they used either a food frequency questionnaire ($n = 2$) or an online dietary intake tool ($n = 1$). Optometrists reported more frequently (i.e., often or always) asking their patients about their nutritional supplement intake than about their diet (43% versus 21%; odds ratio (OR): 2.83, 95% confidence interval, CI: 1.83 to 4.37; $p < 0.001$). Approximately one in three optometrists reported often or always (32%), and one in two, sometimes (52%) providing patients with general or specific dietary advice.

Most respondents (79%) indicated that they recommend the consumption of omega-3 fatty acids to patients, in either supplement form or from food sources, to improve eye health. The remaining (21%) respondents indicated that they did not make any recommendations to their patients regarding omega-3 fatty acids. None of the respondents recommended that their patients avoid consuming omega-3 fatty acids.

For the 44 respondents who reported not making any recommendations relating to omega-3 fatty acids, almost half (47%) indicated that they felt like they did not

know enough about omega-3 fatty acids to justify making recommendations. Thirteen respondents felt there was insufficient published research evidence to support omega-3 fatty acid recommendation for eye health, and three indicated that the published evidence they were aware of showed that omega-3 fatty acids were not beneficial for ocular health. Seven respondents either themselves considered or perceived that their patients considered nutritional advice to be outside the scope of optometric practice.

Four respondents indicated that they previously recommended omega-3 fatty acids to their patients, but were no longer doing so. Reason(s) provided included that: they did not see any clinical benefits in their own patients ($n = 1$), recent evidence had changed their opinions ($n = 1$), or they would recommend other forms of therapy (e.g., intense pulsed light therapy for DED) over dietary interventions ($n = 2$). Of the 44 respondents who did not make any recommendations relating to omega-3 fatty acids, 20% ($n = 9$) indicated recommending supplement formulations based on the results of the Age-Related Eye Disease Studies (AREDS and AREDS II) for their patients with AMD.

Table 3-3 summarizes the factors that predicted whether optometrists recommended omega-3 fatty acid consumption to patients to improve their ocular health, from either dietary modification or oral supplementation. In univariate analysis, optometrists were more likely to recommend omega-3 fatty acids if they worked in an independent practice (OR: 4.06, 95% CI: 1.26–13.06; $p = 0.019$), had a self-declared clinical interest in DED or ocular surface disease (OR: 4.58, 95% CI: 2.12–9.91; $p < 0.001$), or if they recognized that omega-3 fatty acid supplements had potential side effects (OR: 3.94, 95% CI: 1.88–8.25; $p < 0.001$). In multivariate analysis, both a clinical interest in DED or ocular surface disease (OR: 3.10, 95% CI: 1.31–7.36; $p = 0.010$) and recognition that omega-3 fatty acids have side effects (OR: 2.34, 95% CI: 1.05–5.22; $p = 0.037$) were significant factors in predicting whether omega-3 fatty acids were recommended to improve ocular health.

Table 3-3. Factors predicting whether optometrists recommend omega-3 fatty acid consumption (from food sources or supplementation) to improve eye health.

Factor	Univariate Analysis		Multivariate Analysis	
	OR (95% CI)	p Value	OR (95% CI)	P Value
Gender (n = 206)				
Male	Ref			
Female	0.76 (0.38–1.54)	0.450		
Age, in years (n = 206)				
<30	Ref			
31–45	2.04 (0.92–4.49)	0.078		
>45	2.34 (0.93–5.89)	0.070		
Optometric practice experience, in years (n = 206)				
<10 years	Ref			
11–20	2.31 (0.82–6.49)	0.111		
21–30	2.10 (0.74–5.91)	0.162		
>31 years	1.63 (0.51–5.19)	0.412		
Country of practice (n = 206)				
Australia	Ref			
New Zealand	1.48 (0.72–3.04)	0.288		
Therapeutically endorsed (n = 206)				
No	Ref			
Yes	0.27 (0.06–1.21)	0.087		
Principal type of optometric practice (n = 206)				
Academic	Ref		Ref	
Corporate	1.84 (0.57–5.92)	0.306	1.87 (0.49–7.09)	0.356
Hospital or public health clinic	1.05 (0.28–3.99)	0.943	0.96 (0.22–4.30)	0.959
Independent	4.06 (1.26–13.06)	0.019	2.51 (0.67–9.38)	0.171
Average weekly hours spent providing patient care as an optometrist (n = 206)				
1–10	Ref			
11–20	0.90 (0.21–3.94)	0.889		
>20 hours	0.97 (0.30–3.10)	0.959		
Self-declared clinical interest in DED or ocular surface disease (n = 206)				
No	Ref			
Yes	4.58 (2.12–9.91)	<0.001	3.10 (1.31–7.36)	0.010

Self-declared clinical interest in gerontology or aged care (n = 206)				
No	Ref			
Yes	0.62 (0.21–1.88)	0.401		
Self-declared clinical interest in retinal disease (n = 206)				
No	Ref			
Yes	1.07 (0.51–2.27)	0.857		
Pass mark (scoring ≥3 out of 5) on ‘omega-3 knowledge score’ (n = 191)				
No	Ref			
Yes	1.04 (0.37–2.99)	0.936		
Recognised that omega-3 fatty acids have potential side effects (n = 191)				
No	Ref		Ref	
Yes	3.94 (1.88–8.25)	<0.001	2.34 (1.05–5.22)	0.037

DED, dry eye disease; OR, odds ratio; Ref, reference.

3.2.4.1.2 Recommendations Relating to Omega-3 fatty acids in Age-Related Macular Degeneration (AMD)

Figure 3-3A summarizes the self-reported practice behaviors of optometrists relating to omega-3 fatty acid intake, through either diet or supplementation, as a component of clinical management for people with AMD. Overall, 68% of optometrists indicated recommending omega-3 rich foods for AMD (**Figure 3-3B**). Almost all (95%) recommended fish or non-fish seafood as a food source. Almost 80% of optometrists who recommended fish or seafood specified the preferred frequency of consumption, and this was most commonly two to four servings per week (**Figure 3-3C**). Two-thirds of optometrists who recommended omega-3 rich foods for AMD suggested nuts and seeds (63%), and 40%, vegetables or fruits.



3-19

Participants who indicated recommending omega-3 supplements were invited to detail the specific recommendation(s) given to their patients (such as dose, frequency, brand). **Figure 3-3D** provides a visual representation of these free-text responses received from 80 of the 132 practitioners (61%), constructed using a 'word cloud'. Supplements broadly based on the formulation in the AREDS studies (AREDS Research Group, 2001) (Macutec [Stiltec Pty Ltd, Queensland (QLD), Australia], Macuvision [Blackmores, New South Wales (NSW), Australia], or MD Eyes [MD EyeCare, QLD, Australia]) were listed by 21% of optometrists who provided free-text responses ($n = 17$). About one-third of respondents (29%) provided specific dosages in their recommendations ($n = 23$), and these ranged from 250 mg to 5000 mg of omega-3 fatty acids per day (Median [Inter-quartile range, IQR]: 2000 [1000–3000] mg/day). Twelve percent of respondents ($n = 10$) indicated that they do not make specific recommendations relating to the brand or dosage of omega-3 supplement, and 11% percent indicated that they would advise their patients to consult either a pharmacist or a general practitioner for advice relating to specific supplementation products and the dosage.

3.2.4.2 Recommendations Relating to Omega-3 Fatty Acids in Dry Eye Disease (DED)

Figure 3-4A summarizes the self-reported practice behaviors of optometrists who recommended omega-3 fatty acids as a component of DED management. Compared with AMD (68%), a higher proportion of respondents (78%) indicated recommending omega-3 fatty acids from food sources or supplements to manage DED. Almost all (>99%) of respondents who made this recommendation considered omega-3 fatty acids to be appropriate for evaporative and/or mixed etiologies of DED (**Figure 3-4B**). Only one practitioner indicated recommending omega-3 fatty acids specifically for the aqueous-deficient DED subtype. Practitioners were less likely to recommend omega-3 fatty acids to patients with mild, compared to moderate or severe forms of, DED (**Figure 3-4C**, OR: 0.24, 95% CI: 0.16 to 0.37; $p < 0.001$).

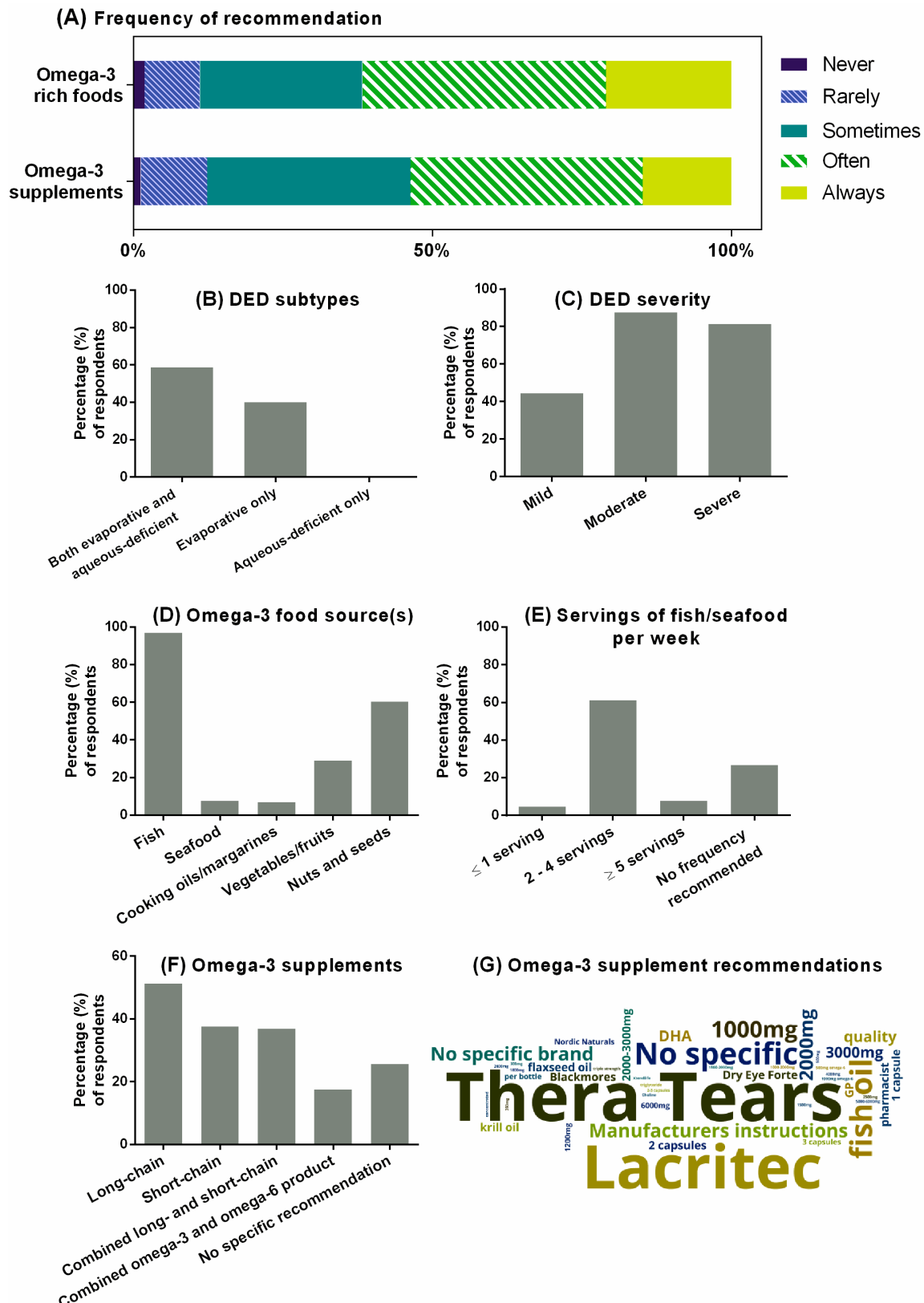


Figure 3-4. Optometrists' self-reported clinical practices as related to omega-3 fatty acids for managing DED. (A) Self-reported frequency of recommending omega-3-rich foods and nutritional supplements. Percentage of respondents shown from $n = 162$ who indicated recommending omega-3 fatty acids for DED, (B) DED subtypes, and (C) severities that participants self-reported making recommendations for omega-3 fatty acids (in either food or supplement forms). Percentages shown from $n = 162$.

(D) Type of omega-3 food source(s) recommended. Percentage shown from $n = 159$ who indicated recommending omega-3-rich foods. (E) Recommended ideal frequency for patients to consume marine-based (long-chain) omega-3-rich foods. Percentages shown from $n = 154$ who indicated recommending fish or seafood as omega-3 food sources in (e). (F) Type of oral omega-3 supplements recommended. Percentages shown from $n = 160$ who indicated recommending omega-3 supplements. (G) Word-cloud representation of specific recommendations for omega-3 supplementation (based on $n = 105$ responses from the 160 participants who indicated recommending omega-3 supplementation).

Self-reported clinical recommendations relating to dietary sources of omega-3-rich foods and frequency of intake in DED were similar to those for AMD (**Figure 3-4D**). Of the 77% of optometrists who recommended for patients to increase their intake of foods rich in omega-3 fatty acids to improve their DED, almost all (97%) specified either fish or non-fish seafood sources, most commonly between two and four servings per week (**Figure 3-4E**). Over half of respondents recommended nuts and seeds (60%), and one in three indicated value in consuming vegetables or fruits to obtain omega-3 fatty acids.

Oral omega-3 supplements were recommended by 78% of optometrists for managing DED (**Figure 3-4F**). The most frequently recommended forms were long-chain omega-3 fatty acids from marine-based sources (40% of all optometrists), short-chain omega-3 from plant-based sources (29% of all optometrists), and combined long- and short-chain omega-3 fatty acids (29% of all optometrists). Omega-3 and omega-6 combination products were recommended by 14% of optometrists for DED.

Figure 3-4G shows a 'word cloud' representation of the free-text responses from the 105 of 160 practitioners (65%) who provided specific recommendations regarding omega-3 supplements for DED. As represented by the size of the lettering, Thera Tears (Akorn Consumer Health, MI, USA) and Lacritec (Stiltec Pty Ltd, QLD, Australia) were the two most frequently recommended commercial brands, listed by 17% and 15% of respondents, respectively. About one-third of respondents provided specific omega-3 dosage recommendations for DED (31%), which ranged from 250 mg to 6000 mg per

day (median [IQR]: 2000 [1000–2750] mg per day), and 10% ($n = 10$) of respondents advised their patients to consult either the instructions on the supplement bottle or the advice of a pharmacist.

3.2.4.2.1 *Practitioner Knowledge of Omega-3 Fatty Acids*

Half of the respondents (50%) were not aware of a difference between the biological effects of plant-based (short-chain) and marine-based (long-chain) omega-3 fatty acids. About one-third (31%) correctly considered plant-based omega-3 fatty acids to have less, and 12% considered plant-based omega-3 fatty acids to have greater, biological effect than marine-based omega-3 fatty acids when consumed at the same dose. Just over half (51%) of respondents considered omega-3 fatty acids to be mostly anti-inflammatory, 15% of optometrists recognized that omega-3 fatty acids are always anti-inflammatory, and 42% of optometrists correctly considered omega-6 fatty acids to be mostly pro-inflammatory. The perceived ideal ratio of omega-6 to omega-3 in the human diet was most frequently selected as 1 to 4 (27% of optometrists), 16% of respondents correctly selected the ideal ratio to be 4 to 1, and 39% of optometrists were unsure of the ideal ratio.

From a forced-choice list of five options ranging from 150 mg/day to 1500 mg/day, 65% of respondents nominated a daily recommended dietary target of long-chain omega-3 fatty acids (the remaining respondents selected 'don't know'). The most common daily targets selected were 1000 mg per day (24% of optometrists) and 1500 mg per day (17% of optometrists). One in 11 respondents correctly selected the ideal approximate target of 500 mg/day for adults. About half of respondents (52%) considered there to be a safety limit for daily omega-3 fatty acid consumption; the perceived limit selected by respondents encompassed a 20-fold dose range. From the four multiple choice options offered, 500 mg per day was considered the upper limit by 4% of all optometrists, 1500 mg per day by 15% of optometrists, 3000 mg per day by 18% of optometrists, and 5000 mg per day by 8% of optometrists.

Over half (53%) of respondents believed that omega-3 fatty acids have potential side effects. Based on multiple-choice options (see **Table 3-1**), these were most commonly believed to be gastric reflux (37%), nose bleeds (23%), and bloating (11%). Forty-six percent of respondents considered possible contraindications to use before recommending omega-3 fatty acids. In open-text boxes, the most commonly suggested contraindications were concurrent anticoagulant medications (26%), allergies to fish/seafood (12%), or a scheduled surgical procedure (3%). Five percent of respondents indicated they would ask the patient to consult their general practitioner or specialist prior to commencing oral omega-3 supplementation.

The level of respondents' knowledge of omega-3 fatty acids was assessed using an 'omega-3 knowledge score', derived from five knowledge-specific questions in the survey (see Table A1 [**Appendix A**]). A 'pass' was considered a score of three or more out of five (i.e., at least 60%). Using this criterion, in both univariate and multivariate analysis (**Table 3-4**), optometrists with a self-reported clinical interest in DED or ocular surface disease were more knowledgeable about omega-3 fatty acids (OR: 2.97, 95% CI: 1.09 to 8.11; $p = 0.034$) relative to those without such interests. Compared with respondents working in academia, those who were based principally in a corporate practice setting had a lower omega-3 knowledge score (OR: 0.080, 95% CI: 0.008 to 0.086; $p = 0.037$).

Table 3-4. Predictive factors for a 'pass' mark on the omega-3 knowledge score, relating to the accuracy of practitioners' general knowledge about omega-3 fatty acids.

Factors (<i>n</i> = 206 for All Categories)	Univariate Analysis		Multivariate Analysis	
	OR (95% CI)	<i>P</i> Value	OR (95% CI)	<i>P</i> Value
Gender				
Male	Ref			
Female	1.35 (0.55–3.34)	0.511		
Age (years)				
≤30	Ref			
31–45	1.93 (0.68–5.50)	0.219		
>45	2.41 (0.81–7.15)	0.113		
Optometric practice experience (years)				
≤10	Ref			
11–20	1.49 (0.48–4.64)	0.492		
21–30	1.66 (0.53–5.21)	0.384		
>31	1.52 (0.40–6.04)	0.549		
Country of practice				
Australia	Ref			
New Zealand	2.04 (0.86–4.83)	0.106		
Therapeutically endorsed				
No	Ref			
Yes	0.55 (0.18–1.62)	0.276		
Principal type of optometric practice				
Academic	Ref			
Corporate	0.07 (0.01–0.76)	0.029	0.08 (0.01–0.86)	0.037
Hospital or public health clinic	1.25 (0.25–6.29)	0.787	1.68 (0.31–9.02)	0.544
Independent	0.77 (0.19–3.06)	0.710	0.72 (0.18–2.94)	0.645
Average weekly hours spent providing patient care as an optometrist (years)				
1–10	Ref			
11–20	0.85 (0.11–6.70)	0.877		
>20 h	1.24 (0.27–5.81)	0.783		
Self-declared clinical interest in DED or ocular surface disease				
No	Ref			
Yes	2.84 (1.12–7.20)	0.028	2.97 (1.09–8.12)	0.034
Self-declared clinical interest in gerontology or aged care				
No	Ref			
Yes	0.54 (0.14–2.08)	0.372		

Self-declared clinical interest in retinal disease			
No	Ref		
Yes	1.45 (0.51–4.10)	0.489	
Omega-3 fatty acids (from diet or supplementation) recommended in their practice for improving ocular health			
No	Ref		
Yes	1.04 (0.37–2.99)	0.936	

DED, dry eye disease; OR, odds ratio; Ref, reference.

3.2.4.2.2 Sources of Evidence and Information

Figure 3-5 summarizes the sources of evidence and information that respondents indicated had informed their current clinical decision-making relating to omega-3 fatty acids. Continuing education conference presentations and articles were the major information sources followed by respondents' university education and personal clinical experiences. Two in five optometrists (40%) indicated using published primary research papers and/or systematic reviews to guide their clinical decision-making in this area of practice.

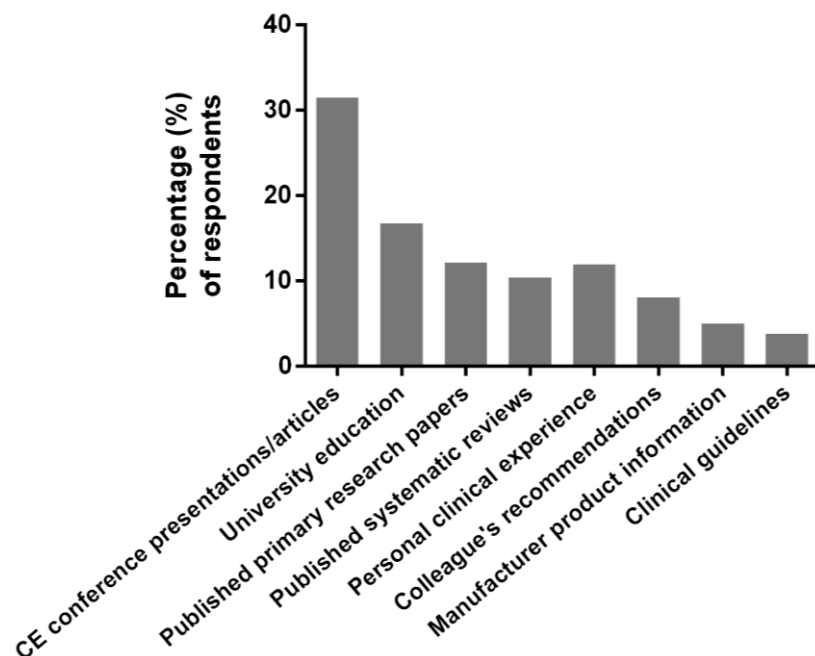


Figure 3-5. Percentage of optometrists who selected each information and/or evidence source (with no limit on the number of sources able to be selected) as informing their clinical decision-making regarding omega-3 fatty acids. CE, continuing education.

3.2.5 Discussion

This study describes the self-reported clinical practices and opinions of Australian and New Zealand optometrists, as related to omega-3 fatty acids and eye health. To our knowledge, this is the first study to consider clinicians' awareness and routine practices relating to omega-3 fatty acids in primary health care. The chosen focus on omega-3 fatty acids for this survey was based on their reported potential to improve outcomes in certain chronic ocular conditions, such as DED and AMD. The current findings provide new insights into optometrists' understanding of current research evidence relating to omega-3 fatty acids in the context of eye health, and their self-reported application of this knowledge in practice. Furthermore, this survey considered the sources of information and evidence used to guide optometrists' clinical decision-making as a foundation for informing the optimal mode(s) of delivering future education programs relating to nutrition in eye care practice.

3.2.5.1 General Recommendations of Optometrists Relating to Diet and Nutrition

This survey, which was made available to an estimated 6500 practicing optometrists via professional optometry networks in Australia and New Zealand, received responses that were considered representative of the major regions in both countries. About half of respondents described their principal practice setting as independent (49%), which indicates a possible over-representation of responses received from independent practices compared to corporate or public health practices (local healthcare workforce data suggest ~30% optometry registrants practice in independent-settings practice (AGDH, 2017)). Globally, there is substantial variation in optometric scope of practice. In some countries, such as France and Japan, optometrists are restricted to providing optical corrections using spectacles and/or contact lenses (George et al., 2019; Naroo & Grit, 2009). In contrast, in some states of the US and Canada, optometric practice includes performing refractive surgery procedures and minor ocular surgery (Black et al., 2019; Harper et al., 2016; Stein et al., 2018).

The role of optometrists in Australia and New Zealand includes the independent diagnosis and management of ocular disorders relating to visual function and eye health (Black et al., 2019; Kiely & Slater, 2015). In addition, optometrists who hold therapeutic medicine endorsement are permitted to prescribe topical, and in New Zealand oral, medications for managing eye disease. Overall, most survey respondents were qualified to prescribe topical ophthalmic therapeutic medications at a minimum (Australia, 86%; New Zealand, 91%). These proportions are higher than the documented percentage of therapeutically endorsed practitioners in both countries (Australia ~63%; New Zealand ~75%) (Black et al., 2019; OBA, 2019); this may be due to a self-selection bias based on clinical skill or interest, and/or that the survey responses were generally from a younger cohort of optometrists (44% under 30 years of age, and 77% under 45 years, compared with ~26% of all Australian optometrists being under the age of 30 and 60% under the age of 45 (OBA, 2019)). Since 2013, all entry-level optometrists from accredited programs in Australia and New Zealand have graduated with therapeutic prescribing rights (Kiely & Slater, 2015).

Optometrists, as major providers of primary eye care, are ideally placed to ask patients about their diet and to offer evidence-based advice about nutrition-based strategies for reducing the risk and/or progression of ocular disease. In a previous survey-based study, it was reported that two in five patients attending for optometric care in Australia expected their optometrists to ask them about their diet and/or nutritional supplement intake, although only one in five recalled being asked about these factors when attending an optometric consultation (Downie et al., 2017). In the present study, most (64%) respondents self-reported generally (i.e., sometimes, often, or always) enquiring about their patients' diet and/or their routine intake of nutritional or vitamin supplements (74%). These values are similar to findings reported in a cohort survey undertaken five years ago, which found that 62% of Australian optometrists self-reported counselling their patients about their diet, and 55% self-reported providing advice about nutritional supplementation (Downie & Keller, 2015).

3.2.5.2 Recommendations Relating to Omega-3 Fatty Acids for Managing AMD

Over 50% of respondents indicated that they would frequently recommend for their AMD patients to increase their intake of omega-3 rich foods. This is comparable to a previous survey, conducted in the UK, which found that about half of eye care professionals (optometrists or ophthalmologists) reported frequently recommending eating oily fish at least twice per week to individuals with AMD and/or individuals considered to be at risk of AMD (Lawrenson & Evans, 2013).

Several prospective cohort studies have associated higher intakes of foods containing long-chain omega-3 fatty acids with a reduced risk of AMD progression (SanGiovanni et al., 2008; Wu et al., 2017). The Blue Mountains Eye Study found that eating oily fish at least once per week, compared with less than once per week, was associated with a lower risk of developing early-stage AMD (de Koning-Backus et al., 2019). Systematic reviews, synthesizing results from primary research studies, have also found that eating approximately two servings of oily fish per week is associated with a reduced risk of both developing early AMD and progressing to late-stage disease (Chong et al., 2008; Zhu et al., 2016). Most optometrists who recommended consumption of fish or seafood, recommended an intake frequency of two or more servings per week, consistent with current evidence (Chong et al., 2008).

With respect to omega-3 fatty acid supplementation, 62% of respondents reported recommending either plant- or marine-based supplements for AMD management. Although it might be argued that a person's diet can be augmented through supplementation if adequate levels of certain nutrients are not being obtained from food sources, the current best-available evidence does not support omega-3 oral supplements for preventing or slowing the progression of AMD (Lawrenson & Evans, 2015). In contrast to many epidemiological studies on diet, a Cochrane systematic review, which combined findings from two randomized controlled trials, reported that long-chain omega-

3 supplementation for a period of up to five years did not reduce the development of vision loss or the risk of progression to advanced AMD (Lawrenson & Evans, 2015). Furthermore, in the AREDS II trial, the addition of xanthophyll carotenoids (lutein and zeaxanthin) and omega-3 fatty acids to the original high-dose antioxidant AREDS formulation did not further reduce the risk of progression to late-stage AMD relative to the original formulation (AREDS2, 2013). This finding is in contrast to observational evidence that has reported the potential benefit of high dietary intakes of lutein and zeaxanthin for lowering the overall risk of developing late-stage AMD (Seddon et al., 1994).

The absorption of EPA and DHA from nutritional supplementation is influenced by the formulation, chemical preparation, and a person's background dietary fats (Dyerberg et al., 2010; Offman et al., 2013). Phospholipid and triglyceride forms are considered to have superior bioavailability compared to ethyl ester forms (Dyerberg et al., 2010). Nonetheless, nutritional supplements do not contain the full spectrum of nutrients present in whole foods. Consuming a supplement, rather than whole foods, may limit key interactions between the fatty acids and other nutrient components that could contribute towards the retinoprotective effect evident with omega-3 rich diets (Downie & Keller, 2014). In the context of AMD, omega-3 supplements are, thus, not an equivalent substitute for omega-3 fatty acids from dietary sources. Our findings suggest that many optometrists may not be aware of this subtle, but important, distinction.

3.2.5.3 Recommendations Relating to Omega-3 Fatty Acids for Managing DED

About 80% of respondents reported recommending omega-3 fatty acids, in either diet or supplement form, for DED management. This proportion is comparable to previously reported values of 70% of Australian optometrists (Downie et al., 2016) and 80% of New Zealand optometrists in surveys of DED practice patterns (Xue et al., 2017). In the present study, we found more respondents reported recommending omega-3 fatty acids for DED management

than for AMD. This is perhaps not surprising given that the anti-inflammatory effects of omega-3 fatty acids are widely promoted, and several clinical trials have demonstrated favorable results supporting the use of omega-3 supplements for improving clinical outcomes in DED (Downie et al., 2019).

Although the pathogenesis of DED is unclear, tear instability, tear hyperosmolarity, and neuro-sensory abnormalities are understood to contribute to a vicious cycle of inflammation that perpetuates on the ocular surface (Bron et al., 2017). The primary etiological factor is considered to differ between the two main DED subtypes: the evaporative form of DED, which is frequently caused by meibomian gland dysfunction, and aqueous-deficient DED, which is often associated with aging and systemic autoimmune conditions (Craig et al., 2017b). Almost all the practitioners who reported recommending omega-3 fatty acids for DED indicated doing so for managing evaporative or mixed DED, rather than for aqueous-deficient DED. The rationale for this practice may relate to the potential for systemic fatty acid consumption to modify lipid production and regulate the lipid-secreting meibomian glands, both of which may be beneficial in evaporative DED (Liu et al., 2016; Macsai, 2008). However, it may be advisable to exercise caution in interpreting the result for aqueous-deficient DED, on account of the relative rarity of this subtype in an unmixed form, without an overlay of the more prevalent evaporative form (Xue et al., 2017). Notwithstanding these practice patterns, the anti-inflammatory benefits conferred by omega-3 fatty acids are not restricted to improving tear-lipid dysfunction, as aqueous-deficient DED may be associated with an immune-mediated inflammatory response (Bron et al., 2017). Notably, a recent Cochrane systematic review found that in DED, long-chain omega-3 supplements, compared to placebo, increased aqueous tear production and improved tear osmolarity, both of which are compromised in aqueous-deficient DED (Downie et al., 2019).

Our study established that practitioners are less likely to report recommending omega-3 fatty acids for milder forms of DED. This trend has been reported in

previous evaluations of self-reported practices relating to recommending omega-3 fatty acids for DED, by eye care practitioners in New Zealand, Australia, and the UK (Downie et al., 2016; Xue et al., 2017). It is unclear why this practice behavior exists given a lack of specific evidence to support this approach. In the DED staged management and therapy algorithm published in the 2017 Tear Film and Ocular Surface Society (TFOS) International Dry Eye Workshop II report (TFOS DEWS II), education regarding potential dietary modification (including oral essential fatty acid supplementation) is listed as a treatment approach for all DED stages, and not limited to only moderate or severe disease (Jones et al., 2017). Whether perceived cost implications for patients at an early disease stage plays a role in this decision-making is not known.

Respondents indicated recommending a variety of daily omega-3 fatty acid dosages for managing DED, ranging from 250 mg to 6000 mg per day (median [IQR]: 2000 [1000–2750] mg/day). Published guidelines exist with regard to the optimal dose of omega-3 fatty acids for several other health conditions. For example, the American Heart Association recommends 4000 mg EPA, or combined EPA and DHA, per day for reducing systemic triglycerides in cases of hypercholesterolemia (Skulas-Ray et al., 2019). The International Society for Nutritional Psychiatry Research recommends 1000 to 2000 mg of EPA per day for treating major depressive disorders (Guu et al., 2019). There are currently no formal recommendations for the optimal supplement dosage that may provide clinical benefit in ocular conditions.

Randomized controlled trials (RCTs), comparing the efficacy of long-chain omega-3 fatty acids relative to placebo or to no intervention for treating DED, have adopted a wide range of dosages, varying across a 20-fold range, from 135 mg to 3000 mg of combined EPA and DHA per day (Downie et al., 2019). This large dosing range could contribute to the apparently contrasting outcomes observed in different intervention trials (Asbell et al., 2018; Deinema et al., 2017; Kangari et al., 2013). Factors that may contribute to heterogeneity

in the reported effect estimates of omega-3 supplement clinical efficacy for treating DED include: differences in study design, population selection, and the choice of outcome measures. There is also lack of certainty in the literature with regard to the optimal form of the long-chain omega-3 fatty acid supplements, which exist in triglyceride, ethyl ester, or phospholipid forms, and the optimal ratio of EPA to DHA for DED treatment (Downie et al., 2019). Compounding these issues is the current lack of consensus on the most appropriate omega-3 fatty acid-prescribing protocol, specifically as related to dose, treatment duration, and composition, to achieve the most clinical benefit (Jones et al., 2017). Of specific relevance to DED, there is emerging evidence to suggest that topical omega-3 fatty acid supplementation also may have a role in modulating ocular surface inflammation (Downie et al., 2018; Downie et al., 2020).

The two commercially available formulations reported to be most frequently recommended by survey respondents were Thera Tears™ (dosage recommended by manufacturer: three capsules daily, each containing 450 mg EPA, 300 mg DHA, 1337.5 mg ALA, and 183 IU vitamin E as an antioxidant) and Lacritec™ (dosage recommended by manufacturer: three capsules daily for six weeks, and two capsules daily thereafter, each capsule containing 134 mg EPA, 66.8 mg DHA, 58.5 mg oleic acid, 58.5 mg linoleic acid (LA), 192 mg linolenic acid, 434 mg *Borago officinalis* seed oil fixed, and 93.5 mg gamma-linolenic (GLA) acid). In addition to omega-3 fatty acids, Lacritec contains short-chain omega-6 fatty acids. Approximately one in five optometrists reported recommending combined omega-3 and omega-6 supplements for managing DED. While eicosanoids derived from the metabolism of the long-chain omega-6 fatty acid, AA, are pro-inflammatory, dietary intake of the short-chain omega-6, GLA, and its precursor, LA, can yield anti-inflammatory effects by acting as precursors to the eicosanoid prostaglandin-E1 (PGE1) (Aragona et al., 2005). The potential effects of omega-6 fatty acids on ocular surface inflammation are, therefore, complex.

The US Women's Health Study found an association between a higher ratio of dietary omega-6 to omega-3 and an elevated risk of DED (Miljanovic et al., 2005). A recent cross-sectional study reported that relatively high omega-3 consumption and moderate omega-6 consumption were protective against meibomian gland dysfunction in postmenopausal women (Ziemanski et al., 2018). However, several RCTs assessing the efficacy of GLA and LA for alleviating ocular surface inflammation have reported inconsistent effects (Barabino et al., 2003; Brignole-Baudouin et al., 2011; Downie et al., 2019). A recent Cochrane systematic review found that relative to placebo, combined omega-3 and omega-6 fatty acid supplements had no benefit on aqueous tear production or ocular surface staining. Although the pooled effect estimate indicated a possible improvement in tear stability with this intervention, the change was not considered clinically meaningful (Downie et al., 2019).

Baseline dietary omega-3 fatty acid intake is another important consideration for identifying patient populations likely to benefit from enhancing their omega-3 fatty acid intake. Supplementation in individuals already achieving sufficient essential fatty acid levels through their diet would not be expected to demonstrate the same biological response as in those who are deficient (Rice et al., 2016). While 99% of surveyed optometrists in this study reported offering dietary advice to their patients, almost none used quantitative tools to survey their patients' existing dietary habits. Techniques that can be used to estimate omega-3 intake include fatty acid assays from blood samples, although testing tends to be invasive, relatively costly and may not be readily accessible to all clinicians, and collection of short-term dietary records, which can be time consuming and, thus, not ideally suited for use in clinical settings. To overcome these barriers, simple food frequency tools may be of value for providing a rapid, non-invasive estimation of an individual's omega-3 intake, and for informing clinical recommendations regarding the potential benefit, or otherwise, of supplementation or dietary modification (Zhang & Downie, 2019).

3.2.5.4 Knowledge of the Potential Benefits and Risks of Omega-3 Fatty Acids

It has been estimated that in Australia, 80% of adults may not be meeting the suggested dietary intake of long-chain omega-3 fatty acids (Meyer, 2016), which, as recommended by the National Health and Medical Research Council (NHMRC) is 430 mg per day for female adults and 610 mg per day for male adults (NHMRC, 2006). Almost half (46%) of practitioners surveyed recommended for their patients to consume between two and four servings of fish or non-fish seafood per week to improve ocular health. However, less than one in 10 respondents estimated the closest to ideal approximate adult dietary target of marine-based omega-3 fatty acids as 500 mg/day (which approximates to consuming two servings of oily fish per week). Furthermore, less than one-third of respondents were aware that short-chain omega-3 fatty acids, obtained from plant-based sources, are less directly biologically active than long-chain omega-3 fatty acids (Burdge, 2006; Calder, 2012). This disparity highlights a potential target area for professional education to raise awareness about the relative biological efficacy of different food sources.

High-dose omega-3 fatty acids, >2000 mg per day, tend to be safe and well tolerated but are not without potential adverse effects. The most common side effects are gastrointestinal (e.g., nausea, bloating), dermatological (e.g., skin itchiness), and hematological (e.g., anticoagulatory effects). Several studies examining the safety profile of omega-3 fatty acids, dosed at up to 4000 mg per day, have concluded that supplementation is generally well tolerated and that associated adverse events are unlikely to be of clinical significance (Bays, 2007; Chang et al., 2018; Guu et al., 2019; Skulas-Ray et al., 2019; Villani et al., 2013). Over half of survey respondents (53%) reported taking the safety profile of omega-3 fatty acids (i.e., side effects and contraindications) into consideration when making clinical recommendations. Optometrists who declared a clinical interest in DED or ocular surface disease were twice as likely to recommend omega-3 fatty acids to their patients (95% CI: 1.05–5.2) than those without such interests. Interestingly, respondents who recognized that omega-3 fatty acids

have potential side effects were approximately four times more likely to recommend their use. While this might initially seem counterintuitive, it may be a testament to optometrists being cautious prescribers, and a likelihood that clinicians with greater awareness about the safety profile of omega-3 fatty acids are also more likely to be knowledgeable about their benefits. More extensive knowledge may bring about greater confidence in making clinical dietary recommendations. This is corroborated by the finding that survey respondents who declared an interest in DED or ocular surface disease were three times more likely to successfully demonstrate their knowledge about the underlying biological effects and dietary targets of omega-3 fatty acids than those who failed to correctly answer 60% or more of the relevant knowledge-focused questions.

3.2.5.5 Sources of Information and Evidence Used to Guide Clinical Decision Making

Four in five respondents selected continuing education conferences and/or articles as the main source of information used to guide their clinical decision making about omega-3 fatty acids. Two in five respondents indicated that they source primary research articles and/or systematic reviews for advice.

The importance of evidence-based practice is well recognized in both optometric training and practice (Suttle et al., 2015). A previous study reported that while attitudes towards evidence-based practice were generally positive among eye care practitioners, a key barrier was a lack of clinician time to access and appraise large volumes of research evidence (Suttle et al., 2015). Specific barriers that appear to challenge the adoption of best practice relating to nutrition and dietary recommendations in practice include the apparently conflicting results from different studies, a perception from some clinicians that nutritional advice is outside the optometric scope of practice, and a paucity of available clinical tools for quantifying diet in clinical practice.

A number of recent initiatives have sought to support optometrists in making evidence-based dietary recommendations that are relevant to eye health. Optometry Australia's 2019 AMD Clinical Practice Guide recommends that optometrists inform patients about how AMD risk can be modified by diet and other lifestyle factors, such as smoking (Optometry Australia, 2019). With respect to DED, the TFOS DEWS II reports provide a peer-reviewed, open-access summary of the current scientific evidence, based on expert consensus (Craig et al., 2017a). In the TFOS DEWS II report, providing patient education about potential dietary modifications (including oral omega-3 supplementation) is listed within the first step of the four-step staged management algorithm for DED.

The current study identified current clinical practice patterns in primary eye care, as related to oral omega-3 fatty acids, and highlighted knowledge gaps and perceived barriers to clinical implementation. There were some limitations to the present study. While the cohort represented a broad range of optometric practice locations and modalities, there was the potential for self-selection bias, and this was reflected in the proportion of participants declaring a clinical interest in DED or ocular surface disease. Also noted was a relative over-representation of younger optometrists, and optometrists working in independent (private) practice. These limitations may affect the generalizability of our findings, but are not uncommon considerations in survey-based research.

3.2.6 Conclusions

Our results suggest that most Australian and New Zealand optometrists routinely provide clinical recommendations about diet and nutritional strategies, as relevant to eye health. While many optometrists indicated recommending diet-based strategies for managing AMD, the potential benefits of whole-food omega-3 sources relative to the limited benefits of omega-3 supplements were not consistently appreciated. Most respondents recommended omega-3 fatty acids for managing DED, despite conflicting data from recent randomized trials. Respondents were more likely to recommend omega-3 fatty acids to patients

with moderate or severe (rather than mild) DED, and to use omega-3 supplementation to treat predominantly evaporative DED. Further research is needed to address uncertainties in the evidence relating to the optimal prescribing regimen (i.e., omega-3 dose and formulation composition) for treating DED. This information will be of value for informing future education programs about eye health and nutrition, particularly at events such as clinical education conferences that were reported to form the main source of information used by optometrists to guide clinical decision-making.

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4

Chapter 4. Assessing omega-3 fatty acid intake: development and validation of a food-frequency questionnaire for eye care practice

4.1 Introduction

In the previous chapter that examined optometrists' recommendations relating to omega-3 PUFAs, 78% of respondents indicated recommending omega-3 rich foods or supplements to patients with dry eye disease (DED). Of those, almost all reported that they would recommend fish and/or seafood, specifically, as a dietary food source. Understanding the need, and monitoring the uptake, of such advice by patients is difficult to confirm, however. Notably, almost none of the respondents in the survey reported using quantitative tools to assess their patients' existing dietary habits. A lack of dietary questionnaires suitable for routine use in eye care practice likely contributed to this outcome.

Failure to establish baseline omega-3 fatty acid levels and to monitor diet has been criticised in previous clinical trials, but existing tools are recognised to have limitations pertaining their scope, applicability and demands on time. This suggests a need for a rapid-to-administer, clinically-applicable tool that can be used to estimate the relative sufficiency of an individual's omega-3 fatty acid intake. Such a tool could be used by practitioners to determine whether their patient might benefit from dietary modification to enhance their omega-3 fatty

acid intake. It could also have application in quantifying and monitoring dietary fatty acid levels in clinical research studies.

The publication in this chapter describes the development and validation of a short food-frequency questionnaire designed specifically for use in eye care practice. **Section 6.2** comprises the published paper in its entirety, formatted in thesis style. The only modifications are re-numbering of tables, figures and appendices within the thesis (indicated in bold) for consistency in style, and reformatting of references to combine with the full reference list for the thesis.

Zhang, A. C., & Downie, L. E. (2019). Preliminary Validation of a Food Frequency Questionnaire to Assess Long-Chain Omega-3 Fatty Acid Intake in Eye Care Practice. *Nutrients*, 11(4). <https://doi.org/10.3390/nu11040817>

The supplementary materials for this publication are provided in **Appendix B** of the thesis.

4.2 Publication: Preliminary Validation of a Food Frequency Questionnaire to Assess Long-Chain Omega-3 Fatty Acid Intake in Eye Care Practice

4.2.1 Abstract

Clinical recommendations relating to dietary omega-3 essential fatty acids (EFAs) should consider an individual's baseline intake. The time, cost, and practicality constraints of current techniques for quantifying omega-3 levels limit the feasibility of applying these methods in some settings, such as eye care practice. This preliminary validation study, involving 40 adults, sought to assess the validity of a novel questionnaire, the Clinical Omega-3 Dietary Survey (CODS), for rapidly assessing long-chain omega-3 intake. Estimated dietary intakes of long-chain omega-3s from CODS correlated with the validated Dietary Questionnaire for Epidemiology Studies (DQES), Version 3.2, (Cancer Council Victoria, Melbourne, Australia) and quantitative assays from dried blood spot (DBS) testing. The 'method of triads' model was used to estimate a validity coefficient (ρ) for the relationship between the CODS and an estimated "true" intake of long-chain omega-3 EFAs. The CODS had high validity for estimating the ρ (95% Confidence Interval [CI]) for total long-chain omega-3 EFAs 0.77 (0.31–0.98), docosahexaenoic acid 0.86 (0.54–0.99) and docosapentaenoic acid 0.72 (0.14–0.97), and it had moderate validity for estimating eicosapentaenoic acid 0.57 (0.21–0.93). The total long-chain omega-3 EFAs estimated using the CODS correlated with the Omega-3 index ($r = 0.37$, $p = 0.018$) quantified using the DBS biomarker. The CODS is a novel tool that can be administered rapidly and easily, to estimate long-chain omega-3 sufficiency in clinical settings.

4.2.2 Introduction

Omega-3 polyunsaturated fatty acids (PUFAs) are essential fatty acids (EFAs) that cannot be synthesized de novo, and thus must be derived from food sources or dietary supplementation. The potential benefit of diets rich in omega-3 fatty acids has been shown in a variety of health conditions, such as hypercholesterolaemia and rheumatoid arthritis (Rajaei et al., 2015; Yokoyama et al., 2007). Omega-3 PUFAs exist in both short- and long-chain forms. The short-chain omega-3 fatty acids and alpha-linoleic acid (ALA) are derived from plant-based sources (e.g., flaxseed and walnuts) and they are a precursor to the more biologically potent long-chain omega-3 EFAs, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Dietary long-chain omega-3 EFAs are found mostly in marine sources (e.g., oily fish and seafood). Once ingested, long-chain omega-3 PUFAs are incorporated into cellular membranes and play a role in cellular signaling, modulating systemic inflammation, and influencing immune function (Calder, 2017; Simopoulos, 2002b).

There is mounting evidence that diets rich in omega-3 EFAs may be beneficial for reducing the risk of development and/or progression of several ocular conditions, such as dry eye disease and age-related macular degeneration (AMD)(Downie & Keller, 2014; Miljanovic et al., 2005; Zhu et al., 2016). The United States (US) Women's Health Study showed that a low dietary intake of omega-3 EFAs is associated with a higher incidence of dry eye disease in women (Miljanovic et al., 2005). The Blue Mountains Eye Study found that eating oily fish once per week, as compared with fewer than once per week, was associated with a lower risk of developing early-stage AMD (Tan et al., 2009). A meta-analysis of prospective cohort, case-control, and cross-sectional studies suggested that consumption of two or more servings of oily fish per week was beneficial in the primary prevention of AMD (Chong et al., 2008). An association has been found between higher dietary intake of omega-3 EFAs and significant risk reduction for developing more advanced, sight-threatening forms of AMD (Christen et al., 2011; Sangiovanni et al., 2009; Seddon et al., 2006).

Omega-3 EFA supplementation may also lower intraocular pressure in adults (Downie & Vingrys, 2018).

Currently, the suggested dietary targets (SDT) for long-chain omega-3 EFA consumption, as recommended by the Australian National Health and Medical Research Council (NHMRC), in diets optimized to lower chronic disease risk, is 430 mg/day for women and 610 mg/day for men (NHMRC, 2005, 2006). These recommendations are consistent with the National Heart Foundation position statement (2015) that recommends two to three servings of fish (serving size: 150–200 g), preferably oily fish, per week to achieve ~250–500 mg/day of combined EPA and DHA consumption (Nestel et al., 2015). The US Department of Health and Human Services Dietary Guidelines (2015–2020) recommend eight ounces of fish/seafood per week, which is approximately equivalent to 250 mg of EPA + DHA per day, and eight to 12 ounces in pregnancy (USDHS, 2015). These recommended values are similar to that of the European Food Safety Authority (EFSA, 2012), while the French Agency for Food, Environmental and Occupational Health and Safety (ANSES) recommends 250 mg/day of each EPA and DHA. However, research suggests that approximately 80% of Australian adults do not meet this recommendation for daily intake (Meyer, 2016). Only ~15% of the French population meet the recommendation for daily intake of EPA, and 8% are estimated to meet the daily DHA recommendation. A study undertaken in the US estimated that low dietary intake of omega-3 fatty acids was a modifiable risk factor contributing to ~84,000 deaths in 2005 (Danaei et al., 2009). Therefore, there is a need for healthcare clinicians to actively enquire about their patients' diet, and preferably quantify their dietary intake of omega-3 EFAs, in order to identify individuals who are likely to benefit from dietary changes and/or supplementation to improve their health.

Various techniques can be used to quantify systemic omega-3 EFA levels, however, most of these methods are not ideally suited for direct application in all clinical settings (Overby et al., 2009). Fatty acid levels present in

subcutaneous adipose tissue are considered to be the most robust long-term marker of fatty acid intake (Baylin et al., 2002). Systemic EFA concentrations can be accurately estimated by assaying fatty acid levels in erythrocytes and plasma phospholipids (Saadatian-Elahi et al., 2009). Although both of these methods provide quantitative data relating to systemic fatty acid levels, the sample collection procedures are invasive, relatively costly, and time consuming (as they require off-site laboratory analyses), and therefore are not currently routinely performed in a clinical setting (Harris, 2008). In addition, these tests are not readily accessible to all clinicians who may be in a position to provide relevant dietary advice regarding the potential benefits of omega-3 EFA intake to their patients (e.g., eye care clinicians, in relation to ocular health). Short-term dietary assessment, such as dietary records requiring self-monitored and detailed recordings over multiple days, and short-term recalls do not account for the day-to-day variation of a habitual diet (Wrieden et al., 2003). Furthermore, both methods have been shown to underestimate average energy intake, particularly in populations with lower socioeconomic status, education, and literacy levels (Institute of Medicine, 2002).

As an alternative, diet questionnaires provide a rapid, non-invasive method for estimating dietary fatty acid intake. Although nutrient intakes derived from several food frequency questionnaires (FFQs) have been shown to correlate with quantitative biological markers of omega-3 EFA intake (Overby et al., 2009), questionnaires designed for use in epidemiology research studies are often exhaustive and relatively time consuming, and not intended for use in routine clinical settings (Rahmawaty et al., 2017; Swierk et al., 2011). Several brief omega-3 dietary questionnaires have been developed for use in clinical settings (Dahl et al., 2011), including for adults with psychological disorders (Lucas et al., 2009; Sublette et al., 2011), and to use for identifying older adults with inadequate omega-3 EFA intake in the context of cardiovascular disease risk (Dickinson et al., 2015). The reliability estimates from different dietary surveys are dependent on the nutritional composition of food sources, which can vary across different geographical locations (Stark et al., 2016).

The aim of this study was to undertake preliminary validation of a novel, clinically applicable questionnaire, the Clinical Omega-3 Dietary Survey (CODS), for assessing dietary long-chain omega-3 EFA intake, as compared with an objective fatty acid blood biomarker and a previously validated survey designed to capture a full dietary profile. Information derived from this tool could then be used by clinicians to estimate whether an individual meets the recommended daily intake of long-chain omega-3 EFAs, or whether they might benefit from enhancing their dietary intake.

4.2.3 Materials and Methods

This cross-sectional research study was conducted in accordance with the tenets of the Declaration of Helsinki, and it was approved by the University of Melbourne Human Research Ethics Committee (HREC #1749830.1) and the St Vincent's Hospital Research Ethics Committee (HREC #HREC/17/SVHM/236).

4.2.3.1 Participants

Adult participants were recruited from the University of Melbourne and St Vincent's Hospital (Melbourne, Victoria, Australia) via advertisements. All participants provided written, informed consent to participate. Eligible participants were aged 18 years or older, and were not pregnant or breastfeeding. There were no eligibility restrictions with respect to general health conditions. Long-chain omega-3 EFA status was quantified using three methods, as detailed below: (a) the CODS; (b) the Dietary Questionnaire for Epidemiological Studies, Version 3.2 (DQES v3.2, Cancer Council Victoria, Australia); and (c) dried blood spot (DBS) testing (Xerion Pty Ltd, Victoria, Australia). For methods (a) and (b), daily long-chain omega EFA intake (mg/day) was calculated as the sum of EPA, DHA, and docosapentaenoic acid (DPA) intake. For method (c), the long-chain omega-3 EFAs present in erythrocytes (%) was calculated as the sum of EPA, DHA, and DPA concentrations (%) present in erythrocytes.

4.2.3.2 Clinical Omega-3 Dietary Survey

A clinically relevant FFQ, the CODS, was developed by the authors of this study to yield an estimate of long-chain omega-3 EFA intake, using the nutrient composition data available in the AUSNUT (2011–2013) food nutrient database (available online at:

<http://www.foodstandards.gov.au/science/monitoringnutrients/ausnut/foodnutrient/Pages/default.aspx>). For natural foods containing the highest concentrations of total long-chain omega-3 fatty acids (ranging from 769.4 mg/100 g to 62 mg/100 g), the fatty acid composition was extracted from the nutritional database, and the food was considered for inclusion in the CODS.

The CODS (Figure S1; **Appendix B**) comprises three main sections, categorized according to the primary type of food as: (i) seafood, (ii) fish, and (iii) meat (including eggs). Seven species of seafood, 17 types of fish (including a generalized description for white-fleshed fish), and seven different types of meat/eggs are incorporated, and are used as a basis for estimating dietary long-chain omega-3 EFA intake. A final section, (iv), considers the consumption of long-chain omega-3 EFAs from dietary supplements. The study participants were required to provide details relating to the supplement formulation (brand, dose, form of omega-3 EFAs, and frequency of intake) and these data were incorporated into the analysis.

The CODS was administered by a single examiner (A.C.Z.), and the participants' responses were recorded on a paper-based survey. The first part of the survey asked participants to recall the average frequency (i.e., number of times per week or per month, as appropriate for the nominated frequency) of fish or seafood consumption (in any form, including fresh and canned) over the past three months. The method provided to the participants for estimating the approximate portion size (in amounts of 50 g, 100 g, 150 g and 200 g) required using the size of the examiner's palm as a reference for a 100 g portion size. Then, the participants were required to estimate the average portion size (in grams) of fish or seafood they typically consumed.

For each food source listed in sections (i), (ii), and (iii) of the CODS, participants were asked to estimate the average portion size per serving (g), and to note how often they consumed that type of food (i.e., average number of times per week or per month). In order to quantify egg intake, the average number of eggs per week or per month was reported. The participants were instructed to only include food sources that they consumed “regularly”, defined as at least once per month over the past three months.

Quantification of the total long-chain omega-3 EFA intake was derived from the composition data available in the AUSNUT (2011–2013) food nutrient database (FSANZ, 2014), using a spreadsheet in Microsoft Excel 2017 (Microsoft Corp., Redmond, WA, USA).

4.2.3.3 Dietary Questionnaire for Epidemiology Studies

An online diet questionnaire, DQES v3.2, was purchased from an independent provider (Cancer Council Victoria, Australia) (Giles & Ireland, 2017). The DQES v3.2 was developed and validated for assessing intake of food and nutrients among Australian adults (Bassett et al., 2016; Hopkins et al., 2017), and it had been previously utilized by the Melbourne Collaborative Cohort epidemiology study, involving a cohort of 810 participants.

The DQES v3.2 consisted of 142 food items, and it used extensive food compositional data derived from two Australian databases, AUSNUT 2007 and NUTTAB 2010 (FSANZ, 2008, 2014), to estimate dietary intake for a spectrum of 98 nutrients, including macronutrients (e.g., carbohydrates, proteins, fats) and micronutrients (e.g., vitamins, minerals). In addition, nutrient intakes from alcoholic beverages are surveyed, although these values were not incorporated into the analysis.

Nutritional information relating to EFAs was estimated from the questionnaire, and included a breakdown of individual fatty acids, derived from the NUTTAB 2010 food database (FSANZ, 2011). The total long-chain omega-3 EFA

intake (mg/day) was calculated as the sum of P205W3FD (EPA), P225W3FD (DPA), and P226W3FD (DHA). As the DQES is proprietary, we were unable to ascertain which specific food items were used to derive the estimate of omega-3 fatty acid intake for this survey.

Participants undertook the DQES online, using a self-administrated delivery method. Once completed, the nutritional analysis for each participant was undertaken by the survey supplier, with the results emailed to the study authors.

4.2.3.4 Dried Blood Spot Testing

The participants' fatty acid profiles were analyzed using dried DBS testing (PUFAcoat™ technology, Xerion Pty Ltd, Victoria, Australia). A single drop of capillary blood (~3 mL) was collected from each participant, using a sterile, single-use lancing device and spotted onto the proprietary PUFAcoat™ test cards, which are designed for long-term stabilization of long-chain PUFAs in dried blood samples. Prior to analysis, the DBS cards were air dried, placed in sealed cellophane bags, and stored in desiccants in a dark, temperature-controlled chamber. As per the manufacturer's instructions, all blood samples were sent for laboratory analyses within four weeks of collection.

The fatty acid analyses were performed in an independent laboratory (Waite Lipid Analysis Service (WLAS), University of Adelaide, South Australia, Australia), using established methods (Liu et al., 2014). In brief, the fatty acid blood spots were transmethylated by mixing with H₂SO₄ (18M AR grade, BDH, Sussex, UK) in anhydrous methanol in a 5 ml sealed vial (Wheaton, Millville, USA), and then were heated for three hours at 70 °C. The fatty acid methyl esters were separated and quantified using gas chromatography (Hewlett-Packard 6890 gas chromatograph, equipped with a 50 m capillary column (0.32 mm internal diameter SGE, Victoria), coated with 70% cyanopropyl polysilphenylene-siloxane (BPX70) (0.25 µm film thickness) and fitted with a flame ionization detector. A helium carrier gas was used and the inlet split ratio was set to 20:1, with the injector temperature at 250 °C and detector

temperature at 300 °C. Fatty acid methyl esters were identified by comparing the retention times and the peak area values of unknown samples to the standards using the ChemStation software (Hewlett Packard, CA, USA), and a normalized percentage was calculated based on the response factors.

The extracted long-chain omega-3 fatty acid output parameters were: 20:5n-3 (EPA), 22:5n-3 (DPA) and 22:6n-3 (DHA). The total long-chain omega-3 EFA erythrocyte concentration (%) was calculated as the sum of: C20:5n-3 (EPA) + C22:5n-3 (DPA) + C22:6n-3 (DHA). The Omega-3 index (%) was defined as the total percentage of EPA and DHA present in erythrocyte phospholipid membranes (Liu et al., 2014).

4.2.3.5 Statistical Analysis

On the basis of an estimated correlation coefficient of 0.45 between quantification methods, as previously reported in a similar study (Sullivan et al., 2006), a sample size of 36 participants was calculated to be required for 80% power at a 5% significance level.

Data normality testing was performed using the D'Agostino–Pearson omnibus normality test. The inter-group comparisons were analyzed using a Student's t-test or Mann–Whitney U test, as appropriate. The Spearman's correlation coefficient (r_s) was used to assess the relationship between outputs from the CODS, DQES, and DBS analyses, with respect to long-chain omega-3 EFA intake levels. The Spearman's correlation coefficients were interpreted with reference to similar studies, where $r_s \leq 0.35$ indicates a weak correlation, $r_s = 0.36–0.67$ indicates a moderate correlation, $r_s = 0.68–1$ indicates a good correlation and $r_s \geq 0.9$ indicating a very good correlation (Rahmawaty et al., 2017).

The 'method of triads' statistical model was used to derive a validity coefficient to estimate the relationship between a dietary measurement (e.g., the CODS) and an estimated "true" intake (Figure S2; [Appendix B]) (da Silva et al., 2018;

Ocke & Kaaks, 1997). Validity coefficients closer to 1 indicated a closer relationship between the estimated dietary score and the estimated “true” intake (Swierk et al., 2011). For this analysis, a validity coefficient <0.2 was considered to represent low validity, values between 0.2 and 0.6 were considered to represent moderate validity, and values >0.6 were regarded as indicative of high validity (Ocke & Kaaks, 1997). For a Heywood case, where a validity coefficient ≥ 1 was estimated, the validity coefficient was set to 1.

A bootstrap procedure was used to estimate the confidence interval (CI) for each validity coefficient (Efron & Tibshirani, 1986; Ocke & Kaaks, 1997). For this method, each bootstrap drew 40 samples with replacement from the original sample, and the validity coefficient for each quantification method was calculated. A total of 1000 bootstrap samples were obtained to build a bootstrap distribution of validity coefficients using Microsoft Excel 2017 (Microsoft Corp., Redmond, WA, USA). The non-Heywood cases were used to calculate the 95th percentile CI for each validity coefficient.

The inter-method agreement, for the estimated daily total long-chain omega-3 EFA intake (mg/day) between the CODS and DQES, was examined using Bland–Altman analysis (Bland & Altman, 1999). The mean difference (bias) and limits of agreement (LoA, defined as the bias ± 1.96 standard deviations of the mean difference) were calculated. A regression analysis was used to analyze the potential relationship between the differences between the methods and the average of the two methods.

Group data are reported as the mean $\pm$ standard deviation (SD), unless otherwise specified.

4.2.4 Results

Forty participants were enrolled in the study, and they completed three independent methods for assessing long-chain omega-3 EFA intake. The mean (SD) age of participants was 45.7 (18.11) years. Among the 40 participants, 26 were female and 14 were male. The study cohort included individuals with diabetes mellitus ($n = 19$), however, there were no statistically significant differences between this subgroup of participants and the remaining subgroup, with respect to age, gender, or Omega-3 index measured using the DBS biomarker ($p > 0.05$ for each comparison).

4.2.4.1 Dietary Assessment of Long-Chain Omega-3 EFA Intake

The average time taken to conduct the CODS was three minutes and the average time taken to complete the DQES (v3.2) was 15 minutes.

Table 4-1 summarizes the estimated daily long-chain omega-3 EFA intake (mg/day), for each of DHA, DPA, and EPA, as assessed using the CODS and DQES (v3.2), for the study cohort ($n = 40$ participants). There was no significant inter-method difference for the estimated intake of omega-3 fatty acids (**Table 4-1**, $p > 0.05$ for all comparisons).

Table 4-1. Comparison of the median and inter-quartile range (IQR) values for long-chain omega-3 fatty acid intake, for the CODS and DQES v3.2 questionnaires, in the study cohort.

	CODS (mg/day)			DQES v3.2 (mg/day)			<i>p</i> -value
	Median	IQR		Median	IQR		
		p25	p75		p25	p75	
Total LC omega-3 EFAs	443.7	204.1	650.0	362.6	200.7	577.9	0.83
EPA (20:5 n3)	140.5	55.9	209.2	91.4	49.9	168.0	0.37
DPA (22:5 n3)	75.2	39.3	102.2	77.8	41.3	116.2	0.41
DHA (22:6 n3)	221.7	95.8	320.2	184.9	98.2	353.7	0.98

Abbreviations: CODS, Clinical Omega-3 Dietary Survey; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; DQES, Dietary Survey for Epidemiology Studies v3.2; EFA, essential fatty acid; EPA, eicosapentaenoic acid; IQR, inter-quartile range; LC, long-chain; p25, 25th percentile; p75, 75th percentile.

4.2.4.2 Dried Blood Spot Analysis of Long-Chain Omega-3 EFAs Levels

Table 4-2 summarizes the estimated long-chain omega-3 EFAs present in erythrocytes, and the Omega-3 index as estimated using the dried blood spot test, for the study cohort.

Table 4-2. Concentration of long-chain omega-3 EFAs present in erythrocytes for the study cohort.

	Median	IQR	
		p25	p75
Total LC omega-3 EFAs (%)	4.15	3.58	5.30
EPA (20:5 n3) (%)	0.60	0.45	0.88
DPA (22:5 n3) (%)	1.25	1.08	1.66
DHA (22:6 n3) (%)	2.16	1.80	2.77
Omega-3 index	5.32	4.58	6.17

Abbreviations: EFA, essential fatty acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; IQR, inter-quartile range; LC, long-chain; total LC omega-3 EFAs, DHA + DPA + EPA; p25, 25th percentile; p75, 75th percentile.

4.2.4.3 Correlation between Quantification Methods

Table 4-3 summarizes the Spearman's correlation coefficients (r_s) for the association between the dietary survey methods (CODS and DQES v3.2, mg/day, **Table 4-1**) and DBS assays (% **Table 4-2**) for quantifying DHA, DPA, EPA, and total long-chain omega-3 EFAs (DHA + DPA + EPA).

Table 4-3. Relationships between the long-chain omega-3 EFA quantification methods, calculated using the Spearman's correlation coefficient (r_s).

	CODS vs. DQES		DBS vs. DQES		DBS vs. CODS	
	r_s	p-value	r_s	p-value	r_s	p-value
Total LC omega-3 EFAs	0.64	<0.0001	0.40	0.0096	0.38	0.017
EPA (20:5 n3)	0.60	<0.0001	0.32	0.042	0.18	0.28
DPA (22:5 n3)	0.53	0.0005	0.12	0.47	0.11	0.48
DHA (22:6 n3)	0.66	<0.0001	0.40	0.011	0.45	0.0038

Abbreviations: CODS, Clinical Omega-3 Dietary Survey; DBS, dried blood spot; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; DQES, Dietary Survey for Epidemiology Studies v3.2; EFA, essential fatty acid; EPA, eicosapentaenoic acid; LC, long-chain; total LC omega-3 EFAs, DHA + DPA + EPA.

Comparing the two dietary methods (CODS and DQES v3.2) there was a moderate-to-strong, positive correlation for each of total long-chain omega-3 EFAs ($r_s = 0.64$; $p < 0.0001$) (**Figure 4-1A**), EPA ($r_s = 0.60$; $p < 0.0001$) (**Figure 4-1B**), the DPA ($r_s = 0.53$; $p = 0.0005$) (**Figure 4-1C**) and DHA ($r_s = 0.66$; $p < 0.0001$) (**Figure 4-1D**).

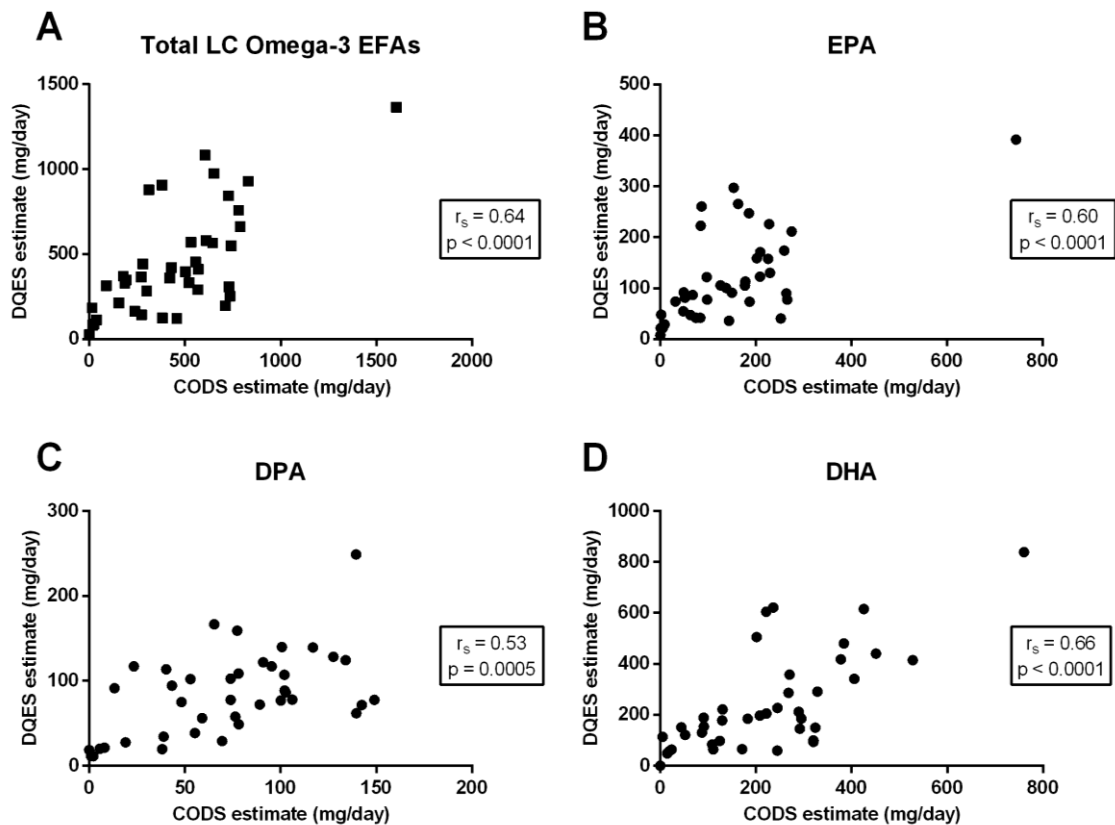


Figure 4-1. The relationship between calculated dietary long-chain (LC) omega-3 essential fatty acid (EFA) intake, quantified using the Spearman's correlation coefficients (r_s), by the Clinical Omega-3 Dietary Survey (CODS) versus the Dietary Questionnaire for Epidemiology Studies (DQES) v3.2, for (A) total LC omega-3 EFAs; (B) eicosapentaenoic acid (EPA); (C) docosapentaenoic acid (DPA); and (D) docosahexaenoic acid (DHA).

Comparing the DQES (v3.2) to the DBS assay, there was a moderate correlation for both total long-chain omega-3 EFAs ($r_s = 0.40$; $p = 0.0096$) (**Figure 4-2A**) and DHA ($r_s = 0.40$; $p = 0.011$, **Figure 4-2D**) and a weak, positive correlation for EPA ($r_s = 0.32$; $p = 0.042$) (**Figure 4-2B**). There was no significant relationship between the DQES (v3.2) and DPA levels measured using the DBS test ($r_s = 0.12$; $p = 0.47$) (**Figure 4-2C**).

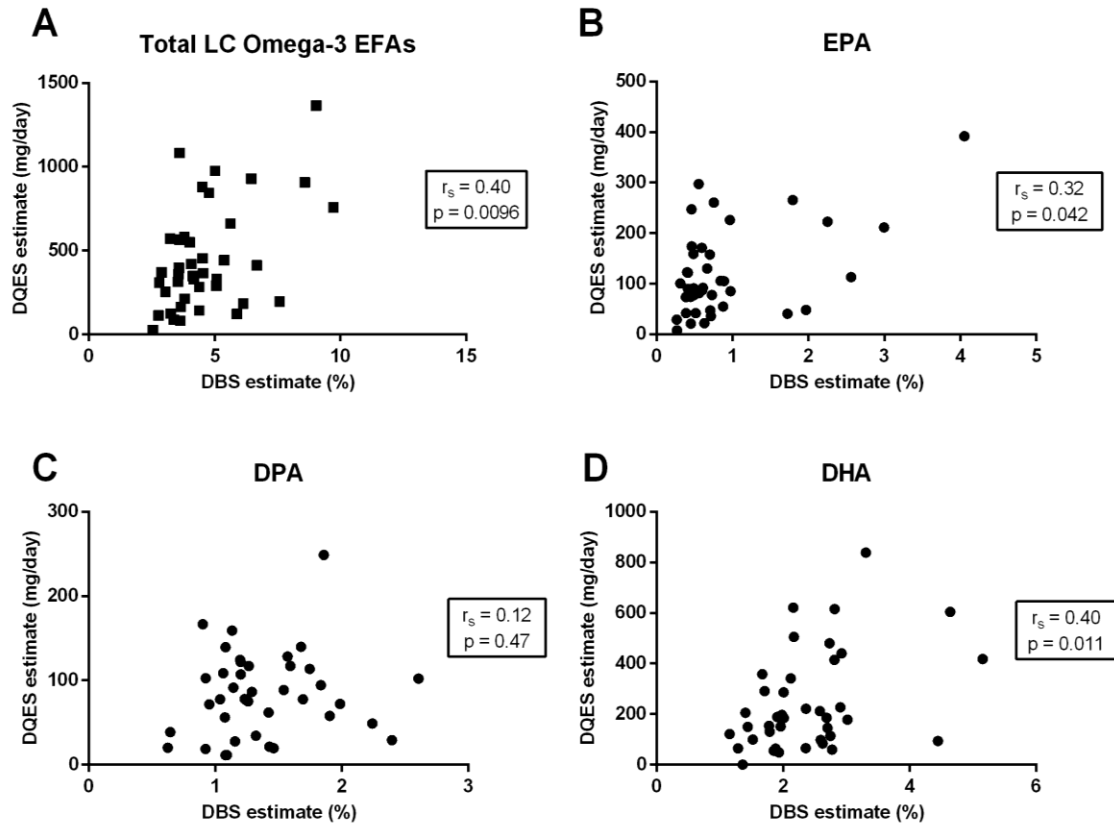


Figure 4-2. The relationship between total long-chain (LC) omega-3 essential fatty acids (EFAs) percentage (%) present in erythrocytes, measured using dried blood spot (DBS) biomarkers, and the dietary LC omega-3 EFA intake estimated using the Dietary Questionnaire for Epidemiology Studies (DQES) v3.2 for (A) total LC omega-3 EFAs, (B) eicosapentaenoic acid (EPA), (C) docosapentaenoic acid (DPA), and (D) docosahexaenoic acid (DHA). Correlation coefficients are calculated using the Spearman's correlation coefficients (r_s).

Comparing the CODS to the DBS fatty acid analysis, there was a moderate positive correlation between the two methods for total long-chain omega-3 EFAs ($r_s = 0.38$; $p = 0.017$) (**Figure 4-3A**) and DHA ($r_s = 0.45$; $p = 0.0038$) (**Figure 4-3D**), and a weak correlation for EPA ($r_s = 0.18$; $p = 0.28$) (**Figure 4-3B**), but no significant correlation for DPA ($r_s = 0.11$; $p = 0.48$) (**Figure 4-3C**).

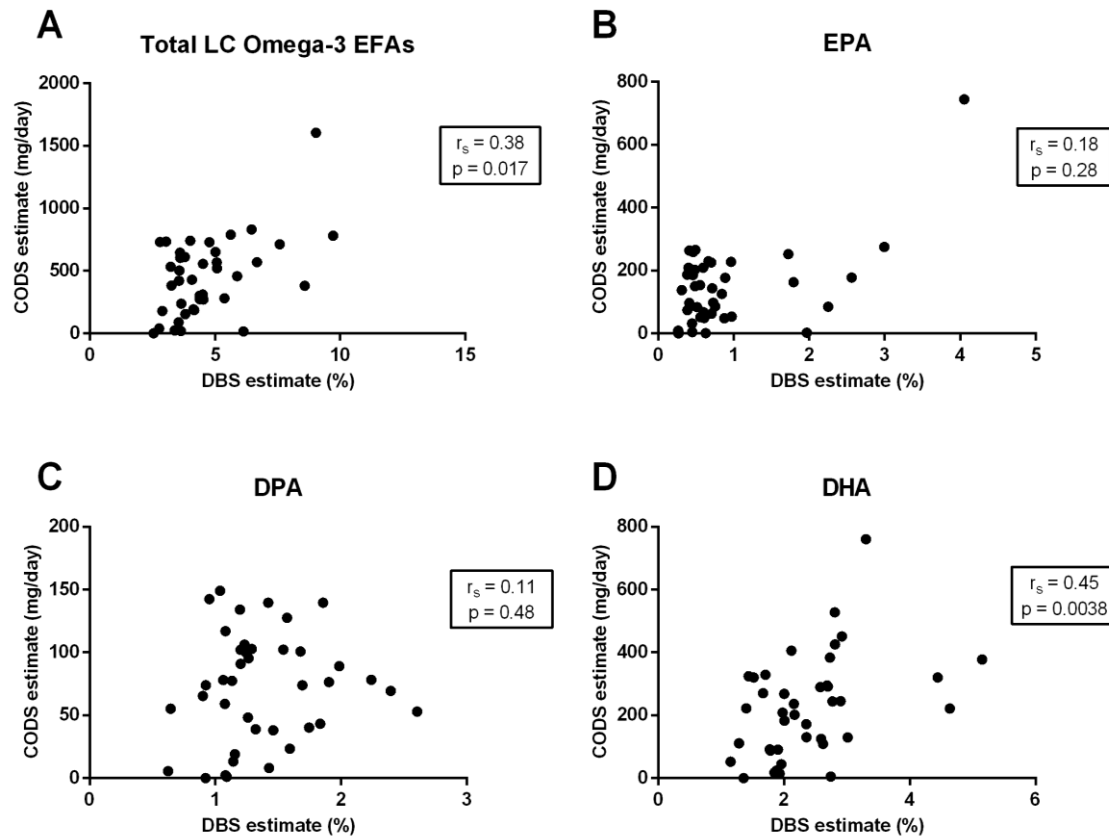


Figure 4-3. The relationship between total long-chain (LC) omega-3 essential fatty acids (EFAs) percentage (%) present in erythrocytes, measured using dried blood spot (DBS) biomarkers, and the dietary LC omega-3 EFA intake estimated using the Clinical Omega-3 Dietary Survey (CODS) for (A) total LC omega-3 EFAs, (B) eicosapentaenoic acid (EPA), (C) docosapentaenoic acid (DPA), and (D) docosahexaenoic acid (DHA). Correlation coefficients are calculated using the Spearman's correlation coefficients (r_s).

For total long-chain omega-3 EFA intake, the relationship between both dietary methods (i.e., CODS and DQES) and the DBS Omega-3 index, which is a measure of the total EPA and DHA present in erythrocyte phospholipid membranes, was considered. There was a moderate, positive correlation between both the CODS ($r_s = 0.37$; $p = 0.018$) (**Figure 4-4A**) and DQES ($r_s = 0.39$; $p = 0.013$) (**Figure 4-4B**) questionnaires compared to the Omega-3 index estimated using the DBS assay.

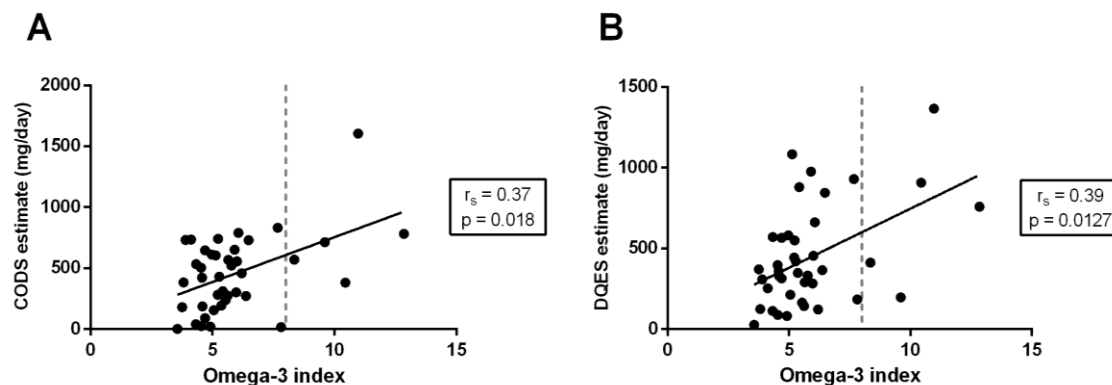


Figure 4-4. The relationship between the overall Omega-3 index, calculated as the total percentage (%) of EPA and DHA present in erythrocytes, and estimated using the dried blood spot (DBS) biomarker, and the dietary estimate of total long-chain omega-3 intake using (A) the Clinical Omega-3 Dietary Survey (CODS) and (B) the Dietary Questionnaire for Epidemiology Studies (DQES) v3.2. Correlation coefficients are calculated using the Spearman's correlation coefficients (r_s).

4.2.4.4 Method of Triads Analysis

Table 4-4 summarizes the validity coefficient (ρ) for each of the methods used to estimate the dietary omega-3 EFAs (CODS, DQES, and DBS), relative to an estimated true intake (T), calculated using the method of triads (Ocke & Kaaks, 1997).

Table 4-4. Validity coefficients (ρ) calculated using the method of triads for each of the methods vs. the estimated true intake (T) for each of the long-chain omega-3 EFAs.

	CODS Validity Coefficient vs. T [ρ_{QT}] (95% CI)	DQES Validity Coefficient vs. T [ρ_{RT}] (95% CI)	DBS Validity Coefficient vs. T [ρ_{BT}] (95% CI)
Total LC omega-3 EFAs	0.77 (0.31–0.98)	0.83 (0.39–0.98)	0.49 (0.12–0.73)
EPA (20:5 n-3)	0.57 (0.21–0.93)	1.00 * (0.39–1.00)	0.31 (0.07–0.65)
DPA (22:5n-3)	0.72 (0.14–0.97)	0.73 (0.16–0.97)	0.16 (0.03–0.50)
DHA (22:6n-3)	0.86 (0.54–0.99)	0.77 (0.39–0.97)	0.52 (0.21–0.74)

* Validity coefficients >1 were set to 1.00 (Heywood cases). Abbreviations: CI, confidence interval; CODS, Clinical Omega-3 Dietary Survey; DBS, dried blood spot; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; DQES, Dietary Survey for Epidemiology Studies v3.2; EFA, essential fatty acid; EPA, eicosapentaenoic acid; LC, long-chain; total LC omega-3 EFAs, DHA + DPA + EPA; Q, questionnaire; R, reference method; T, true intake.

The CODS obtained high validity coefficients for estimating each of total long-chain omega-3 EFAs ($\rho = 0.77$, 95% CI: 0.31 to 0.98); DHA ($\rho = 0.86$, 95% CI:

0.54 to 0.99); and DPA ($\rho = 0.72$, 95% CI: 0.14 to 0.97). A moderate validity coefficient was obtained for estimating EPA ($\rho = 0.57$, 95% CI: 0.21 to 0.93).

The DQES obtained high validity coefficients for estimating total long-chain omega-3 EFAs ($\rho = 0.83$, 95% CI: 0.39 to 0.98); DHA ($\rho = 0.77$, 95% CI: 0.39 to 0.97); and DPA ($\rho = 0.73$, 95% CI: 0.16 to 0.97). A Heywood case was met for the validity coefficient estimate for EPA ($\rho = 1.04$), and thus the validity coefficient was set to 1.0 (95% CI: 0.39 to 1.00).

For the DBS assay, moderate validity coefficients (relative to the estimated true intake T) were obtained for estimating each of total long-chain omega-3 EFAs ($\rho = 0.49$, 95% CI: 0.12 to 0.73); DHA ($\rho = 0.52$, 95% CI: 0.21 to 0.74); and EPA ($\rho = 0.31$, 95% CI: 0.07 to 0.65). A low validity coefficient was obtained for estimating DPA ($\rho = 0.16$, 95% CI: 0.03 to 0.50).

4.2.4.5 Bland–Altman Analysis

The inter-method agreement was assessed using a Bland–Altman analysis (**Figure 4-5**) for the CODS- and the DQES-derived estimates for total daily long-chain omega-3 EFA intake (mg/day). Regression analysis indicated the absence of a significant inter-variable relationship across the spectrum of quantified values ($p = 0.97$). There was no significant global bias (mean difference) between these two assessment methods (mean $\pm$ standard error: 6.1 ± 38.6 mg/day, $p > 0.05$).

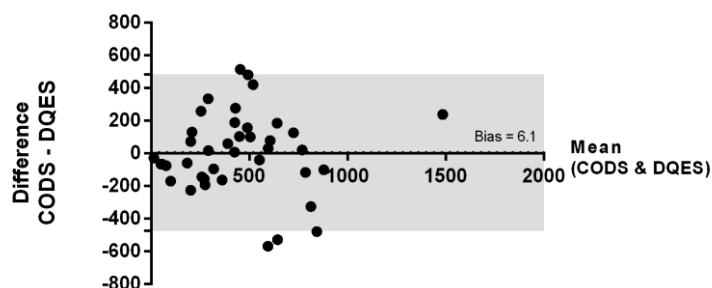


Figure 4-5. Bland–Altman plot comparing the estimated total daily intake of long-chain omega-3 EFAs (mg/day), measured using the CODS and DQES. The dotted line shows the bias (6.1 mg/day) for the comparison between the two methods, which was not statistically significant (95% CI: -32.5 to 44.7 mg/day). The grey shaded area highlights the limits of agreement (LoA).

4.2.5 Discussion

This preliminary validation study shows that a novel FFQ, the CODS, is a simple, valid tool for assessing long-chain omega-3 EFA intake in Australian adults. The CODS, which considers foods containing the highest concentrations of long-chain omega-3 EFAs, extracted from an Australian food compositional database (AUSNUT 2011–2013) (FSANZ, 2014), only requires a few minutes to complete. The dietary estimates derived from the CODS correlated moderately well with long-chain omega-3 fatty acid intake quantified using the comprehensive, validated DQES (v3.2), as well as the systemic fatty acid profiles derived from dried blood spot analyses. Given that the CODS is straightforward to use, and can be rapidly completed in clinical settings, we propose that this survey can be applied clinically to estimate patients' long-chain omega-3 EFA intake, particularly in eye care settings where no similar tools currently exist. Therefore, this may provide information to inform clinical advice with respect to the sufficiency of a patient's estimated intake of dietary long-chain omega-3 EFAs, and thus any potential recommendations surrounding dietary modification and/or supplementation.

Knowledge of a patient's baseline omega-3 status is essential to develop informed, best-practice clinical recommendations relating to the relative appropriateness of dietary adjustment(s) and/or supplementation. Biomarker-based assessments of systemic omega-3 EFA levels, typically derived from subcutaneous adipose or blood assays, although accurate, are costly and time consuming, and therefore are not routinely applied in clinical settings. In addition, these types of biomarker assessments may not be readily available to all clinicians who require knowledge of patients' omega-3 fatty acid intake to provide informed clinical care. For example, as major providers of primary eye care, optometrists frequently provide clinical care to individuals who are at risk of, or who have existing, eye conditions where the natural history of the disease may be influenced by omega-3 EFA intake. Recent research demonstrates that eye care clinicians have identified a need for validated clinical tools to assess

the degree of dietary omega-3 fatty acid sufficiency for their patients (Downie & Keller, 2015). Furthermore, when attending eye examinations, there is an expectation among patients that the provision of comprehensive clinical care includes an assessment and relevant evidence-based advice surrounding dietary risk factors for ocular disease (Downie et al., 2017). A major limitation with generic clinical recommendations regarding omega-3 EFA intake (e.g., to eat oily fish at least twice a week) is that such advice does not take into consideration the substantial variability of omega-3 fatty acid concentrations across different species of fish. For example, a 100 g serving of salmon provides approximately seven-fold more long-chain omega-3 EFAs than that of 100 g of lean fish, such as whiting (Soltan & Gibson, 2008). In this regard, tailored questionnaires that are designed to specifically assess EFA intake may have the capacity to more accurately capture dietary intakes for specific nutrients (e.g., fatty acids) compared with generic questionnaires (i.e., questionnaires that assess total dietary intake but do not consider the specific fatty acid sources) (Meyer et al., 2013).

Several FFQs have been developed to assess dietary omega-3 intake (Overby et al., 2009; Serra-Majem et al., 2009), which range from long (Swierk et al., 2011) to short in length (Dahl et al., 2011; Lucas et al., 2009; Sublette et al., 2011). Questionnaires may perform differently in different geographic locations and patient populations, and as such, instruments should be validated by recruiting participants who are representative of the primary target population (Cade et al., 2002). In terms of relatively short, clinically applicable FFQs, Sublette et al. developed and validated a 21-item questionnaire, which took approximately five minutes to complete, administered with a sample of 61 US adults with and without major depressive disorders (Sublette et al., 2011). Dahl et al. developed and validated another brief, 10-minute questionnaire, administered with a sample of healthy Norwegian population (Dahl et al., 2011). A nine-item FFQ was developed based on the 2005 Canadian Nutrient File of Health Canada, conducted in a sample of women with low marine food intakes and with psychological stress (Lucas et al., 2009). It showed poor agreement

with an Australian-based FFQ, when administered in an Australian population, however, estimates of the nutritional intake improved when nutritional composition data were replaced with Australian equivalents (Dickinson et al., 2015).

We validated the CODS relative to both an erythrocyte biomarker and a previously validated food frequency questionnaire (DQES v3.2). Nutritional information provided by the DQES differed from the CODS as it comprehensively considers all food groups available in Australia, in order to create a full dietary profile of consumed nutrients. In contrast, the CODS only assesses foods that are rich in omega-3 EFAs. Information relating to long-chain omega-3 EFA intake was extracted from the DQES based upon food compositional data from the NUTTAB (2010) database (FSANZ, 2011), whereas dietary estimates for the CODS were extracted from the independent AUSNUT (2011–2013) database (FSANZ, 2014). Other potential dietary reference methods included 24-hour food recalls, and short-term and long-term food diaries. These techniques provided an alternative, but not necessarily more accurate, method of assessing dietary intake (Cade et al., 2002). The limitation of short-term food diaries is the day-to-day variation of foods consumed, and as such, a single observation may provide a poor measure of overall dietary intake (Overby et al., 2009). Furthermore, although the food records provided a precise measure of dietary habits and portion sizes over the short monitoring period (e.g., seven days), bias may be induced as dietary behaviors of a participant may be influenced over the capture period (Thompson & Byers, 1994).

The limitation of not capturing a baseline omega-3 status, which in practice may inform clinical recommendations, is similar to the confound of not quantifying this parameter in omega-3 EFA intervention trials, in order to consider this factor in the evaluation of therapeutic efficacy. For example, numerous clinical studies have sought to investigate the therapeutic efficacy of omega-3 EFA supplements for treating dry eye disease, with many reporting apparently

differing results (Asbell et al., 2018; Chinnery et al., 2017; Deinema et al., 2017; Jones et al., 2017). However, as recently reported in a Cochrane systematic review of 34 randomized controlled trials assessing the effect(s) of oral omega-3 and/or omega-6 supplements on dry eye symptoms and signs (Downie et al., 2019), the vast majority of studies to date have not surveyed (through food questionnaires) or quantified (e.g., through blood testing) baseline systemic fatty acid levels. It is critical to have an understanding of basal levels, as individuals already achieving sufficient levels of PUFAs from food sources may not demonstrate the same response to a given dose of fatty acid supplementation as those with a diet deficient in some, or all, PUFAs (Rice et al., 2016; Silva & Singer, 2015). The assessment of post-treatment omega-3 status is also a means of evaluating participant compliance (in addition to other measures such as returned capsule counts, compliance diaries, etc.), however, in this systematic review, it was only assessed in two of the included trials (Asbell et al., 2018; Deinema et al., 2017). While the quantification of baseline omega-3 EFA levels is recommended as a standard for cardiovascular trials (Rice et al., 2016), currently, there is no such guidance in relation to ocular studies. If the cost of biological assays is prohibitive, the findings of our study suggest that the CORDS may be a relevant surrogate marker for estimating dietary intake of long-chain omega-3 EFAs.

A potential advantage of using methods based on surveys, rather than biological biomarkers, to assess omega-3 EFA intake is the ability to disambiguate fatty acids consumed from food sources and nutritional supplements. This is of particular relevance to the clinical recommendations for omega-3 EFAs in the context of eye disease, where the source of omega-3 EFAs may influence clinical outcomes. For example, several epidemiological studies have shown that a high (food-sourced) dietary intake of omega-3 fatty acids is associated with a significantly reduced risk of developing age-related macular degeneration (AMD) (Chong et al., 2008; Tan et al., 2009), and a decreased risk of AMD progression in individuals with the established disease (Christen et al., 2011; Sangiovanni et al., 2009; Seddon et al., 2006). However, perhaps counter

intuitively, a Cochrane collaboration systematic review of randomized controlled trial evidence reported that the use of omega-3 fatty acid nutritional supplements, for a follow-up period of up to five years, did not reduce the incidence of progression to late-stage AMD or the development of moderate-to-severe vision loss, compared to placebo supplementation (Lawrenson & Evans, 2015). There are currently no randomized controlled trials on dietary omega-3 EFA supplementation for the primary prevention of AMD. Therefore, whole food sources containing high levels of long-chain omega-3 EFAs have been shown to be retinoprotective, whereas, omega-3 supplementation does not appear to confer the same benefit. The specific mechanism(s) underlying the benefits of omega-3 EFAs in AMD have not been established, but likely derive from the anti-inflammatory and/or anti-oxidative effects of omega-3 EFAs (Lawrenson & Evans, 2015), and the potential interaction of these fatty acids with other nutrients found in whole foods rich in these components.

The Omega-3 index is a measure of the relative concentration of EPA and DHA in erythrocyte membranes, and it is considered an acceptable marker for evaluating the risk of coronary heart disease (Harris, 2008). An association has been reported between Omega-3 indices <4% and a high incidence of cardiovascular disease; shifting the Omega-3 index from 4% to 8% is also estimated to reduce the relative incidence of fatal coronary heart disease by 30% (Harris et al., 2017). In the present study, we compared the DQES and CODS (survey-based) estimates of daily long-chain omega-3 EFA intake to the Omega-3 index quantified using the DBS biomarker assay. Total long-chain omega-3 EFA intake, estimated with both the CODS and DQES v3.2, showed a moderately strong, positive correlation with the Omega-3 index (CODS: $r_s = 0.39$, $p = 0.013$; DQES v3.2: $r_s = 0.37$, $p = 0.018$). Of note, on the basis of interpolation of the data shown in **Figure 4-4**, targeting a desired Omega-3 index of 8% corresponds to an intake of ~600 mg/day of long-chain omega-3 EFAs (Harris et al., 2017), as estimated using the CODS. Consistently, this estimate agrees with NHMRC SDT recommendations for lowering chronic disease risk (Meyer, 2016; NHMRC, 2006).

In general, the accuracy of dietary surveys depends on the recall ability of the participant and the accuracy and availability of nutritional composition data in the geographical region (Stark et al., 2016). We acknowledge a limitation of the CODS is that it does not consider the conversion of ALA and short-chain omega-3 fatty acid to long-chain metabolites, or the consumption of omega-6 fatty acids, being the other major class of PUFAs. Foods containing ALA from nutrition composition databases were not considered due to the heterogeneity in ALA concentrations reported across foods in the same groups (e.g., the amount of ALA present in margarine varies from 1 g/100 to 8 g/100 g, depending on the brand and type i.e., monounsaturated or polyunsaturated) (FSANZ, 2014), and the additional time that would have been required to complete a questionnaire that incorporated these food sources. The decision to omit ALA estimates from the CODS was also based on the known efficiency of conversion of short- to long-chain omega-3 fatty acid forms *in vivo*, which has been reported to range from 4–20% for EPA and estimated at 5–9% for both DPA and DHA (Brenna, 2002; Burdge & Wootton, 2002; Gerster, 1998). This conversion is further reduced in the presence of increasing circulating levels of omega-6 EFAs (Gerster, 1998). Long-chain omega-6 EFAs also competitively inhibit the incorporation of omega-3 EFAs into phospholipid membranes (Simopoulos, 2008). The poor validity coefficient between DPA intake estimated from survey methods and a biological biomarker has also been observed in other studies in the same region (Sullivan et al., 2006; Swierk et al., 2011), and may be related to the selective uptake of DPA in tissues (Kaur et al., 2011; Miller et al., 2013). DPA, being an intermediate metabolite, is also highly interconvertible with EPA and less readily metabolized to DHA (Kaur et al., 2011; Miller et al., 2013). These factors may contribute to the lack of correlation between the DPA estimate using CODS and the DBS biomarker assay. Nevertheless, we observed a significant correlation between the CODS and the DBS biomarker assay, for total long-chain omega-3 fatty acids, DHA, and EPA. Further validation of the CODS, in particular within a larger population of patients in clinical practice, would be of value to confirm the generalizability of these findings.

In this preliminary investigation, we demonstrated that the CODS is a potentially useful tool for assessing long-chain omega-3 intake, validated in a population of Australian adults, factoring in both food sources and dietary supplementation. In addition, the CODS estimated daily intake of long-chain omega-3 EFAs was moderately well correlated to the Omega-3 index, which is a validated marker for cardiovascular disease risk. We propose that the CODS could provide a rapid, non-invasive tool (as a no-cost alternative application as compared with more costly investigations) for evaluating the relative sufficiency of a patient's dietary omega-3 EFA intake in a clinical setting. Future directions will include repeatability assessment and validation of the CODS against another well-controlled dietary reference method (e.g., a multiple-day food record), in a larger, homogenous population of participants. These additional investigations will be of value for strengthening the rigor of the CODS, and deriving further data to support its utility for implementation in eye care practice.

Supplementary Materials (Appendix B): The following are available online at www.mdpi.com, Figure S1: The Clinical Omega-3 Dietary Survey, Figure S2: Diagram adapted from Ocke & Kaaks, to describe the method of triads (Ocke & Kaaks, 1997).

Author Contributions: Conceptualization, A.C.Z and L.E.D.; developing the methodology and collecting the data, A.C.Z.; supervised by L.E.D.; analysis was undertaken by A.C.Z and L.E.D.; writing, including original draft preparation, review and editing, were undertaken by A.C.Z and L.E.D.; funding acquisition, L.E.D.

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5

Chapter 5. The relationship between systemic omega-3 PUFA levels and corneal nerve structure and function

5.1 Introduction

The previous chapters have examined clinical practice patterns relating to omega-3 PUFAs and reported the development of a tool for assessing dietary omega-3 PUFA intake. At present, the clinical application of omega-3 PUFAs for ocular surface disease primarily focuses on reducing inflammation. In addition to these effects, pre-clinical models show systemic omega-3 PUFAs can be used to both prevent and treat corneal neuropathy in diabetes (Coppey et al., 2020). Furthermore, in dry eye disease, oral omega-3 PUFA supplementation (1500 mg/day) for three months imparted corneal nerve regeneration in a randomised controlled trial (Chinnery et al., 2017). However, it is currently unclear whether there is a relationship between systemic omega-3 PUFA levels and corneal nerve health.

Systemic omega-3 fatty acid levels can be quantified using the Omega-3 Index, which was first proposed as a therapeutic marker for cardiovascular health (Harris & Von Schacky, 2004). Shifting from an Omega-3 Index of 4% to 8% is estimated to reduce the risk of fatal heart disease by 30% (Harris et al., 2017). In overweight men, the Omega-3 Index was positively associated with insulin sensitivity, and negatively associated with nocturnal blood pressure and

C-reactive protein concentrations (Albert et al., 2014). A plausible relationship between the Omega-3 Index and peripheral nerve health, which to date has not been investigated in a clinical setting, could indicate a potential role for omega-3 PUFAs in modulating peripheral nerve health in diabetes.

In diabetes, degeneration of the corneal sub-basal nerve plexus, visualised using IVCN, occurs prior to clinically detectable symptoms and signs of diabetic sensorimotor polyneuropathy (DSP) (Breiner et al., 2014; Ziegler et al., 2014). An associated loss of corneal sensation might be expected to occur in parallel with anatomical sub-basal nerve loss; however, reduced corneal sensitivity is only consistently reported in populations with moderate-to-severe DSP, but not in those without DSP symptoms (Pritchard et al., 2014; Tavakoli et al., 2011a). If the pathophysiology of diabetes-associated corneal nerve damage in humans is similar to that in mice, corneal cold thermoreceptors, expressing TRPM8 receptors, could be preferentially affected in early diabetes (Alamri et al., 2019). Corneal cold thermoreceptors are activated by cooling (>0.5 °C relative to ocular surface temperature) and tear hyperosmolarity (>310 mOsm/L) (Alcalde et al., 2018). This chapter uses a modified model of Murphy's non-contact corneal aesthesiometer (Murphy et al., 1996), which enables corneal sensitivity to be tested using air stimuli at two temperatures: room-temperature (~ 23 °C) and cooled (~ 18 °C). In this chapter, corneal sensitivity thresholds to each temperature stimulus are correlated with corneal nerve structural parameters, to examine the structure-function relationship.

Changes to corneal sensory nerves are also potentially associated with immune-mediated responses (Gao et al., 2016). Corneal dendritic cell (DC) maturation has been postulated as an early indicator of immune activation in type 2 diabetes (Lagali et al., 2018). However, changes in DC density and morphology in diabetes without DSP symptoms is not well understood. It is also unclear whether this occurs differentially in the central and peripheral corneal regions. Along with corneal nerve architecture, the densities and morphologies of DCs in the sub-basal nerve plexus are examined in this chapter.

The aim of this study was to investigate central and peripheral corneal sensitivity and the associated corneal structure-function relationship in individuals with diabetes without neuropathy symptoms. Furthermore, we sought to investigate the association between systemic fatty acid levels and corneal morphological and functional parameters, in both healthy individuals and those with diabetes without symptomatic neuropathy.

5.2 Methods

5.2.1 Ethics

This project was approved by the University of Melbourne Human Research Ethics Committee (HREC #1749830.2) and performed in accordance with the principles of the Declarations of Helsinki. All participants provided written informed consent to participate, prior to any study related procedures.

5.2.2 Participant selection

This study was conducted in the Department of Optometry and Vision Sciences at the University of Melbourne (Victoria, Australia) between March 2018 and January 2019. Participants were eligible to participate if they were aged 18 years or over and either i) had self-reported type 1 or type 2 diabetes, or ii) were healthy with no uncontrolled systemic or ocular conditions. Peripheral neuropathy symptoms were assessed using the Norfolk Quality of Life-Diabetic Neuropathy (QoL-DN) questionnaire (Vinik et al., 2005b). Participants reporting neuropathy symptoms, using a cut-off score of 16 or above, were not eligible to participate (Vinik et al., 2005b). Participants with diabetes reported their most recent HbA1c values.

Participants meeting any of the following criteria were ineligible to participate: i) a known bleeding disorder; ii) a history of rigid contact lens wear; iii) a known allergy or previous hypersensitivity to any ocular agents required for the study (i.e., topical ocular anaesthetic, sodium fluorescein dye, lissamine green dye);

iv) any of: active ocular inflammation or infection, history of recurrent herpetic keratitis or active disease within six months of baseline, corneal disorders or abnormalities that may affect the integrity of the corneal nerves; v) any of: active ocular infection or active ocular surface inflammation except dry eye; vi) a diagnosis of neuropathy from any cause other than diabetes; vii) best spectacle corrected visual acuity of worse than 6/12 in either eye; viii) women who were pregnant or breastfeeding.

Participants who wore soft contact lenses were asked to refrain from lens wear for at least 24 hours before examinations. Participants using lubricating eye drops were asked to not instil these for at least two hours before examination procedures.

5.2.3 Clinical examinations

5.2.3.1 Ocular Surface Disease Index (OSDI) Questionnaire

The OSDI, a 12-item questionnaire, was used to capture the presence and severity of symptoms relating to dry eye disease, as well as impact on daily function (Schiffman et al., 2000). Each question answered was scored from 0 (none of the time) to 4 (all of the time). The OSDI score is calculated as $[(\text{the sum of scores for all questions answered} \times 100)/(\text{total number of questions answered} \times 4)]$. The total score ranges from 0 (no disease) to 100 (maximum severity of disease).

5.2.3.2 Norfolk quality of life-diabetic neuropathy (Norfolk QOL-DN)

Participants' self-reported symptoms of diabetic neuropathy were captured using the Norfolk QoL-DN, a validated, 47-item questionnaire (Vinik et al., 2005b). This two-part questionnaire assesses the symptoms experienced by the patient, and the impact of the patient's neuropathy on activities of daily life. The impact of diabetic neuropathy is assessed in five domains: activities of daily living, symptoms, small fibre neuropathy, large fibre neuropathy, and autonomic

neuropathy. A score of 16 and above identifies diabetic neuropathy with 79% sensitivity and 85% specificity (Vinik et al., 2005b).

5.2.3.3 Habitual visual acuity

Habitual visual acuity was measured using a Snellen visual acuity chart calibrated for testing at three metres. If the habitual visual acuity in each eye was equal to or better than 6/12, this correction was used. If the habitual visual acuity in either eye was worse than 6/12, subjective refraction was performed to obtain the best spectacle corrected visual acuity in each eye.

5.2.3.4 Ocular surface examination

5.2.3.4.1 Tear osmolarity

Tear osmolarity was measured using the TearLab™ Osmolarity System (TearLab™ Corp., San Diego, CA). Prior to testing, appropriate instrument calibration was performed, including both monthly (using hyperosmolar control solutions) and daily (using electronic test cards) procedures, as per manufacturer recommendations.

Measurements were taken once in each eye, beginning with the right eye. Immediately after a blink, a 50 nL tear sample was collected from the inferior temporal tear meniscus approximately one-third of the distance from the lateral canthus, without making conjunctival or eyelid contact. If the testing pen touched any eyelashes, the testing card was discarded, and the measurement was repeated in that eye. Following sample collection, the test pen was returned to the cradle to obtain the tear osmolarity reading from the device (in mOsm/L). Readings from both eyes were recorded; the higher reading from the two eyes was used for analysis. Interocular differences were calculated for each participant for the purpose of deriving the presence of dry eye disease in accordance with the TFOS DEWS II diagnostic criteria (Wolffsohn et al., 2017).

5.2.3.4.2 Slit lamp examination

5.2.3.4.2.1 Ocular surface examination

Slit lamp biomicroscopy was performed in both eyes using white light and 10-16X magnification. The Efron grading scale was used for grading: i) anterior blepharitis; ii) meibomian gland dysfunction; iii) conjunctival redness (temporal and nasal); iv) limbal redness (Efron, 1998; Efron, 2012). Grades ranged from 0 to 4, and were judged in 0.1 step increments for increased clinical sensitivity (Bailey et al., 1991). Clinically notable findings for the tear film, cornea, conjunctiva, and the anterior chamber were also recorded.

5.2.3.4.2.2 Tear stability

Tear break-up time (TBUT) was measured using sodium fluorescein strips (Amcon dry eye tests, DET Nomax Inc., St Louis MO) in both eyes. Individual strips were moistened with non-preserved saline, shaken to remove excess fluid, and applied onto the inferior bulbar conjunctiva to deliver an approximate fluid volume of 1 μ L to each eye. A single instillation of sodium fluorescein was used for both TBUT assessment and corneal staining.

TBUT was assessed three times in each eye using the cobalt blue light illumination and a yellow Wratten filter on a slit lamp biomicroscope at 10X magnification. Participants were first asked to blink normally two to three times, then to refrain from blinking for as long as they could tolerate. Using a stopwatch, timing started immediately after blink and stopped on the first occurrence of an observed true tear break up (i.e., not just local thinning or tear film irregularity). An average of three measures from each eye was recorded.

5.2.3.4.2.3 Corneal and conjunctival staining

Corneal fluorescein staining was evaluated in each eye immediately after TBUT measurements, using cobalt blue illumination and a Wratten yellow barrier filter. Corneal fluorescein staining was graded using the five-point Oxford scale for a single area, ranging from 0 to 4 (Bron et al., 2003), in 0.1 increments.

Conjunctival staining using lissamine green strips (GreenGlo, HUB pharmaceuticals, CA, USA), instilled in both eyes, was examined under white light. Individual strips were moistened with non-preserved saline, and a sizable drop (~20-40 μ L) was applied onto the inferior bulbar conjunctiva in each eye. Conjunctival staining was graded using the 5-point Oxford scale separately for temporal and nasal conjunctiva in both eyes.

5.2.3.5 Non-contact corneal aesthesiometry

5.2.3.5.1 Instrument

A custom non-contact corneal aesthesiometer (SDZ Aesthesiometer, SDZ Electronics, Auckland, New Zealand), designed and constructed based on the prior work of Murphy, Patel, and Marshall, was used to measure corneal sensation thresholds (Murphy et al., 1996). The slit lamp-mounted instrument has a brass stimulus jet with a 0.5 mm bore that delivers an air-stimulus (ranging from 0.1 to 5.0 mbar) over an approximate corneal area of 0.8 mm². The stimulus presentation is 0.9 seconds in duration and varies in increments of 0.1 mbar. Lower threshold values indicate higher corneal sensitivity. Similar corneal aesthesiometry instruments have been described in the literature and validated (Murphy et al., 1998; Swaneveldt et al., 2019).

The aesthesiometer was modified from Murphy's model with a built-in cooling device, to allow for the release of air stimuli at room temperature (23-24 °C) and cooled temperature (18-19 °C). A built-in video camera, located inferior to the bore, allowed for monitoring of participants' blink and eye movements during testing (**Figure 5-1**). Markers on the camera screen allowed for accurate instrument alignment for all measurements. Test-retest repeatability of the corneal sensitivity measurements derived from the modified NCCA was evaluated; findings are presented in **Appendix C**.

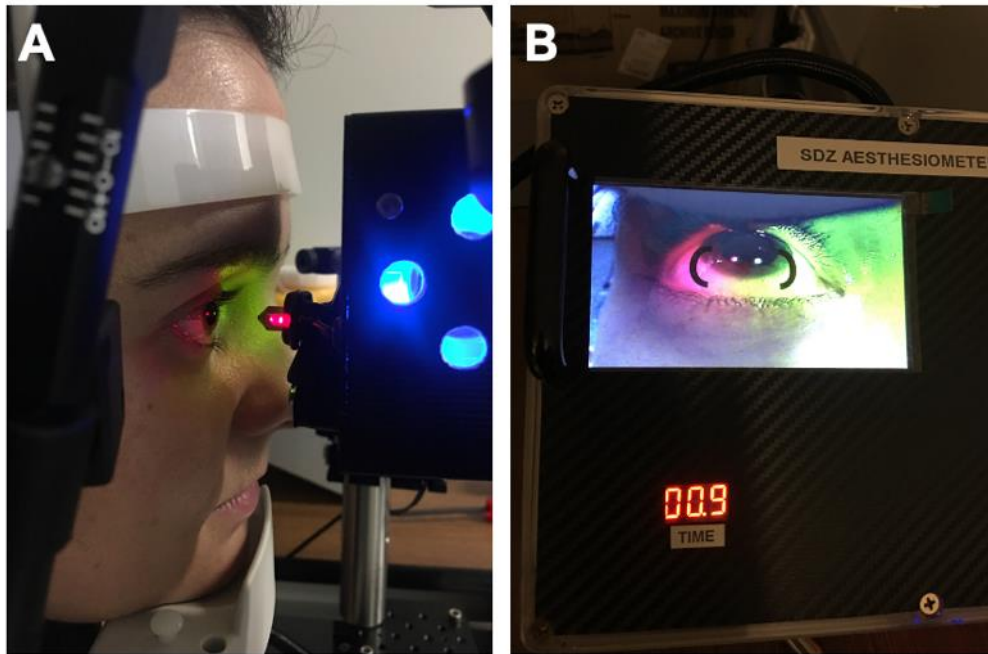


Figure 5-1. Non-contact corneal aesthesiometer. A) Position of the instrument bore for release of air stimulus relative to the participant positioned on the slit-lamp. B) Display of the built-in camera for monitoring of participants' eye movements and position during corneal sensitivity assessment of the peripheral cornea.

5.2.3.5.2 Testing procedure

Corneal sensitivity thresholds were measured using a double-staircase testing protocol with a forced-choice selection (i.e., 'felt' and 'not felt'). An algorithm for the double-staircase was written in MATLAB (MATLAB, version 9.2 (R2017a), The MathWorks Inc. Natick, MA, USA). For the purpose of this study, a 'trial' was defined to involve testing of a single stimulus. The final sensitivity threshold was taken as the average threshold after three reversals (for each staircase), ignoring the first reversal. Corneal sensitivity thresholds were measured for both the central and peripheral cornea with room-temperature and cooled stimuli.

To account for the lowest measurable threshold (i.e., instrument floor), the algorithm entered a decision tree (see **Appendix C**) if two consecutive detections were detected at the lowest possible stimulus level (0.1 mbar). In the decision tree, false-positive testing was delivered in combination with stimuli at 0.1 mbar. For false-positive testing, the examiner would set up the trial but not press the release button (which was obscured from the participant). In the

decision tree, if the participant responded 'felt' to two stimuli at 0.1 and 'not felt' to two false-positive checks (i.e., trials with no stimulus), the threshold was recorded as "<0.1 mbar". If the participant responded 'felt' to one of the two stimuli at 0.1 mbar and none of the false positive checks, the final threshold was recorded as 0.1 mbar. If the participant responded 'felt' to both false-positive checks, the test was deemed unreliable. For unreliable tests, testing was stopped, participants were re-instructed, and the entire staircase was repeated for that measurement.

An eight-second audio cue recording was developed and used for each trial. The recoding had a white-noise background to mask any surrounding noise that could potentially confound responses. Participants were instructed to blink a few times upon hearing a short beep (at 2 seconds), before keeping their eyes open for a subsequent long, continuous beep (at 5 seconds, lasting 3 seconds). The audio cue was played to each participant via noise-cancelling headphones (Philips Active Noise Cancelling headphones, SHL3850NC, China). The timing of the cues was indicated to the examiner via an accompanying, linked video.

Participants were instructed that for each trial they may, or may not, detect a stimulus, at any time during the long continuous beep, and that not every trial would necessarily generate a stimulus. For each trial, participants verbally indicated either 'felt' or 'not felt' immediately after the recording ended.

5.2.3.5.3 Threshold determination

The right eye of all participants was assessed, except for two participants who had histories of unilateral ocular injuries in the right eye; in these participants the left eye was assessed.

The determination of corneal sensitivity threshold was performed in a quiet, temperature- and humidity-controlled room (20 ± 30 °C; with a humidity of $50 \pm 5\%$), under standard room illumination. Ceiling vents were partitioned from the testing area to prevent any extraneous air flow affecting measurement

accuracy. The participant was positioned comfortably at the slit lamp and instructed to fixate on a central target using the non-testing eye. The bore tip of the aesthesiometer device was placed 10 mm from the anterior corneal surface, in line with the corneal apex, and the limbal markers on the camera screen were positioned to align with the temporal and nasal limbus (**Figure 5-1**).

Testing commenced from a predetermined threshold in each staircase. A supra-threshold stimulus was first shown to demonstrate the presence of a detectable stimulus and to determine the starting threshold. Subsequent stimuli levels were randomised according to the double-staircase algorithm. Trials were repeated until a final threshold for the central cornea was determined.

For the peripheral inferior cornea, participants fixated on a superior location. The testing bore tip was positioned 1 mm above the inferior limbus (aligned using the built-in camera), at a 10 mm distance from the cornea.

The instrument's cooling mechanism was then activated for three minutes, as per manufacturer's instructions, to allow for the release of a cooled air stimulus (approximately 18-19 °C). Corneal sensitivity threshold measurements were repeated for both the central and peripheral cornea to the cooled stimulus.

Evaluation of the test-retest repeatability of corneal sensitivity measurements (**Appendix C**) showed that corneal sensitivity threshold measurements are repeatable for assessments evaluated on different days, at a different time, or both. Mean inter-condition difference between measurements for all comparisons was less than 0.05 mbar, which is half of the minimum step size of the instrument (of 0.1 mbar).

5.2.3.6 Corneal *in vivo* confocal microscopy

5.2.3.6.1 Examination procedure

Corneal *in vivo* confocal microscopy (IVCM) was performed with the Heidelberg Retinal Tomograph III with Rostock Corneal Module (Heidelberg Engineering

GMB, Dossenheim, Germany). The instrument uses a 670 nm red wavelength Helium-Neon diode laser source and is equipped with a 63x immersion objective lens with a numerical aperture of 0.9 (Olympus, Tokyo, Japan). Captured images represent a field of view of 400 x 400 μm , over 384 x 384 pixels.

A large, bubble-free droplet of viscous, hydroxypropyl methylcellulose 2.5% (GenTeal gel; Novartis Ophthalmics, East Hanover, NJ) was placed on the objective lens tip, and a disposable, sterile polymethylmethacrylate cap (TomoCap; Heidelberg Engineering GmbH, Heidelberg, Germany) was mounted over the objective lens, coupled by the gel. The depth of the microscope measurement was then calibrated, to focus on the anterior surface of the TomoCap, prior to imaging.

The same eye of the participant was examined for both the NCCA and IVCM procedures, with NCCA measurements conducted first. For IVCM, topical anaesthetic (Proxymetacaine hydrochloride, 0.5%, Alcon laboratories, NSW, Australia) was applied to both eyes to aid comfortable fixation, by the non-testing eye on a central fixation target. This same target, placed at a fixed distance of 135 cm from the anterior surface of the Tomocap, was used for all participants. A side-mounted camera allowed the examiner to manually position the objective lens on the central cornea.

The objective lens of the IVCM device was slowly advanced until the Tomocap made gentle contact with the central corneal apex, guided by the red-laser centration beam. The examiner focussed the objective lens until the corneal epithelial sub-basal nerve plexus became visible. This required depth plane was typically 40-50 μm from the anterior surface of the Tomocap.

Participants were then asked to progressively re-fixate across 12 fixed locations, arranged in a 3-down and 4-across matrix with the corneal apex as the reference point. Starting from the top left corner of the fixation target sequence, multiple video sequences were captured over an approximate area of 1800 μm^2 .

During image capture, the examiner applied a constant pressure between the Tomocap and the cornea to minimise compression lines in the captured image.

For the peripheral cornea, participants were instructed to maintain fixation on a similar target that was located 60 cm higher than the central fixation target. The reflection of the red-laser light was adjusted to 1mm above the inferior limbus. Participants were asked to re-fixate across the 12 fixation points, and multiple video sequences were captured using the same method as for the central cornea. Images in the peripheral cornea were captured from an area 1-1.5 mm above the inferior limbus. A total of 300-600 images were captured in each corneal region, from each participant. Continuous video mode was used, as opposed to section mode, to capture a wider field from each fixation point. Using this method, the overlap between images from adjacent fixation points can then be minimised.

5.2.3.6.2 Image analysis

5.2.3.6.2.1 Corneal nerves

Images that were blurred, had an imaging depth that was inconsistent, or had vignetting effects that obstructed nerves were removed from the potential analysis sample pool. No post-capture image enhancements (e.g., brightness, sharpening) were performed.

Twelve images of the central cornea and eight images of the peripheral cornea were randomly selected for each participant using a random number generator (Microsoft Excel, v16.0, Microsoft Corporation, Redmond, WA, UA); selected images were visually checked to ensure any overlap was minimal (< 20%). As reported by Vagenas *et al.*, the average value for corneal nerve fibre length (CNFL), estimated from a minimum of eight randomly selected IVCM images overlapping by less than 20%, will fall, with 95% confidence, within 13% of the true mean, and for CNBD, within 30% of the true mean (Vagenas *et al.*, 2012).

Central corneal SBNP parameters were quantified using automatic image analysis software (ACCMetrics v2; University of Manchester; UK) (**Figure 5-2**) (Dabbah et al., 2011). Although automated quantification is recognised to underestimate CNFL compared with manual quantification (Dehghani et al., 2014a), the method is consistent and objective, and, thus, not prone to observer bias. Furthermore, CNBD evaluation using manual quantification has been shown to have low inter- and intra-observer repeatability (Petropoulos et al., 2013).

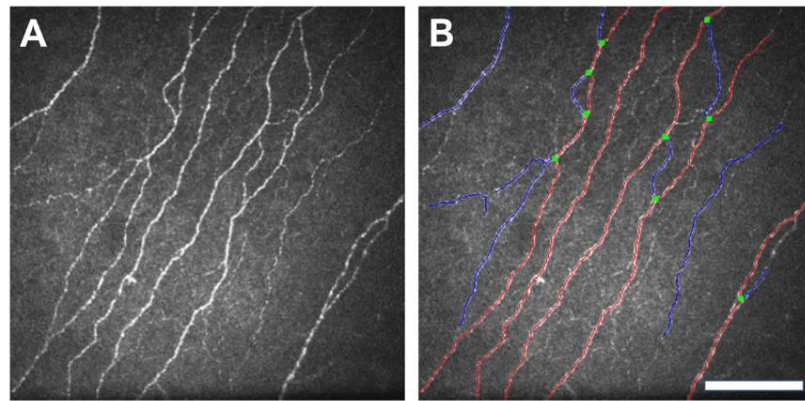


Figure 5-2. Corneal sub-basal nerve plexus assessment. (A) *In vivo* confocal microscopy image. (B) Annotated image assessed using the ACCMetrics software (Dabbah *et al.*, 2011). Red=main nerve fibre. Blue=nerve branch. Green=branch point. Scale bar=100 μm .

Table 5-1 shows the corneal nerve metrics obtained from the automated image analysis (ACCMetrics software):

Table 5-1. Main corneal sub-basal nerve plexus parameters quantified using ACCMetrics

Parameter	Unit	Definition (Petropoulos et al., 2013)
Corneal nerve fibre length (CNFL)	mm/mm ²	The total length of all nerve fibres in the image capture frame
Corneal nerve fibre density (CNFD)	fibres/mm ²	The total number of main fibres divided by the area of the image frame
Corneal nerve branch density (CNBD)	branches on main fibre/mm	The total number of main nerve branches that stem directly from a nerve fibre, divided by the area of the image frame
Corneal nerve total branch density (CTBD)	total branches/mm	The total number of branches within the area of the image frame

Peripheral CNFL was quantified using the NeuronJ plugin on FIJI (ImageJ; US National Institutes of Health, Bethesda, Maryland, USA) (Meijering et al., 2004; Schindelin et al., 2012). This method minimises measurement errors in the peripheral cornea where nerves are thinner and fainter, and avoids mistaking of putative DCs with longer extensions (dendrites) for nerve structures (Dehghani et al., 2014a). The beginning of the nerve of interest was located by the examiner, and the tracing algorithm was set to automatically compute the “optimal” path of the nerve. The examiner manually guided the nerve tracing in areas of low contrast to ensure corneal nerves were accurately captured. CNFL (in mm/mm²) was calculated as the sum of total nerve length (in mm) divided by 0.16 mm², the area of the IVC image.

5.2.3.6.2.2 Dendritic cells

The evaluation of immune cells in the central and peripheral cornea was performed from the same set of randomly selected images as for corneal nerve measurements, using the cell count plugin on FIJI (<https://imagej.net/plugins/cell-counter.html>; ImageJ; US National Institutes of Health, Bethesda, Maryland, USA) (Schindelin et al., 2012). These cells were classified putatively as DCs, identified as highly reflective bodies, with or without associated dendrites, in the corneal sub-basal nerve plexus.

There is no universally-accepted corneal DC classification system for stratifying cellular phenotypes identified using IVC. Ferdousi *et al.* classified cells <50 µm in length without associated dendritic structures to be ‘immature’ DCs, and cells >50 µm in length and associated dendritic structures to be ‘mature’ DCs (Ferdousi et al., 2019). In contrast, both Mastropasqua *et al.* and Lagali *et al.* considered mature DCs as slender cells with evidence of individual dendritic processes >25 µm in total end-to-end length, and immature DCs as cells with corpuscular cell bodies and shorter or no processes, up to 25 µm in end-to-end length (Lagali et al., 2018; Mastropasqua et al., 2006).

In the current study, corneal DCs were classified into three morphological phenotypes (**Table 5-2**), as described by Lagali *et al.* (Lagali *et al.*, 2018):

Table 5-2. Definition of corneal dendritic cell morphological phenotypes

Putative dendritic cell subtype	Description
Mature dendritic cells (mDCs)	Slender bodies and extensions of long-arm-like processes (dendrites), >25 μm in total end-to-end length.
Immature dendritic cells (imDCs)	Small, reflective bodies, either with short-dendrites or without discernible dendrites, with end-to-end length of <25 μm .
Globular cells	Larger than DCs and have round or oval cells bodies and no visible dendrites.

Definitions based upon Lagali *et al.* (2018)

These morphological phenotypes are demonstrated in **Figure 5-3**.

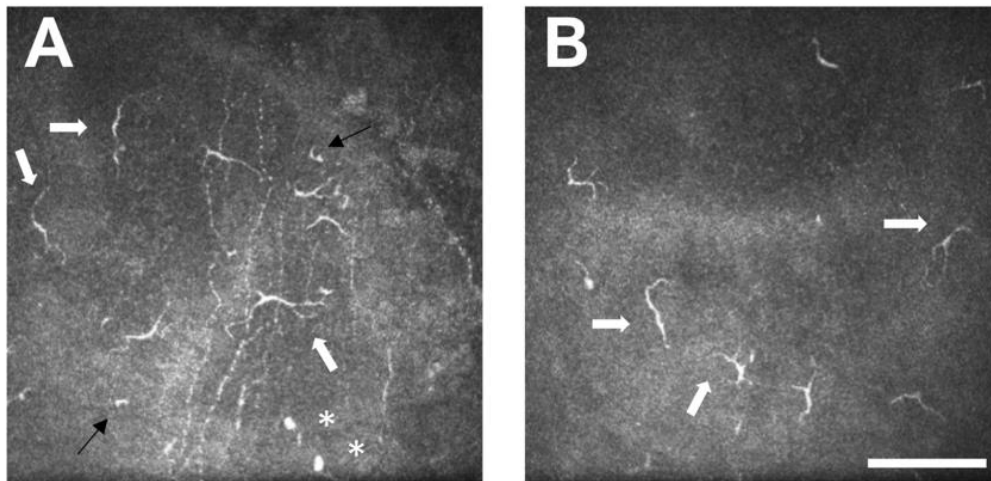


Figure 5-3. Dendritic cells in the sub-basal nerve plexus of the peripheral cornea, captured using IVCN, in a: A) healthy participant, and B) participant with diabetes. White arrows indicate mature DCs with long dendrites; black arrows indicate immature DCs with small cell bodies and short dendrites; and white asterisks indicate globular cells, with larger, rounded cell bodies and no visible dendrites. Scale bar=100 μm

Appendix D describes the assessment of the intra- and inter-observer agreement in DC quantification for total DC counts and by cell morphology.

5.2.3.7 Systemic omega-3 fatty acid testing

Systemic fatty acid profile analyses were undertaken using dried blood spot (DBS) testing methods, as described in **Chapter 4**. Analyses were performed by an independent laboratory (Waite Lipid Analysis Service (WLAS), University of Adelaide, South Australia, Australia) using previously established methods (Liu et al., 2014). In brief, a finger-prick method was used to obtain capillary blood samples, according to manufacturer's instructions. Approximately 3 mL of capillary blood was collected and spotted onto the proprietary PUFAcoat™ test cards. The cards were air dried, placed in sealed cellophane bags and stored with desiccants in a dark, temperature-controlled chamber (21-22 °C). All DBS testing kits were labelled with the participant's study identification code, as per the manufacturer's instructions, and sent for laboratory analyses within four weeks of collection.

For the DBS testing method, **Table 5-3** shows the coefficients of variation for omega-3 and omega-6 PUFAs, calculated using repeated analysis data in the manufacturer's published study (Liu et al., 2014).

Table 5-3. Coefficient of variation for omega-3 and omega-6 polyunsaturated fatty acids, for the systemic fatty acid analysis, calculated from published data by Liu *et al.* (2014)

	Fatty acid concentration (%) calculated for each triplicate*	Coefficient of variation (%)
Linoleic acid (LA; 18:2n-6)	19.8 ± 0.2	1.0
Arachidonic acid (AA; 20:4n-6)	7.4 ± 0.1	1.35
Alpha-linolenic acid (ALA; 18:3n-3)	0.4 ± 0.01	2.5
Eicosapentaenoic acid (EPA; 20:5n-3)	6.3 ± 0.1	1.59
Docosahexaenoic acid (DHA; 22:6n-3)	6.8 ± 0.1	1.47

Data are presented in mean (SD), as percentage of total blood fatty acids.

*Data for triplicates are extracted from Table 2 by Liu *et al.* (2014)

5.2.4 Statistical analysis

Sample size was calculated using CNFL, based on data from a published study that used automated IVCN image analysis (Petropoulos et al., 2014). Using an estimated difference in population means between control and diabetes groups for CNFL of 4.1 mm/mm², for 80% power at a confidence level of 95% and an estimated standard deviation of 4.5 mm/mm², 20 participants were calculated to be required in each study group.

Demographic characteristics and corneal nerve parameters were compared using R for statistical computing version 4.0.0 (R Core Team, 2019). As the study population was included based on the absence of DSP symptoms, assessment of corneal nerve parameters combined data from participants with both diabetes subtypes to ensure that groups are age-matched and to retain adequate statistical power. For evaluation of DCs and the relationship between corneal nerve parameters and systemic fatty acid profiles, additional analyses were performed separating type 1 and type 2 diabetes to evaluate whether outcomes were influenced by diabetes subtype.

For corneal sensitivity thresholds, sub-threshold values (<0.1 mbar) were replaced by the lower limit divided by two (i.e., 0.05 mbar), based upon the approach recommended by Beal (Beal, 2001). The presence of dry eye disease was classified dichotomously based on the TFOS DEWS II diagnostic criteria: OSDI score ≥ 13 and one of: i) tear osmolarity ≥ 308 mOsm/L in either eye or an interocular difference >8 mOsm/L; ii) non-invasive TBUT <10 seconds; or iii) clinical signs of >5 corneal spots with sodium fluorescein or >9 conjunctival spots with lissamine green (interpreted as grade 1.5 or higher using the Oxford grading in the present study) (Wolffsohn et al., 2017).

For measures of anterior ocular surface parameters, the average value of data from both eyes was used to provide inter-group comparisons of ocular surface inflammation. For corneal structure and function parameters, data for each participant were captured and analysed from one eye only (as specified in

5.2.3.5). Data normality was assessed using the Shapiro-Wilk test. Descriptive statistics are summarised as mean $\pm$ standard deviation (SD) for normally distributed data, or median (inter-quartile range, IQR) for non-normally distributed data. Inter-group comparisons were analysed using either the independent sample t-test for normally distributed variables, or the Mann-Whitney-U test for non-normally distributed variables. Fisher's exact test (two-tailed) was used to compare frequencies of categorical variables between groups. Paired testing was used for comparing central vs peripheral corneal parameters within the same individual.

The association between CNFL and corneal sensitivity thresholds was investigated, for normally distributed data using Pearson's correlation coefficient (R), and for non-normally distributed data using Spearman's correlation coefficient (ρ). Outliers were evaluated as observations lying $\geq 1.5 \times \text{IQR}$ from the mean (Hubert & Vandervieren, 2008). Outlier values were verified against the source data; values not attributing to data or measurement errors were retained in the dataset. Analyses were verified to ensure that significant results were not due to the presence of any outliers. For all comparisons, an alpha value of 0.05 was adopted to define statistical significance.

The relationship between systemic PUFA levels and corneal nerve parameters was evaluated using multiple linear regression (IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp.), adjusted for age, sex, neuropathy symptom score, presence of diabetes, and presence of dry eye disease. For the diabetes group, univariate models examined whether diabetes duration, diabetes type and HbA1c were factors influencing the dependent variables to determine model selection. For dependent variables that were not normally distributed, a log-transformation was performed prior to model fitting. For corneal sensitivity thresholds as the dependent variable, tear osmolarity was incorporated as an additional predictor, as a factor potentially affecting corneal sensation (Alcalde et al., 2018).

5.3 Results

5.3.1 Participant characteristics

Table 5-4 summarises the demographics, ocular surface parameters, and systemic fatty acid profiles of the study population. Eligible participants included 26 individuals with diabetes and 21 age- and sex-matched controls.

Table 5-4. Participant demographic and clinical characteristics

	Control (n=21)	Diabetes (n=26)	p-value
Demographics			
Age, years	48 (26–64)	49.0 (30–63)	0.86
Sex, F/M	14/7	15/11	0.56
HbA _{1c} , %	-	7.0 (6.6–8.0)	-
Diabetes duration, years	-	13 (6–22)	-
Norfolk QoL-DN score, /156	1.0 (0.0–2.0)	4.5 (1.0–7.8)	0.032
Ocular surface parameters			
Diagnosis of DED, n (%)	2 (9.5)	4 (15.4)	0.68
OSDI score, /100	6.7 ± 7.1	7.4 ± 8.0	0.76
Tear osmolality, mOsm/L*	299.7 ± 6.9	301.9 ± 10.81	0.42
Anterior blepharitis, /4.0 [†]	0.50 (0.30–1.50)	0.88 (0.45–1.71)	0.34
MGD, /4.0 [†]	0.70 (0.40–1.45)	0.80 (0.53–1.51)	0.70
Conjunctival redness, /4.0 [†]	0.83 (0.70–1.03)	0.80 (0.59–1.05)	1.00
Limbal redness, /4.0 [†]	0.35 (0.25–0.65)	0.43 (0.25–0.75)	0.52
TBUT, seconds	10.40 (8.94–11.66)	7.40 (5.80–8.97)	0.0007
Corneal staining, /4.0 [‡]	0.15 (0.10–0.40)	0 (0–0.19)	0.039
Conjunctival staining, /4.0 [‡]	0.15 (0.03–0.30)	0.05 (0–0.28)	0.47
Systemic fatty acid profiles			
Total omega-6, %	28.55 ± 5.44	22.58 ± 5.04	0.0003
Total AA (22:4n-6), %	6.77 ± 1.50	5.61 ± 1.63	0.016
Total omega-3, %	4.90 (3.91–5.07)	4.21 (3.49–4.97)	0.36
Total EPA (20:5n-3), %	0.60 (0.45–0.73)	0.62 (0.51–0.82)	0.52
Total DHA (22:6n-3), %	2.05 (1.88–2.60)	1.96 (1.49–2.31)	0.26
Omega-3 Index, %	5.54 (4.54–6.06)	5.00 (4.25–5.68)	0.32
Omega-6:omega-3 ratio	6.69 (5.09–7.67)	5.27 (4.49–6.36)	0.093

Data are in mean ± SD or median (IQR) as appropriate, unless otherwise indicated. *Shown as the highest value of the two eyes. [†]Using the Efron Grading Scale. [‡]Using the Oxford grading scale. **Abbreviations:** AA, arachidonic acid; EPA, eicosapentaenoic acid; DED, dry eye disease; DHA, docosahexaenoic acid; HbA_{1c}, glycosylated haemoglobin; MGD, meibomian gland dysfunction; Norfolk QoL-DN, Norfolk Quality of Life–Diabetic Neuropathy questionnaire; OSDI, Ocular Surface Disease Index questionnaire; TBUT, tear break-up time.

All participants (n=47) scored less than 16 on the Norfolk QoL-DN questionnaire. Overall, the diabetes group had higher neuropathy symptoms scores compared with the control group; however, this statistically significant difference is not deemed clinically significant (Vinik et al., 2005b). There were no significant intergroup differences for age or sex. The diabetes group included 16 participants with type 1 diabetes and 10 participants with type 2 diabetes. The median duration of diabetes was 13 (IQR: 6-22) years. Participants with type 1 diabetes were younger than those with type 2 diabetes (median [IQR]: 31 [24–56] vs 61 [54–70] years; $p=0.005$). There was no difference between type 1 and type 2 diabetes for disease duration (type 1 diabetes: 17 [7–27] vs type 2 diabetes 10 [7–18] years; $p=0.53$) or self-reported HbA1c (type 1 diabetes: 7.5% [6.1–8.1%] vs type 2 diabetes (7.0% [6.7–7.7%]; $p=0.28$).

With respect to anterior eye parameters, overall, the diabetes group had lower TBUT and higher corneal staining compared with controls (**Table 5-4**). However, the proportion of individuals classified as having DED, based upon the TFOS DEWS II criteria (Wolffsohn et al., 2017), was similar in both groups.

Total omega-3 fatty acid profiles were similar in both groups. One person in the diabetes group reported taking oral omega-3 PUFA supplements (2000 mg/day); their Omega-3 Index was in the upper quartile. The diabetes group had lower concentrations of both total omega-6 PUFAs and AA; however, the omega-6 to omega-3 ratios were similar between groups.

5.3.2 Corneal structure and function

Figure 5-4 shows representative IVCN images of study cohort.

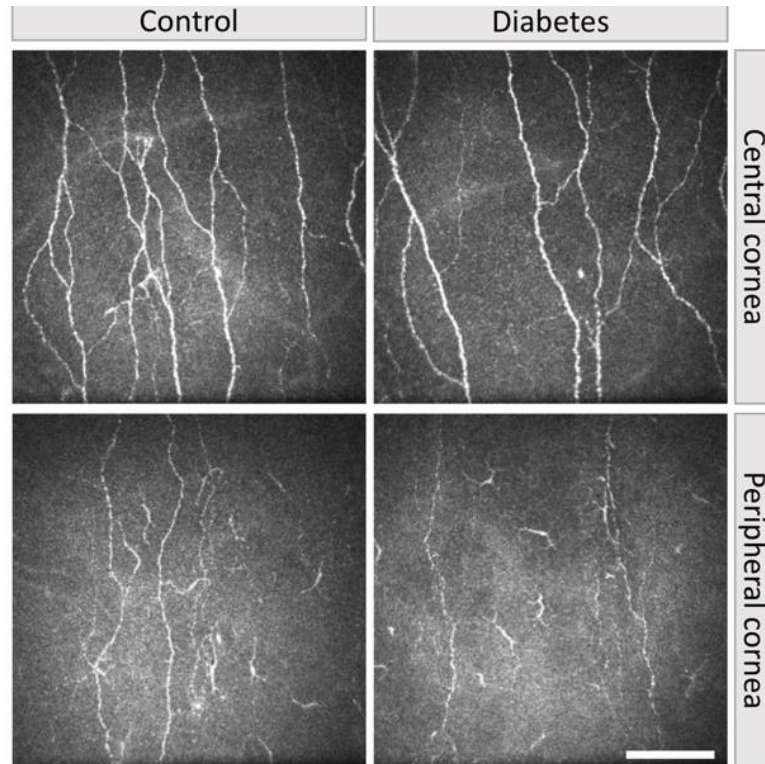


Figure 5-4. Representative central and peripheral corneal sub-basal nerve plexus images captured by IVCN, for individuals with and without diabetes. Scale bar=100 μ m

Table 5-5 and **Table 5-6** shows intergroup comparisons of corneal sub-basal nerve plexus parameters, and corneal sensitivity thresholds, respectively.

Table 5-5. Corneal sub-basal nerve plexus parameters quantified from *in vivo* confocal microscopy images

	Control (n=21)	Diabetes (n=26)	p-value
Central cornea			
CNFL (mm/mm ²)	14.71 $\pm$ 2.99	12.60 $\pm$ 3.41	0.030
CNFD (nerves/mm ²)	24.67 $\pm$ 6.05	20.99 $\pm$ 6.67	0.054
CNBD (branches/mm ²)	35.00 $\pm$ 12.57	23.60 $\pm$ 11.45	0.002
CTBD (total branches/mm ²)	50.88 $\pm$ 18.72	35.48 $\pm$ 16.49	0.005
Peripheral cornea			
CNFL (mm/mm ²)	5.00 $\pm$ 2.84	4.42 $\pm$ 2.39	0.46

Data are reported as mean $\pm$ SD. **Abbreviations:** CNBD; corneal nerve branch density CNFD, corneal nerve fibre density; CNFL, corneal nerve fibre length; CTBD, corneal nerve total branch density.

Table 5-6. Corneal sensitivity thresholds measured using non-contact corneal aesthesiometry

Stimulus	Central cornea			Peripheral cornea		
	Control (n=21)	Diabetes (n=26)	p- value	Control (n=21)	Diabetes (n=26)	p- value
Room- temperature (mbar)	0.28 (0.18–0.35)	0.59 (0.42–0.75)	<0.001	0.33 (0.25–0.40)	0.56 (0.43–0.80)	<0.001
Cooled (mbar)	0.15 (0.05–0.23)	0.48 (0.35–0.69)	<0.001	0.25 (0.05–0.30)	0.46 (0.36–0.54)	<0.001

Data are reported as median (IQR).

Central corneal sub-basal plexus nerve parameters were lower in the diabetes group than in the control group for: CNFL, CNBD, and CTBD. There was no inter-group difference for central CNFD or peripheral CNFL (**Table 5-5**).

Central corneal sensitivity thresholds were higher in the diabetes group compared with controls for both room-temperature and cooled stimuli. The same difference was observed, in the peripheral cornea, to stimuli of both temperatures (**Table 5-6**). Although corneal sensitivity thresholds to the cooled stimulus were generally lower than those for stimuli at room-temperature, a significant inter-temperature difference was found only in the control group for the central cornea ($p=0.030$). Within the diabetes group, corneal sensitivity thresholds to both temperatures were similar in all corneal regions ($p>0.05$ for both comparisons). There was also no difference between central and peripheral corneal sensitivity thresholds for stimuli at a given temperature.

Corneal structure-function relationship

Figure 5-5 shows the relationship between CNFL and corneal sensitivity thresholds to both room-temperature and cooled stimuli, in the central and peripheral cornea.

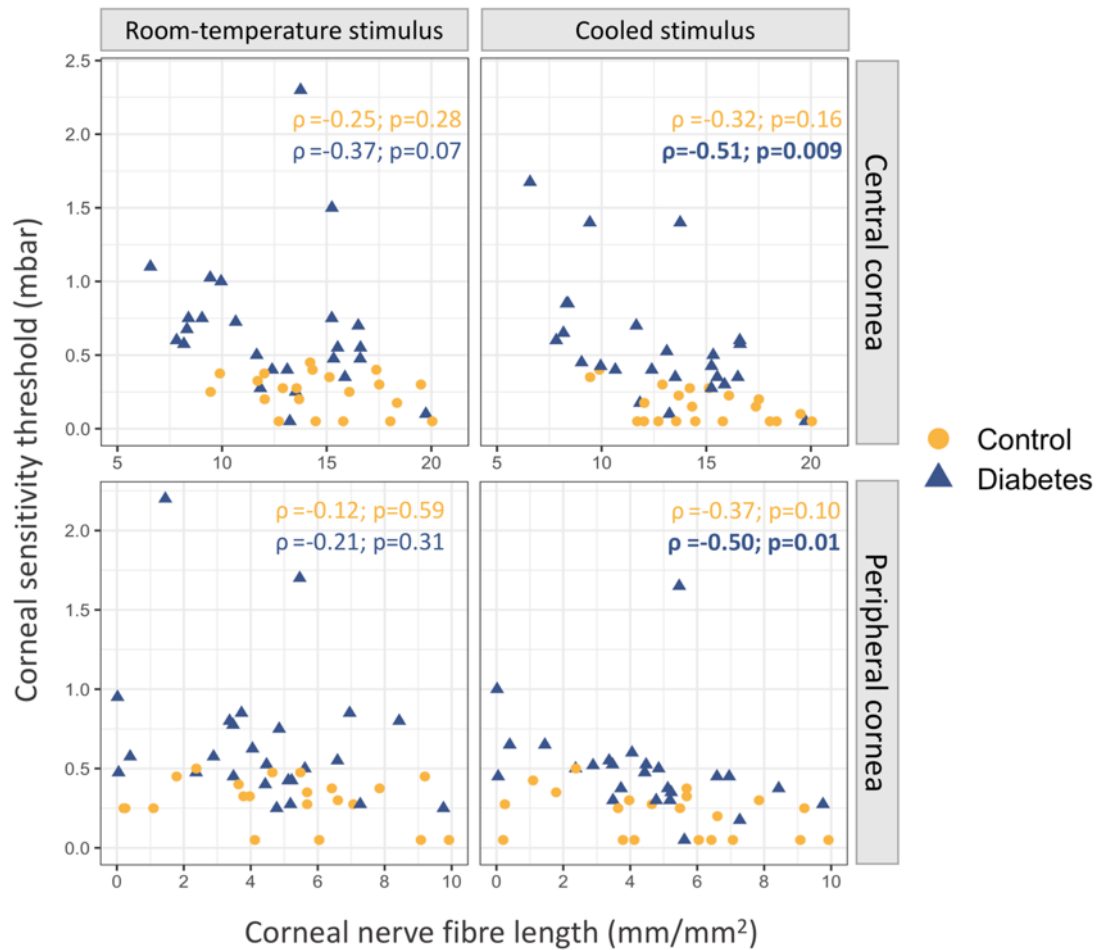


Figure 5-5. Comparison of corneal structure-function relationships in individuals with diabetes and controls. Spearman's correlation coefficient (ρ) is reported for the relationship between corneal nerve fibre length (CNFL) and corneal sensitivity thresholds to room-temperature and cooled stimuli, in the central and peripheral cornea.

There was a negative correlation between CNFL and corneal sensitivity threshold to the cooled stimulus in the diabetes group, in both central ($\rho = -0.50$; 95% CI: -0.75 to -0.12; $p = 0.009$) and peripheral ($\rho = -0.50$; 95% CI: -0.75 to -0.11; $p = 0.01$) regions. This relationship was not evident in the control group (Central: $\rho = -0.32$; 95% CI: -0.66 to 0.15; $p = 0.16$. Peripheral: $\rho = -0.37$; 95% CI: -0.70 to 0.09; $p = 0.10$). In both participant groups, CNFL did not correlate with corneal sensitivity to room temperature stimuli in either the central or peripheral cornea.

5.3.3 Corneal dendritic cells

Data relating to the density of immune cells at the sub-basal nerve plexus, putatively termed DCs, are summarised in **Table 5-7**. Densities include the total number of DCs in each corneal region and the subclassification of DCs based on the morphological subtypes described in **Table 5-2** (Lagali et al., 2018).

Table 5-7. Corneal epithelial dendritic cell (DC) density

	Central cornea			Peripheral cornea		
	Control (n=21)	Diabetes (n=26)	p- value	Control (n=21)	Diabetes (n=26)	p- value
Total DCs (cells/mm ²)	20 (14-34)	33 (11-66)	0.60	85 (43-118)	93 (39-142)	0.66
Mature DCs (cells/mm ²)	10 (4-18)	11 (4-26)	0.76	52 (38-96)	46 (24-78)	0.77
Immature DCs (cells/mm ²)	12 (5-22)	12 (4-42)	0.83	18 (5-23)	16 (7-42)	0.37
Globular cells (cells/mm ²)	0 (0-1)	0 (0-1)	0.65	0 (0-1)	0 (0.0-1)	0.84

Data are shown as median (IQR). **Abbreviations:** DC, dendritic cell.

Total DC density was similar in the diabetes and control groups in both the central and peripheral cornea. There were also no inter-group differences between diabetes and controls for DC densities when stratified by morphological phenotype (**Table 5-7**)

In the diabetes group, there were no differences in total central DC density between type 1 (median [IQR]: 33 [14 to 84] cells/mm²; n=16) and type 2 (32 [10 to 47] cells/mm²; n=10) diabetes (p=0.054). However, individuals with type 1 diabetes had more DCs in the peripheral cornea (130 [111 to 81] cells/mm²) compared to those with type 2 diabetes (43 [36 to 51] cells/mm²; p=0.009).

Overall, as expected, DC density was higher in the peripheral corneal compared with the central cornea in both participant groups. Compared to the central cornea, the peripheral cornea had a higher proportion of mature DCs as a percentage of total cells, both in the diabetes group (median [IQR]: central vs

peripheral: 38% [26 to 54%] vs 66% [44 to 81%]) and in the control group (40% [27 to 61%] vs 73% [60 to 87%]).

5.3.4 Corneal nerve parameters and systemic omega-3 fatty acid levels

Table 5-8 to **Table 5-11** summarise the relationship between systemic PUFA profiles and each of CNFL, CNFD, and corneal sensitivity thresholds (log-transformed), adjusting for age, sex, diabetes status (absent/present), presence of dry eye disease, and Norfolk DN-QoL score. In the diabetes group, there was no significant association between any dependent variables (CNFL, CNFD, or corneal sensitivity thresholds) and HbA1c, diabetes duration, or diabetes type.

The Omega-3 Index ($\beta=0.33$; $p=0.02$), age ($\beta=-0.46$; $p=0.001$) and diabetes status ($\beta=-0.30$; $p=0.03$) were significant factors associated with CNFL (**Table 5-8**; Model 1). The relationship between CNFL and systemic levels of each of DHA, EPA and total omega-6 PUFAs were explored in separate models (Models 2 to 5). DHA level ($\beta=0.32$; $p=0.027$) and participant age ($\beta=-0.41$; $p=0.003$) were associated with CNFL ($R^2=0.37$, $p=0.003$). However, neither EPA nor omega-6 PUFA levels were related to CNFL.

The Omega-3 Index, age, and diabetes status were also significantly associated with CNFD (**Table 5-9**; Model 1: $R^2=0.36$; $p=0.004$). Systemic DHA level (Model 3: $R^2=0.37$ $p=0.003$) was a significant factor for predicting CNFD, independent of age and diabetes status. Neither EPA nor total omega-6 PUFA levels were associated with CNFD parameters (Models 2 and 4).

For corneal sensitivity thresholds, participant age and diabetes status were the only factors associated with corneal sensitivity thresholds to both room-temperature (**Table 5-10**) and cooled stimuli (**Table 5-11**). Neither systemic omega-3 nor omega-6 PUFA levels were related to corneal sensitivity thresholds with stimuli of either temperature.

Table 5-8. Multiple linear regression models for variables predicting central corneal nerve fibre length (CNFL)

Variable	B (95% CI)	β	p-value	B (95% CI)	β	p-value	B (95% CI)	β	p-value	B (95% CI)	β	p-value
	Model 1: Predictor variable: Omega-3 Index			Model 2: Predictor variable: EPA level			Model 3: Predictor variable: DHA level			Model 4: Predictor variable: Total omega-6 PUFAs		
Fatty acid predictor variable	0.83 (0.16 to 1.50)	0.33	0.02	1.31 (-0.82 to 2.70)	0.25	0.064	1.97 (0.24 to 3.71)	0.32	0.027	-0.02 (-0.20 to 0.17)	-0.03	0.86
Age (years)	-0.08 (-0.13 to -0.04)	-0.46	0.001	-0.09 (-0.14 to -0.04)	-0.48	0.001	-0.07 (-0.12 to -0.03)	-0.41	0.003	-0.08 (-0.13 to -0.03)	-0.44	0.004
Diabetes (present)	-1.99 (-3.78 to -0.20)	-0.30	0.03	-2.20 (-4.04 to -0.36)	-0.33	0.02	-1.82 (-3.64 to 0.006)	-0.27	0.051	-2.22 (-4.39 to -0.05)	-0.33	0.045
Sex (Male)	0.88 (-0.98 to 2.74)	0.13	0.35	0.48 (-1.37 to 2.33)	0.07	0.61	0.89 (-1.01 to 2.80)	0.13	0.348	0.11 (-1.78 to 2.00)	-0.02	0.90
Dry eye disease (present)	1.03 (-1.53 to 3.60)	0.10	0.42	0.77 (-1.85 to 3.39)	0.08	0.56	1.18 (-1.43 to 3.79)	0.12	0.367	0.70 (-2.04 to 3.44)	-0.07	0.61
Norfolk QoL-DN score	-0.02 (-0.24 to 0.19)	-0.03	0.82	-0.01 (-0.23 to 0.21)	-0.02	0.90	-0.04 (-0.25 to 0.18)	-0.05	0.739	-0.03 (-0.26 to 0.21)	-0.04	0.81
Model statistics	R=0.62. R ² =0.39. F=4.22. p=0.002.			R=0.59. R ² =0.35. F=3.60. p=0.006.			R=0.61. R ² =0.37. F=3.99. p=0.003.			R=0.54. R ² =0.30. F=2.75. p=0.025.		

B, unstandardised regression coefficient. **β** , standardised regression coefficient. **Abbreviations:** CI, confidence interval; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; Norfolk QoL-DN, Norfolk Quality of Life-Diabetic Neuropathy questionnaire; PUFAs, polyunsaturated fatty acids.

Table 5-9. Multiple linear regression models for variables predicting central corneal nerve fibre density (CNFD)

Variable	B (95% CI)	β	p-value	B (95% CI)	β	p-value	B (95% CI)	β	p-value	B (95% CI)	β	p-value
	Model 1: Predictor variable: Omega-3 Index			Model 2: Predictor variable: EPA level			Model 3: Predictor variable: DHA level			Model 4: Predictor variable: Total omega-6 PUFAs		
Fatty acid predictor variable	1.71 (0.37 to 3.04)	0.35	0.01 *	2.42 (-0.40 to 5.23)	0.24	0.09	4.42 (1.00 to 7.85)	0.37	0.013 *	-0.18 (-0.55 to 0.19)	-0.16	0.33
Age (years)	-0.16 (-0.25 to -0.065)	-0.44	0.001 **	-0.17 (-0.27 to -0.067)	-0.47	0.002 *	-0.14 (-0.23 to -0.044)	-0.39	0.005	-0.16 (-0.26 to -0.06)	-0.45	0.003
Diabetes (present)	-3.51 (-7.08 to 0.06)	-0.27	0.054	-3.92 (-7.64 to -0.21)	-0.30	0.039 *	-3.10 (-6.69 to 0.50)	-0.24	0.09	-4.76 (-9.06 to -0.46)	-0.36	0.031
Sex (Male)	1.63 (-2.09 to 5.35)	0.12	0.38	0.72 (-3.02 to 4.46)	0.054	0.70	1.81 (-1.95 to 5.56)	0.13	0.34	-0.15 (-3.60 to 3.90)	0.01	0.94
Dry eye disease (present)	2.55 (-2.57 to 7.66)	0.13	0.32	1.99 (-3.31 to 7.29)	0.10	0.45	2.93 (-2.22 to 8.07)	0.15	0.26	1.92 (-3.51 to 7.35)	0.10	0.48
Norfolk QoL-DN score	1.71 (-0.43 to 0.42)	-0.004	0.98	0.009 (-0.43 to 0.45)	0.006	0.97	-0.037 (-0.46 to 0.39)	-0.024	0.86	-0.055 (-0.52 to 0.41)	-0.04	0.81
Model statistics	R=0.60. R ² =0.36. F=3.79. p=0.004.			R=0.56. R ² =0.31. F=2.97. p=0.017.			R=0.60. R ² =0.36. F=3.82. p=0.004.			R=0.52. R ² =0.27. F=2.51. p=0.037.		

B, unstandardised regression coefficient. **β** , standardised regression coefficient. **Abbreviations:** CI, confidence interval; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; Norfolk QoL-DN, Norfolk Quality of Life-Diabetic Neuropathy questionnaire; PUFAs, polyunsaturated fatty acids.

Table 5-10. Multiple linear regression models for variables predicting central **corneal sensitivity threshold to room-temperature air stimuli** (log transformed)

Variable	B (95% CI)	β	p-value	B (95% CI)	β	p-value	B (95% CI)	β	p-value	B (95% CI)	β	p-value
	Model 1: Predictor variable: Omega-3 Index			Model 2: Predictor variable: EPA level			Model 3: Predictor variable: DHA level			Model 4: Predictor variable: Total omega-6 PUFAs		
Fatty acid predictor variable	-0.01 (-0.10 to 0.08)	-0.03	0.83	0.002 (-0.17 to 0.17)	0.003	0.98	-0.03 (-0.25 to 0.19)	-0.04	0.79	-0.01 (-0.03 to 0.02)	-0.08	0.59
Age (years)	0.01 (0.001 to 0.01)	0.31	0.025	0.01 (0.001 to 0.01)	0.30	0.03	0.01 (0.001 to 0.01)	0.30	0.03	0.01 (<0.001 to 0.01)	0.28	0.04
Diabetes (present)	0.52 (0.30 to 0.75)	0.61	<0.001	0.53 (0.30 to 0.75)	0.61	<0.001	0.52 (0.30 to 0.75)	0.60	<0.001	0.49 (0.24 to 0.75)	0.57	<0.001
Sex (Male)	0.03 (-0.21 to 0.27)	0.04	0.79	0.04 (-0.19 to 0.27)	0.05	0.72	0.03 (-0.21 to 0.27)	0.03	0.82	0.05 (-0.18 to 0.27)	0.05	0.68
Dry eye disease (present)	-0.05 (-0.40 to 0.30)	-0.04	0.77	-0.05 (-0.41 to 0.30)	-0.04	0.77	-0.05 (-0.41 to 0.30)	-0.04	0.76	-0.05 (-0.40 to 0.30)	-0.04	0.76
Tear osmolarity	0.004 (-0.01 to 0.02)	0.08	0.89	0.004 (-0.01 to 0.02)	0.09	0.54	0.004 (-0.01 to 0.02)	0.08	0.59	0.004 (-0.01 to 0.02)	0.10	0.51
Norfolk QoL-DN score	-0.02 (-0.05 to 0.004)	-0.23	0.09	-0.02 (-0.05 to 0.004)	-0.23	0.09	-0.02 (-0.05 to 0.004)	-0.23	0.09	-0.02 (-0.05 to 0.003)	-0.24	0.08
Model statistics	R=0.66. R ² =0.44. F=4.37. p=0.001.			R=0.66. R ² =0.44. F=4.36. p=0.001.			R=0.66. R ² =0.44. F=4.38. p=0.001.			R=0.67. R ² =0.44. F=4.43. p=0.001.		

B, unstandardised regression coefficient. **β** , standardised regression coefficient. **Abbreviations:** CI, confidence interval; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; Norfolk QoL-DN, Norfolk Quality of Life-Diabetic Neuropathy questionnaire; PUFAs, polyunsaturated fatty acids.

Table 5-11. Multiple linear regression models for variables predicting central **corneal sensitivity threshold to cooled air-stimuli** (log transformed)

Variable	B (95% CI)	β	p-value	B (95% CI)	β	p-value	B (95% CI)	β	p-value	B (95% CI)	β	p-value
Fatty acid predictor variable	-0.05 (-0.14 to 0.05)	-0.11	0.36	-0.05 (-0.23 to 0.12)	-0.07	0.55	-0.09 (-0.31 to 0.14)	-0.10	0.44	0.002 (-0.02 to 0.02)	0.02	0.88
Age (years)	0.004 (-0.003 to 0.01)	0.14	0.26	0.004 (-0.003 to 0.01)	0.15	0.24	0.003 (-0.003 to 0.01)	0.13	0.29	0.004 (-0.003 to 0.01)	0.14	0.29
Diabetes (present)	0.64 (0.41 to 0.86)	0.67	<0.001	0.64 (0.41 to 0.87)	0.68	<0.001	0.63 (0.40 to 0.86)	0.66	<0.001	0.65 (0.39 to 0.91)	0.68	<0.001
Sex (Male)	-0.01 (-0.25 to 0.23)	-0.01	0.93	0.014 (-0.22 to 0.25)	0.01	0.91	-0.01 (-0.25 to 0.24)	-0.007	0.95	0.03 (-0.20 to 0.26)	0.03	0.80
Dry eye disease (present)	-0.10 (-0.25 to 0.23)	-0.07	0.59	-0.10 (-0.46 to 0.26)	-0.07	0.58	-0.11 (-0.47 to 0.25)	-0.08	0.53	-0.10 (-0.47 to 0.26)	-0.07	0.56
Tear osmolarity	0.01 (-0.004 to 0.02)	0.20	0.15	0.01 (-0.003 to 0.03)	0.21	0.13	-0.01 (0.004 to 0.02)	0.20	0.15	0.01 (-0.002 to 0.03)	0.22	0.10
Norfolk QoL-DN score	-0.01 (-0.04 to 0.01)	-0.12	0.31	-0.01 (-0.04 to 0.01)	-0.13	0.31	-0.01 (-0.04 to 0.01)	-0.12	0.34	-0.01 (-0.04 to 0.02)	-0.12	0.35
Model statistics	R=0.72. R ² =0.52. F=6.07. p<0.001.			R=0.72. R ² =0.52. F=5.92. p<0.001.			R=0.72. R ² =0.52. F=5.99. p<0.001.			R=0.72 R ² =0.51. F=5.83. p<0.001.		

B, unstandardised regression coefficient. **β** , standardised regression coefficient. **Abbreviations:** CI, confidence interval; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; Norfolk QoL-DN, Norfolk Quality of Life-Diabetic Neuropathy questionnaire; PUFAs, polyunsaturated fatty acids.

5.4 Discussion

This cross-sectional study found reduced corneal sensitivity in individuals with diabetes without neuropathy symptoms compared with healthy controls. While a corneal structure-function relationship was not evident in individuals without diabetes, a negative correlation was identified between CNFL and corneal sensitivity thresholds to a cooled stimulus in the diabetes group, in both the central and peripheral cornea. Furthermore, this study newly reports a positive association between both the systemic Omega-3 Index and erythrocyte DHA levels, with each of CNFL and CNFD, independent of age or diabetes status.

Corneal nerves play an essential role in regulating ocular surface homeostasis and tear function. In diabetes, progressive corneal nerve fibre loss and tear abnormalities predispose individuals to dry eye disease and neurotrophic keratopathy (Shih et al., 2017). The present findings of reduced CNFL in the diabetes group are in agreement with prior research that found altered corneal sub-basal nerve plexus parameters in individuals with diabetes without DSP (Pritchard et al., 2014). The present study also found increased corneal sensitivity thresholds to both room-temperature and cooled air stimuli in individuals with diabetes compared with healthy controls. It should be acknowledged that for our study cohort, the presence of DSP was excluded based on symptomology using a validated questionnaire, as clinical assessment could not be accommodated within the logistics of this study. The lack of clinical DSP evaluation and the reliance of DSP classification based on reported symptoms, using the Norfolk QoL-DN score, is a limitation of the present study.

In the diabetes group, CNFL correlated only with corneal sensitivity thresholds to cooled, but not room-temperature air stimuli. No structure-function relationship to either stimulus temperature was identified in healthy individuals. The observed lack of correlation in healthy individuals agrees with finding by Patel *et al.*, and suggests that in normal eyes, corneal sub-basal nerve plexus anatomical parameters do not reflect the functional characteristics of nerve

endings that are responsible for mediating corneal sensitivity at the ocular surface (Patel et al., 2009). Notably, IVCN only visualises the anatomical structure of the corneal sub-basal nerve plexus and not of the superficial nerve terminals; whereas corneal sensitivity is influenced by nerve receptors in both the sub-basal nerve plexus and the superficial terminals. Corneal stromal nerves are also difficult to quantify owing to the transverse orientation of the nerve compared to the *en face* distribution of the corneal sub-basal nerve plexus (Patel & McGhee, 2009). In diabetes, only Misra *et al.* has reported a positive association between central corneal sub-basal nerve plexus density and corneal sensitivity thresholds to a room-temperature air stimulus (Misra et al., 2015). However, the population in this study had a substantially lower average corneal sub-basal nerve density (11.04 ± 3.8 mm/mm²) and higher sensitivity thresholds (1.3 ± 13 mbar) compared to the present study, suggesting that the structural-functional relationship may alter with progressive corneal nerve loss in diabetes.

The observed altered structure-function relationship in diabetes is potentially indicative of disease-related effects on the sensory function of cold thermoreceptors. A putative mechanism could relate to differential effects of TRPM8 containing axons that have been shown to be preferentially affected in early experimental diabetes in mice (Alamri et al., 2019). Although the mechanisms inducing TRPM8 dysfunction are not clear, it has been hypothesised to involve protein kinase-C activation, which is implicated in the pathophysiology of DSP (see **Chapter 2**) (Rivera et al., 2020). In the cornea, TRPM8-expressing cold thermoreceptors also regulate basal tearing and blinking responses; impaired cold thermoreceptor function can induce a loss of basal tear secretion (Parra et al., 2010). A similar mechanism could contribute to tear abnormalities in individuals with diabetes. Notably, the same structure-function relationship was observed in the central and peripheral cornea for both groups. Conversely, in mice with systemic hyperglycaemia, TRPM8 expressing nerves are more markedly reduced in the central than the peripheral cornea (Alamri et al., 2019).

Only one other study has assessed temperature-modulated corneal sensitivity thresholds in individuals with diabetes. This study found that in individuals with diabetic retinopathy, 61% of whom were undergoing retinal laser photocoagulation, all forms of corneal sensation, including responses to cooled, warm and mechanical stimuli, were lower than in controls (Neira-Zalentein et al., 2011). However, corneal nerve density was not assessed concurrently in this population.

In the lower limbs of a similar population with diabetes who had normal nerve conduction thresholds and no neuropathy symptoms, Løseth *et al.* found that only cold, and not warm, perception thresholds were reduced compared to healthy controls (Løseth et al., 2008). Furthermore, a decrease in CNFD in diabetes has also been associated with an increase in cold detection thresholds in the skin (Quattrini et al., 2007). These findings support the hypothesis losses of small nerve fibres may associated with changes in sensitivity to cooled temperatures.

There was no difference in total DC density in the central or peripheral cornea between the diabetes and control groups. This finding differs from several other studies that have reported a higher central DC density in adolescents with type 1 diabetes (Ferdousi et al., 2019), and in people with diabetes without neuropathy or with mild neuropathy relative to controls (Tavakoli et al., 2011a). It is possible that this difference exists between type 1 and type 2 diabetes owing to the immune-mediated involvement in type 1 diabetes; however, Tavakoli *et al.*, found differences in corneal immune cell density only with regard to the severity of neuropathy, and not between individuals with type 1 and type 2 diabetes (Tavakoli et al., 2011a). As all participants reported no neuropathy symptoms in the present study, data on type 1 and type 2 diabetes were pooled for analysis. This method also facilitated selection of an age-matched control group, as those with type 2 diabetes are generally older than those with type 1 diabetes. Although our study found a higher DC density in the peripheral corneas of people with type 1 compared with type 2 diabetes, the reason for this

difference is unknown. Whether the age difference between the subgroups may have played a role is not clear, and further investigation in larger, age- and sex-matched cohorts is warranted to confirm whether DC density or morphology differ between diabetes types.

Presumed mature DCs, with extended dendrites and elongated cell bodies, were more abundant in the peripheral cornea than in the central cornea as anticipated. However, there was no inter-group difference in the number or proportion of DC morphological subtypes between controls and diabetes in either corneal region. In contrast, Lagali *et al.* reported a higher proportion of mature cell clusters earlier in type 2 diabetes with disease onset of less than 10 years (Lagali et al., 2018). As shown in the analysis of DC repeatability assessment in **Appendix D**, both intra- and inter-observer variability increases with increasing DC numbers in the captured image. Furthermore, the quantification of immature cells shows superior intra- and inter-observer agreement compared with mature DCs and globular cells. It is also worth noting that comparison of DC morphologies across studies is limited by both potential observer bias and the lack of concordance of cellular classification systems used. The current study adopted the classification system described Lagali *et al.*, for consistency (Lagali et al., 2018). However, there is a need to establish a universally-accepted classification system for quantifying DCs visualised using IVCN. Such system, which could be developed from a consensus process, would improve consistency in DC quantification and concordance in DC classification to allow outcomes from different studies to be better compared.

Of the long-chain omega-3 PUFAs, only systemic DHA level was related to structural parameters in the corneal sub-basal nerve plexus. The association between systemic DHA, but not EPA, levels with corneal nerve architecture could relate to the role that DHA plays in modulating neural membrane properties (Bazan, 2006), and the neurotrophic effects of the DHA-derived docosanoids mediator, neuroprotectin D1 (NPD1) (Hong et al., 2014). Pre-clinical studies have shown that topical treatment with both DHA and NPD1 can

elicit corneal nerve regeneration after trigeminal nerve injury (Cortina et al., 2013). Both oral long-chain omega-3 and DHA supplements have also demonstrated to reduce neuropathic pain in rodents (Heng et al., 2015; Silva et al., 2017).

The results of the present study suggest that the Omega-3 Index, which reflects the total dietary intake of omega-3 fatty acids, may be a potential marker of corneal nerve density. With respect to the current thesis, dietary omega-3 fatty acid intake was not captured using the clinical omega-3 dietary survey (CODS) as the tool was developed and validated after recruitment commenced for the present study. Systemic PUFA levels were quantified using biological markers rather than through diet surveys to provide an objective metric. However, systemic markers cannot differentiate omega-3 PUFAs consumed from food sources from those ingested via supplementation. In this study, most participants were presumed to derive PUFAs from food sources as only one person reported taking omega-3 PUFA supplements. Further research including more individuals who consume omega-3 supplements could potentially inform whether the dietary source could influence neurological outcomes. This strategy may also allow for the inclusion of individuals with a wider range of Omega-3 Indices.

Our study population had a low and relatively narrow range of Omega-3 Indices (median 5.2 [IQR 4.4 to 5.9]). This value is consistent with the findings of a systematic review of global fatty acid concentrations that showed Australians tend to possess Omega-3 Indices between 4–6% (Stark et al., 2016). Low Omega-3 Indices in our population are not surprising, given that 80% of Australians do not meet the recommended omega-3 fatty acid dietary intake recommended by the National Health and Medical Research Council (approximately 500 mg per day) (Meyer, 2016). However, this relatively narrow range of values limited the ability to determine a target Omega-3 Index for maintaining optimal peripheral nerve health. Recruitment of a larger cohort with a wider range of Omega-3 Indices (particularly under 4% and over 8%) would

better inform whether the same cut-off used for cardiovascular markers can be used for assessing neuropathy risk. Furthermore, although the Omega-3 Index reliably reflects dietary DHA and EPA intake over the preceding three months (Katan et al., 1997), it does not represent fatty acid intake over the longer term. Unlike the dynamic nature of the Omega-3 Index, CNFL changes would likely have occurred over years, rather than months (Pritchard et al., 2015). This indicates the need for further studies examining longitudinal changes in corneal nerve architecture in individuals with high omega-3 food intake and/or omega-3 PUFA supplementation.

A limitation of the present study is that the sample size did not provide statistical power to allow stratification of diabetes into subtypes. Furthermore, clinical examinations were not performed to more definitely exclude DSP in the study population because of the logistical confines of this study. As HbA1c values were self-reported rather than derived using laboratory methods, there is the potential for recall bias, and the reporting of inaccurate values could have been influenced by participant apprehension. The potential for misclassification bias also cannot be eliminated, as HbA1c was not measured in the control group.

In conclusion, this study found an altered corneal structure-function relationship to cooled air stimuli in diabetes, which may point to disease-related effects that are specific to sub-population(s) of corneal nerves. A positive correlation was identified between the Omega-3 Index and CNFL that was independent of age and diabetes status. These findings raise a promising notion that increasing systemic omega-3 PUFA levels could improve corneal nerve health. High-quality intervention studies, in clearly defined populations, are required to examine whether dietary interventions with omega-3 PUFAs have the potential to improve peripheral nerve health.

Chapter 6. Omega-3 PUFA supplementation for peripheral nerve health: a systematic review and meta-analysis

6.1 Introduction

Chapter 5 identified a positive relationship between the systemic Omega-3 Index and corneal sub-basal nerve structure, independent of age or diabetes status. This finding raises the possibility that increasing systemic omega-3 PUFA levels may offer a means of promoting corneal nerve health. However, the observed relationship does not imply causality for omega-3 PUFAs in improving peripheral nerve outcomes; whether dietary intervention with omega-3 fatty acids can improve these outcomes requires evaluation in controlled intervention studies with minimal bias and confounding factors.

This chapter describes the protocol and findings of a systematic review, undertaken to comprehensively synthesise and appraise the existing clinical evidence relating to the use of omega-3 fatty acid supplements as an intervention for improving peripheral nerve outcomes. To maximise the capture of all literature relevant to this topic, this review includes RCTs examining oral omega-3 fatty acid supplementation on any direct assessment of peripheral nerve structure and/or function, studied in any study population.

This chapter comprises two publications: the first publication reports the methodology (systematic review protocol) that was written prior to undertaking systematic review; the second publication describes the outcomes of the systematic review.

6.2 Publication: Omega-3 polyunsaturated fatty acid supplementation for improving peripheral nerve health: protocol for a systematic review

The citation for the systematic review protocol publication included in this chapter is:

Zhang, A. C., MacIsaac, R. J., Roberts, L., Kamel, J., Craig, J. P., Busija, L., & Downie, L. E. (2018). *Omega-3 polyunsaturated fatty acid supplementation for improving peripheral nerve health: protocol for a systematic review*. *BMJ Open*, 8(3), e020804. <https://doi.org/10.1136/bmjopen-2017-020804>

The systematic review search strategies are included in the online supplementary appendices of the publication, accessible at: <https://bmjopen.bmj.com/content/8/3/e020804.full>. For completeness, these search strategies are also provided in **Appendix E** of the thesis.

Section 6.2 is the verbatim content of the published protocol paper, formatted in thesis style. The only modifications are re-numbering of tables, figures and appendices within the thesis (indicated in bold) for consistency in style, and the reformatting of references to combine with the full reference list for the thesis.

6.2.1 Abstract

Introduction: Damage to peripheral nerves occurs in a variety of health conditions. Preserving nerve integrity, to prevent progressive nerve damage, remains a clinical challenge. Omega-3 polyunsaturated fatty acids (PUFAs) are implicated in the development and maintenance of healthy nerves and may be beneficial for promoting peripheral nerve health. The aim of this systematic review is to assess the effects of oral omega-3 PUFA supplementation on peripheral nerve integrity, including both subjective and objective measures of peripheral nerve structure and/or function.

Methods and analysis: A systematic review of randomised controlled trials that have evaluated the effects of omega-3 PUFA supplementation on peripheral nerve assessments will be conducted. Comprehensive electronic database searches will be performed in OVID Medline, Embase, the Cochrane Central Register of controlled Trials (CENTRAL), US National Institutes of Health Clinical Trials Registry and the World Health Organisation International Clinical Trials Registry Platform (WHO ICTRP). The title, abstract and keywords of identified articles will be assessed for eligibility by two reviewers. Full text articles will be obtained for all studies judged as eligible or potentially eligible; these studies will be independently assessed by two reviewers to determine eligibility. Disagreements will be resolved by consensus. Risk of bias assessment will be performed using the Cochrane Collaboration risk of bias tool to appraise the quality of included studies. If clinically meaningful, and there are a sufficient number of eligible studies, a meta-analysis will be conducted and a summary of findings table will be provided.

Ethics and dissemination: This is a systematic review that will involve the analysis of previously published data and therefore ethics approval is not required. A manuscript reporting the results of this systematic review will be published in a peer-reviewed journal and may also be presented at relevant scientific conferences.

6.2.2 Strengths and limitations of this study

- This will be the first systematic review to consider the efficacy and safety of omega-3 PUFA supplementation on peripheral nerve structure and function.
- This systematic review will only consider data from randomised controlled trials, which provide the highest level of evidence for single intervention studies.
- This review will be conducted according to the Cochrane Handbook for Systematic Reviews of Interventions, and in accordance with the Preferred Reporting Items for Systematic Reviews and Meta Analyses (PRISMA) statement.
- As we will include studies that have evaluated the use of omega-3 PUFA supplementation for treating any form of peripheral nerve damage, there may be limited scope to perform a meta-analysis due to clinical heterogeneity.
- There are currently no gold standard outcome measures for assessing peripheral neuropathy, which may affect the capacity to quantitatively synthesise data from individual studies to derive clear estimates of treatment effect(s).

6.2.3 Background

6.2.3.1 Description of the condition

The peripheral nervous system encompasses the nerves mediating sensory, motor and autonomic functions that are located outside of the brain and spinal cord. Alterations to the anatomical integrity of the peripheral nerves can adversely affect their function, presenting clinically as abnormal or loss of sensation, weakness and/or as changes to autonomic function (Hughes, 2002). England describes peripheral neuropathy as “a general term that indicates any disorder of the peripheral nervous system (England & Asbury, 2004);” this is a broad definition that includes nerve damage due to a variety of aetiologies. The pathophysiological mechanisms underlying both the development and progression of peripheral neuropathy are complex and may depend on the cause. Some of these mechanisms include altered metabolism and intracellular signaling (Callaghan et al., 2012a), vascular and inflammatory stress (Cameron & Cotter, 2008), and reactive oxygen species formation (Cashman & Hoke, 2015).

The most common systemic cause of peripheral neuropathy, which is evident in over 50% of individuals affected by the condition, is diabetes mellitus; the risk of peripheral neuropathy increases with longer disease duration (Dyck et al., 1993). and may be correlated with the degree of glycaemic control, particularly in type-1 diabetes (Charles et al., 2011; Gerstein et al., 2008; Nathan, 2014; Pop-Busui et al., 2017). Other causes include hereditary neuropathies (e.g., Charcot-Marie-Tooth Syndrome), post-infectious and inflammatory neuropathies (e.g., Guillain-Barré Syndrome) and drug-induced neuropathies (e.g., platinum analogues, thalidomide, alcohol) (Martyn & Hughes, 1997). Up to one-third of cases do not have an identified aetiology, and are thus defined as idiopathic peripheral neuropathies (Gordon Smith & Robinson Singleton, 2006).

Clinical evaluations of peripheral nerve integrity generally include a combination of symptoms, signs and electrodiagnostic studies, which aim to evaluate the

extent of nerve damage (England & Asbury, 2004). Symptoms and signs of nerve damage are often assessed using validated neuropathy scales or composite scores (which combine symptomatology with clinical measures of nerve function). Nerve biopsies are invasive and, as a result, not easily repeatable, and are therefore not frequently used as an outcome parameter in longitudinal studies, but are rather reserved for diagnostic purposes (Sommer et al., 2010).

Electrodiagnostic testing examines the characteristics of the conduction of an electrical signal that travels through a single nerve. These tests are useful in providing diagnostic information and for longitudinally monitoring disease progression (Mallik & Weir, 2005). Nerve conduction studies are reproducible and correlate well with underlying structural abnormalities (Dyck et al., 1985). but the precision of these tests is limited to detecting changes in large myelinated nerve fibres, as they are not sufficiently sensitive to detect small nerve fibre damage (Azhar et al., 2010; Lauria & Lombardi, 2007). Quantitative sensory tests, which quantify thermal and pain thresholds, can be used to evaluate small nerve fibre function (Lauria & Lombardi, 2007). Skin biopsies offer an alternative method to accurately diagnose and classify the extent of small fibre neuropathy, even in the absence of large fibre nerve damage (Hoeijmakers et al., 2012; Lauria & Lombardi, 2007). Cutaneous silent period (CSP) testing is a reproducible measurement of the nonceptive spinal reflex where thinly myelinated A-delta fibres are the afferent arm. Quantitative sudomotor axonal reflex testing (QSART) assesses the function of unmyelinated post-ganglionic sudomotor C-fibres (Floeter, 2003; Sletten et al., 2010). These are amongst several other methods to assess various small fibre types, and as each individual test may have a relatively low sensitivity a combination of modalities is usually preferable to better assess small fibre function (Kamel et al., 2015).

Recently, corneal confocal microscopy has been applied to visualise small nerve fibres *in vivo* (De Silva et al., 2017). This technique has been shown to correlate

well with intraepidermal nerve fibre biopsy results and is useful for detecting and documenting various types of small fibre neuropathies (Asghar et al., 2014; Ferdousi et al., 2015; Quattrini et al., 2007; Sturniolo et al., 2015; Tavakoli et al., 2009). Corneal confocal microscopy has also been suggested to be useful for monitoring disease progression, and as a marker for improvements in nerve function, in the investigation of therapeutic targets for diabetic peripheral neuropathy (Pritchard et al., 2014; Tavakoli et al., 2011b; Tavakoli et al., 2013).

Peripheral neuropathies are typically treated based on their subtype and/or underlying cause(s). Treatments primarily aim to manage the underlying condition to prevent progressive nerve damage and to treat any associated symptoms (Azhary et al., 2010; Boulton et al., 2004; England & Asbury, 2004). The consequences associated with symptoms of neuropathic impairment do not only affect an individual's quality of life, but remain an economic burden in the cost of healthcare and medical resources (Alleman et al., 2015; McCarberg & Billington, 2006; Mehra et al., 2014). This is especially true in chronic conditions such as diabetes, where lifetime care is required (Callaghan et al., 2012a).

6.2.3.2 Description of the intervention

Omega-3 polyunsaturated fatty acids (PUFAs) are essential fatty acids (EFAs) with multiple double bonds, the first of which is located at the third carbon from the methyl end of the molecule. Short-chain omega-3 PUFAs, alpha-linolenic acid (ALA), found in plant sources, is a metabolic precursor to the long-chain omega-3 PUFAs docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), which are present in high abundance in oily fish. As humans do not have the enzymes to synthesise these fatty acids *de novo*, omega-3 PUFAs must be obtained from the diet or through supplementation (Nettleton, 1991). The other major class of EFAs are the omega-6 fatty acids, which derive from the diet in the form of linolenic acid (LA) and are elongated *in vivo* to gamma-linoleic acid (GLC) and arachidonic acid (AA). Most eicosanoids derived from the omega-6 dependent AA-pathway are pro-inflammatory; in contrast, long-chain omega-3

fatty acids bias prostaglandin metabolism towards the production of anti-inflammatory eicosanoids.

As omega-3 PUFAs competitively inhibit the metabolic conversion of omega-6 PUFAs (Calder & Grimble, 2002), the balance of omega-3 to omega-6 fatty acid consumption can affect systemic inflammatory processes and immune activity. The ratio of consumed omega-6 to omega-3 in typical Western diets is approximately 15 to 1, whereas a ratio of 4 to 1 is considered optimal (Simopoulos, 2002a). Increased consumption of omega-3 PUFAs is considered to provide a range of potential general health benefits, including a reduced risk of cardiovascular disease (Simopoulos, 2008; Yokoyama et al., 2007) and lowered systemic triglycerides (Chauhan et al., 2017). DHA, as an integral component in cellular membrane structures of the brain and retina, has been implicated in perinatal visual and neural development (Birch et al., 1998; Hibbeln et al., 2007; McNamara, 2010). In ocular conditions, omega-3 fatty acids supplements can reduce the symptoms and clinical signs associated with ocular surface inflammation in dry eye disease (Deinema et al., 2017). The American Heart Foundation recommends a daily intake, for adults, of 500 mg of long-chain omega-3 PUFAs (Kris-Etherton et al., 2002), and up to 4 g/day in hypertriglyceridemia (Miller et al., 2011).

6.2.3.3 How the intervention might work

Once consumed, omega-3 PUFAs alter membrane protein activity and cellular signaling response, to reduce immune activity and the concentration of systemic lipid inflammatory mediators (Gibney & Hunter, 1993). The incorporation of omega-3 PUFAs into cellular membranes, and their subsequent effect on membrane activity, has been shown to alter vascular function, improve sciatic nerve blood flow and enhance nerve conduction velocity in a rat model of experimental diabetic neuropathy (Coste et al., 2003).

Omega-3 PUFAs also affect intracellular signalling pathways and the expression of genes, some of which may be associated with the regulation of neuron

growth and neuroprotection (Kitajka et al., 2004). In animal models of diabetes, omega-3 PUFA supplementation has been shown to attenuate adverse changes in nerve structure and function (Gerbi et al., 1999; Yee et al., 2010). Mice enriched with genes that increase endogenous profiles of omega-3 PUFAs have been shown to have reduced neuronal cell death and increased recovery to mechanical stress and peripheral nerve injury (Gladman et al., 2012). Omega-3 PUFAs have also been demonstrated to promote neurite growth in rat sensory neurons (Robson et al., 2010).

Derivatives of omega-3 PUFA metabolism, resolvins and protectins, which are oxygenated metabolites from EPA and DHA respectively, may further promote neuronal function. Neuroprotectin D-1 has been shown to facilitate the regeneration of corneal nerves following refractive surgery and neurite growth from the trigeminal ganglion of mice (Cortina et al., 2013) and to prevent neuropathic pain after peripheral nerve injury (Xu et al., 2013b).

6.2.3.4 Why it is important to do this review

This will be the first systematic review to consider the potential effects of omega-3 PUFA supplementation on peripheral nerve integrity. Omega-3 PUFA supplementation has been shown to reduce neuronal damage and enhance recovery following nerve injury in experimental animal models of peripheral neuropathy. Confirmation of these effects in clinical populations would contribute significantly towards enhancing the clinical management of peripheral neuropathy. A therapeutic agent to prevent the pathogenesis of, or slow the progression of, peripheral nerve damage has the potential to greatly improve clinical outcomes. Furthermore, the potential for omega-3 PUFA supplements to alleviate neuropathy-associated symptoms would be predicted to reduce impairment on an individual's quality of life and lessen the economic burden of peripheral neuropathy in the community.

6.2.4 Objectives

The primary objective of this systematic review is to evaluate the efficacy and safety of oral omega-3 PUFA supplements for improving peripheral nerve health. Efficacy outcomes will consider both subjective endpoints (i.e., symptoms) and objective clinical measures, including changes to peripheral nerve structure and function.

6.2.5 Methods and analysis

We will conduct the proposed systematic review and meta-analyses according to the recommendations stated in the *Cochrane Handbook for Systematic Reviews of Interventions* (Higgins et al., 2011), and following the Preferred Reporting Items for Systematic Reviews and Meta Analyses (PRISMA) statement (Moher et al., 2009). The protocol for this review has been registered in the PROSPERO International prospective register of systematic reviews (CRD42018086297).

6.2.5.1 Eligibility criteria

All studies published from the date of database inception until 21st November 2017 will be included. Studies will be selected according to the following eligibility criteria:

Types of studies

We will include randomised controlled trials (RCTs) where participants were allocated to consume oral omega-3 PUFA supplements. We will exclude quasi-randomised trials. We will not exclude studies based upon language, publication status, year, or the number of participants. In cases where more than one publication reporting data from the same cohort of participants exist (i.e., from the same trial), the study reporting on the largest number of participants will be included. Published conference abstracts will be eligible for inclusion.

Types of participants

We will include studies involving adults (i.e., aged 18 years or older), recruited from within any study setting, where the structure and/or function of peripheral nerves was assessed. To be eligible for inclusion in the review, studies need to include at least one subjective measure of peripheral neuropathy (e.g., symptom score), one composite measure of peripheral neuropathy (i.e., combining subjective and objective measures), or one objective measure of peripheral nerve structure (e.g., nerve biopsy) or function (e.g., nerve conduction studies).

Types of interventions

We will consider interventions where participants were randomised to oral supplementation with short-chain and/or long-chain omega-3 PUFAs. We will accept studies that administered omega-3 supplements in any form or dosage. We will exclude studies where the intervention was administered in the form of dietary manipulation (i.e., a food-based intervention), and where omega-3 PUFA supplements were administered in combination with another intervention (including other nutritional interventions), unless the intervention was administered in the same dose and frequency in the comparator group. We will consider studies where omega-3 PUFA supplements were compared to placebo or no treatment.

Types of outcome measures

There are no gold standard or universally accepted outcome measures for peripheral nerve assessment. In selecting the outcome measures for this review, we considered the recommendations provided by the European Neuromuscular Centre (ENMC) International workshop: Selection of Outcome Measures for Peripheral Neuropathy Clinical Trials (10-12 December 2014), taking into account both subjective and clinical measures (Merkies & Lauria, 2006).

We will assess all outcome measures at six months of follow-up, with an acceptable follow-up range of between three and nine months from baseline. If

studies do not report the change from baseline, we will utilise data reported at the end of the follow-up period.

Primary outcomes

The primary outcome will be the change, from baseline, in peripheral neuropathy impairments, as quantified by validated, composite (i.e., combining symptoms plus objective measures) neuropathy measures. We have not been prescriptive in our selection of particular scales as there are no universally agreed scoring systems; examples of validated, composite neuropathy assessment scales include the Michigan Diabetic Neuropathy Score (MDNS) (Feldman et al., 1994), Neuropathy Impairment Score (NIS) (Dyck et al., 1995), and Total Neuropathy Score (TNS) (Cornblath et al., 1999). For the purpose of this review, we define a ‘validated’ measure being a survey instrument that has been psychometrically tested.

Secondary outcomes

We will consider the following secondary outcomes:

1. Symptoms: change, from baseline, in symptoms of peripheral neuropathy, measured by a validated, patient-assessed symptom score.
2. Pain: change, from baseline, in mean scores of pain, measured by a validated, patient-assessed pain scale. Examples of validated scales include the visual analogue scale (VAS) (Wewers & Lowe, 1990), Likert scales (Farrar et al., 2001; Hasson & Arnetz, 2005), and the McGill Pain Questionnaire (MPQ) (Melzack, 1975).
3. Disability: change, from baseline, in the mean score of a patient-reported disability measure. Examples of validated disability measures include the Overall Neuropathy Limitation Scale (Graham & Hughes, 2006) and the Overall Disability Sum Score (ODSS) (Merkies et al., 2002).
4. Anatomical markers:
 - a. Change, from baseline in central corneal nerve fibre length (CNFL), defined as the total length of nerves in a given area, measured in

- mm/mm², using a laser-scanning in vivo confocal microscope (IVCM);
- b. Change, from baseline in intraepidermal nerve fibre density (IENFD).
5. Nerve Conduction Studies (NCS): Change, from baseline, in nerve conduction study parameters, as recommended by England (2005) (England et al., 2005):
- a. Sensory nerve action potential (SNAP) amplitudes of the sural, median and ulnar nerves;
 - b. SNAP latencies of the sural, median and ulnar nerves;
 - c. Sensory nerve conduction velocity (NCV) of the sural, median and ulnar nerves;
 - d. Distal compound motor action potential (CMAP) amplitude of the peroneal, tibial, median and ulnar nerves;
 - e. CMAP latency of the peroneal, tibial, median and ulnar nerves;
 - f. Motor NCV of the peroneal and ulnar motor NCV of the peroneal, tibial, median and ulnar nerves;
 - g. Minimum F-wave latency of the peroneal, tibial, median and ulnar nerves.
6. Sensory function in the cornea: change, from baseline, in corneal sensation, as quantified using:
- a. Contact aesthesiometry, to quantify mechanical detection thresholds using the Cochet-Bonnet aesthesiometer (measured in millimetres);
 - b. Non-contact aesthesiometry, to quantify corneal sensation quantified using an air-based aesthesiometer (measured in millibars).
7. Sensory function in the skin: Change, from baseline, in sensory function test scores:
- a. Mechanical detection thresholds, measured using pressure aesthesiometry (e.g., von Frey hair aesthesiometer);
 - b. Cold detection thresholds measured using quantitative sensory testing (QST) methods;

- c. Warm detection thresholds measured using QST methods;
 - d. Thermal pain thresholds, for cold and hot stimuli, measured using QST methods.
8. Adverse events: we will consider all adverse events, and will analyse them in the following categories: (i) any adverse events, (ii) adverse events leading to discontinuation of the interventions, and (iii) serious adverse events, being those leading to hospitalization or prolonged admission, a life-threatening event or death.

6.2.5.2 Search methods for identification of studies

We will conduct comprehensive electronic database searches in: the Cochrane Central Register of Controlled Trials (CENTRAL) (Issue 10, October 2017), Ovid MEDLINE, Ovid MEDLINE Epub Ahead of Print, In-Process & Other Non-Indexed Citations, Ovid MEDLINE Daily (January 1946 to November 2017), EMBASE (January 1947 to November 2017). We will also search the US National Institutes of Health Clinical Trials Registry (www.clinicaltrials.gov) and the World Health Organization (WHO) International Clinical Trials Registry Platform (ICTRP) (www.who.int/ictcp/en/). We will not impose any restrictions on date or language in our search strategies. Search strategies for all electronic searches are included in Appendices 1 – 5 (**Appendix E** of thesis). We will additionally search the bibliographies of included RCTs to identify any other potentially relevant studies.

6.2.5.3 Data collection and analysis

After the search strategies are performed within each electronic database, the reference lists will be imported into EndNote. Duplicate entries will be identified and removed. The final reference library will be imported into Covidence (Covidence), the standard production platform for Cochrane reviews, for the study selection process.

6.2.5.4 Selection of studies

The titles, keywords and abstracts of all unique articles, identified by the search strategies, will be independently reviewed by two review authors to identify potentially eligible studies. The full text papers of articles identified as relevant or potentially relevant, by at least one reviewer, will be retrieved for full text screening. Full text articles will be independently screened by two review authors and assessed for eligibility according to the pre-specified inclusion and exclusion criteria. Reasons for exclusion will be identified and recorded for ineligible studies that progress to the full text screening stage. Any disagreements in eligibility assessment will be adjudicated between the two reviewers; if consensus cannot be achieved, a third independent author will be consulted to reach consensus. We will include a PRISMA flow diagram (summarising the article selection process) and a 'Characteristics of excluded studies' table (with reasons for study exclusion).

6.2.5.5 Data extraction and management

Two review authors will independently extract outcome data and key study characteristics for all included studies. Any discrepancies will be resolved by discussion and consensus. We will contact the study authors of relevant trials, by email, if further information or clarification is required. If we fail to receive a response from the corresponding author within four weeks, or if the authors are unable to provide us with the requested information, we will use the information that is available.

For each study, we will extract the following information:

1. Article details: year of publication, journal of publication, language, publication status;
2. Study details: dates study conducted, trial registration number, country, study setting, corresponding author details (name, institution, email, address) and whether the study investigators were contacted for further information;

3. Methods: exclusions after randomisation, losses to follow-up, how missing data were handled, whether a sample size calculation was reported;
4. Participants: number of participants in each intervention group, participant baseline characteristics (i.e., age, gender and/or sex (as specified by the study authors), underlying conditions, peripheral neuropathy diagnostic criteria), participant inclusion criteria and exclusion criteria, comparison of groups at baseline;
5. Interventions: intervention(s) and comparator (type (long- or short-chain), dose (milligrams/day), duration of treatment), concomitant medications or treatments, compliance measures (i.e., whether compliance was assessed, and the method used, e.g., returned capsule counts, red blood cell fatty acid profiles, etc.);
6. Outcomes: pre-specified primary and secondary outcome measures, time points of assessments;
7. Other: sources of funding statement (i.e., present or absent), actual source of funding (e.g., industry funding), conflicts of interest statement (i.e., present or absent), nature of conflict of interest (e.g., industry employee).

6.2.5.6 Assessment of risk of bias in included studies

Two review authors will independently assess the risk of bias for each included study using *the Cochrane Handbook for Systematic Reviews* guidelines.

Assessment of bias will be considered using the following domains:

1. Selection bias (random sequence generation and allocation concealment);
2. Performance bias (blinding of participants and all study personnel);
3. Detection bias (blinding of outcome assessors);
4. Attrition bias (incomplete outcome data);
5. Reporting bias (selective reporting of outcomes);
6. Other sources of bias (funding source, conflicts of interest).

Each review author will judge the risk of bias in each domain as: (i) low risk, (ii) unclear risk or (iii) high risk. Disagreements in bias assessment will be resolved by consensus between the two reviewers.

6.2.5.7 Measures of treatment effect

We will analyse data according to the methods described in Chapter 9 of the *Cochrane Handbook for Systematic Reviews of Interventions* (Higgins et al., 2011).

As all of the pre-defined outcomes are continuous measures, we will extract information on the change in mean from baseline, and standard deviations of change, for the intervention and comparison groups. If change from baseline is not reported, we will extract information on the mean and standard deviation of the outcome, for the intervention and comparator groups, at the specified follow-up period. The effects of the interventions will be expressed as the mean difference (MD), with 95% confidence intervals (CIs), between the intervention and comparator groups for significant outcomes ($p < 0.05$); exact p-values will be reported.

6.2.5.8 Unit of analysis issues

The unit of analysis for this review will be the individual participant. In studies where outcomes were measured in ocular tissues, the unit of analysis will be the enrolled study eye of the participant. In studies where participants were randomly allocated to treatment, there will be no unit of analysis issues if only one eye per person is included in the trial, or if both eyes per person are included and the average value of both eyes are reported. In studies where participants were randomly allocated to treatment and both eyes were included, but reported separately, we will analyse this as clustered data (i.e. adjusted for within-person correlation). We may have to contact the trial investigators for further information to do this.

6.2.5.9 Dealing with missing data

For any studies where missing outcome data (e.g., omitted standard deviations, standard errors) are identified, we will attempt to contact the study authors. If we fail to receive a response from the corresponding authors in four weeks, or if the authors are unable to provide us with the requested information, we will use the information that is available. We will use imputed data, if this has been derived by the trial investigators using an appropriate method, but will not impute missing data ourselves.

6.2.5.10 Assessment of heterogeneity

We will assess clinical and methodological heterogeneity in the included studies by examining differences in the intervention (e.g., type, dose, form), participant characteristics at baseline (e.g., age, gender/sex, cause of neuropathy, eligibility criteria, etc.), and risk of bias. We will quantify statistical heterogeneity using the I^2 statistic, as outlined in Chapter 9 of the *Cochrane Handbook for Systematic Reviews of Interventions* (Higgins et al., 2011); we will consider an I^2 statistic of 60% or more as consistent with a moderate level of heterogeneity. In measuring heterogeneity, we will also consider the: (i) magnitude and direction of the effects of individual studies, and (ii) strength of evidence for heterogeneity (using a p-value < 0.10 from the Chi-squared test as the criterion for significant heterogeneity).

6.2.5.11 Assessment of reporting biases

If at least ten studies are included in a meta-analysis, we will use a funnel plot to assess for any potential publication bias. We will interpret any asymmetries in the funnel plot in association with the trial characteristics, considering relevant factors such as sample size.

6.2.5.12 Data synthesis

We will undertake meta-analyses, for the primary and/or secondary outcomes, when this would be clinically meaningful (i.e., for studies where the treatment,

participants and the underlying clinical questions are similar). If fewer than three RCTs are to be included in a meta-analysis, we will use a fixed-effect model, otherwise we will use a random-effects model.

If there is inconsistency between individual study results, such that the pooled results may not provide a fair representation of the trial findings (e.g., the effects are in opposite directions, or $I^2 > 60\%$ or the Chi-squared test p-value is <0.10), we will not pool the study data but will instead describe the pattern of the individual study results. If there is statistical heterogeneity but all of the effect estimates are in the same direction, such that a pooled estimate would seem to provide a good summary of the individual trial results, we may pool the data.

If a meta-analysis is not deemed appropriate, we will provide a descriptive or tabulated results summary.

6.2.5.13 Subgroup analysis and investigation of heterogeneity

If sufficient data are available, we will perform subgroup analyses by prognostic factors (e.g., type of disease (including sub-type of diabetes), severity of peripheral neuropathy at baseline, and age) and by potential intervention effect modifiers (e.g., dose, duration and type of omega-3 PUFA supplement), as these factors are potentially important to any observed treatment effects.

6.2.5.14 Sensitivity analysis

Provided there are sufficient data available, we will perform a sensitivity analysis for the primary outcome (i.e., change in peripheral neuropathy impairment score), to assess the impact of excluding studies that: i) were appraised as having a high risk of bias due to lack of allocation concealment or lack of blinding of participants and study personnel, ii) had more than 20 percent of participants that were lost to follow-up, iii) were unpublished, and iv) were funded by industry.

6.2.5.15 Summary of findings table

Provided that sufficient data are available, we will provide a “Summary of findings” table for the primary outcome (change in peripheral neuropathy as measured using a validated composite neuropathy assessment) and the following secondary outcomes (as previously defined): (i) Symptoms, (ii) Pain, (iii) Corneal nerve fibre length (CNFL), (iv) Intraepidermal nerve fibre density (IENFD), (v) SNAP amplitudes of the sural nerve, and (vi) Motor NCV of the peroneal nerve. We will follow the recommendations specified in Chapter 11 of the *Cochrane Handbook for Systematic Reviews of Interventions* (Higgins et al., 2011). The strength and quality of the evidence for each outcome will be assessed using the Grading of Recommendations Assessment, Development and Evaluation (GRADE) approach (Puhan et al., 2014).

6.2.6 Conclusions

The term “peripheral neuropathy” describes a heterogeneous group of disorders that cause damage to the peripheral nervous system. Currently, management approaches for peripheral neuropathy are aimed primarily at addressing the underlying cause and/or managing symptoms. For many causes of peripheral neuropathy, including diabetes, reversing or even limiting the progression of nerve damage remains a challenge with currently available therapeutics.

Omega-3 PUFAs are reported to be associated with a range of general health benefits that include reducing the risk of cardiovascular disease (Colquhoun et al., 2008; Simopoulos, 2008; Yokoyama et al., 2007), lowering systemic triglycerides and improving clinical symptoms of dry eye disease (Chauhan et al., 2017; Deinema et al., 2017). In animal models of experimental peripheral nerve injury, increasing endogenous levels of omega-3 PUFAs have been shown to improve sciatic blood flow and accelerate the recovery of neuronal function (Gerbi et al., 1999; Gladman et al., 2012; Robson et al., 2010),

The aim of this systematic review is to assess the safety and efficacy of oral omega-3 PUFA supplementation for improving peripheral nerve health. If it is demonstrated that omega-3 supplements can improve measures of peripheral nerve function and/or quality of life, it is anticipated that this therapy would make a valuable contribution to the current clinical management of peripheral neuropathy.

Authors' contributions: All authors (ACZ, LED, LB, JK, RJM, LR and JPC) made contributions to the conception and/or design of the work; and drafted (ACZ & LED) or revised (LB, JK, RJM, LR and JPC) the protocol; and approved the final version of the manuscript; and agree to be accountable for all aspects of the submitted work.

Funding statement: This work was supported by 2018 Melbourne Neuroscience Institute (MNI) Interdisciplinary Seed Funding (LED, RJM, LR, JK); the funder had no role in the design of this protocol and will not have any role in the undertaking, data analyses, or reporting of the systematic review.

Competing interests statement: All authors declare no conflicts of interest relevant to this review.

Acknowledgements: We acknowledge the advice of Iris Gordon, Cochrane Eyes and Vision Group, who provided feedback on the search strategies for this review.

6.3 Publication: Omega-3 polyunsaturated fatty acid (PUFA) oral supplements for improving peripheral nerve health: a systematic review and meta-analysis

The publication reporting the findings of the systematic review is presented in this section. The citation details are:

Zhang, A. C., De Silva, M. E. H., MacIsaac, R. J., Roberts, L., Kamel, J., Craig, J. P., Busija, L., & Downie, L. E. (2019). *Omega-3 polyunsaturated fatty acid oral supplements for improving peripheral nerve health: a systematic review and meta-analysis*. *Nutrition Reviews*, 78(4), 323-341.

<https://doi.org/10.1093/nutrit/nuz054>

Section 6.3 is the verbatim content of this published paper, formatted in thesis style, with tables, figures, and appendices re-numbered for consistency (indicated in bold). For readability, the reference style in this section has been retained in the preferred style of the publisher and presented in numbered format. The corresponding reference list is thus provided separately at the end of this chapter.

Supplementary materials associated with this publication are provided in **Appendix E** of this thesis.

6.3.1 Abstract

Context: Peripheral nerve damage can occur in a variety of systemic conditions and can have a profound impact on functional and psychological health, and there are currently limited therapeutic interventions.

Objective: The aim of this systematic review, conducted in accordance with the Cochrane Collaboration handbook and reported according to the PRISMA checklist, was to evaluate the efficacy and safety of omega-3 oral supplements for improving peripheral nerve structure and function.

Data sources: PubMed, Embase, the Cochrane database and trial registries were searched from inception to February 2019, for randomized controlled trials assessing the effects of omega-3 oral supplementation on outcomes of peripheral nerve structure and/or function.

Data extraction: Evidence was identified, critically appraised and synthesized, and the certainty of evidence was appraised using the Grading of Recommendations Assessment, Development and Evaluation approach.

Data analysis: Fifteen trials were included. Two clinically-similar studies, investigating the effect of omega-3 supplementation in individuals receiving chemotherapy, were meta-analyzed. Pooled data showed a reduced incidence of peripheral neuropathy (RR 0.58, 95% confidence interval [CI] 0.43 to 0.77) and preservation of sensory nerve action potential amplitudes compared with placebo (MD: 4.19 μ V, 95%CI: 2.19 to 6.19).

Conclusion: This review finds, with low certainty, that omega-3 supplementation attenuates sensory loss and reduces the incidence of neuropathy secondary to oxaliplatin and paclitaxel, relative to placebo. There is currently limited evidence to ascertain whether omega-3 supplementation is beneficial in other systemic conditions characterized by peripheral nerve damage.

6.3.2 Background

The peripheral nervous system (PNS) mediates sensory, motor and autonomic functions outside of the brain and spinal cord.¹ PNS function depends upon its anatomical integrity and integrated neural communication across the body.² Damage to the PNS can result in peripheral neuropathy (PN), which adversely affects nerve function, leading to sensory deficits, motor imbalance and/or autonomic dysfunction.³ PN is a potential complication of multiple systemic conditions, most commonly diabetes mellitus. Other potential causes include infection, inflammation, hereditary conditions, neurotoxicity and trauma.³

Current management regimes for PN are typically based upon treating the underlying condition(s) and addressing their functional impact. There are some specific conditions, such as chronic inflammatory demyelinating polyneuropathy, Guillain-Barre syndrome and the vasculitic neuropathies, that respond to immunomodulatory treatment.⁴ However, for most etiologies, including diabetes mellitus, there are currently no established disease-modifying treatments for limiting progressive peripheral nerve damage.⁵ PN induced by neurotoxic substances, such as in chemotherapy, has long-term negative impacts on the quality of life of cancer survivors.⁶ Epidemiological studies show that progressive PN can be associated with lifestyle factors, including cigarette smoking and obesity, due to their effects on systemic inflammatory pathways and vascular regulation.⁷ There is also scientific rationale that an individual's diet may influence PN progression. A potassium restrictive diet has shown to be neuroprotective.⁸ Additionally, omega-3 fatty acid consumption has the potential to affect peripheral nerve health.

Omega-3 fatty acids are one of two major classes of 'essential' polyunsaturated fatty acids (PUFAs) that cannot be synthesized *in vivo*, and thus must derive from the diet or supplementation. Short-chain omega-3 fatty acids, alpha-linoleic acid (ALA), found in plant sources, are a metabolic precursor to the long-chain omega-3 PUFAs (docosahexaenoic acid [DHA] and eicosapentaenoic acid [EPA]), which are mainly found in marine sources. Once ingested, omega-3

PUFAs impart a range of biological effects, including favoring prostaglandin metabolism towards the production of anti-inflammatory and neuroprotective metabolites; this occurs in competition with the omega-6 pathway, which yields mostly pro-inflammatory prostaglandins.⁹

The balance of omega-3 to omega-6 consumption regulates systemic inflammatory processes and immune activity.¹⁰ Systematic reviews indicate that diets rich in omega-3 PUFAs are associated with a range of potential health benefits, including lowering systemic triglycerides and improving early cognitive development.¹¹⁻¹³ DHA is an integral component of neuronal phospholipid membranes and is implicated in cortical and visual development.¹⁴ DHA deficiency has been associated with impaired neuronal function and in the development of neurodegenerative disorders.¹⁵ Pre-clinical evidence supports a role for omega-3 fatty acids in promoting neuronal survival and axonal regeneration following peripheral nerve injury.¹⁶ Neuroprotectin D-1 (NPD-1), a DHA metabolite, reduces oxidative stress-induced cellular apoptosis by suppressing pro-apoptotic signalling, and attenuating pro-inflammatory responses.¹⁷ Both DHA and NPD-1 have therapeutic potential in neurodegenerative conditions, such as Alzheimer's disease and spinal cord injury,^{15,18} and in the cornea, for promoting small nerve fiber regeneration following injury.¹⁹ Various pre-clinical studies have associated omega-3 supplementation with neuroprotection, mitigating functional losses and promoting neurite growth.^{16,20} If similar benefits are evident in clinical populations, omega-3 fatty acids may provide a means for improving peripheral nerve health, and potentially have application in the clinical management of PN.

The aim of this systematic review, conducted according to the Cochrane Collaboration handbook guidelines and reported according to the PRISMA checklist,²¹ was to synthesize the current, best-available evidence, relating to the efficacy and safety of oral omega-3 supplementation for improving peripheral nerve health, to inform clinical practice and future research in the field.

6.3.3 Methods

This systematic review was reported in reference to the Preferred Reporting Items for Systematic Reviews and Meta Analyses (PRISMA) statement (see Appendix S2 in the supporting information published online)²¹ and conducted according to recommendations in the Cochrane Handbook for Systematic Reviews of Interventions.²² The protocol was published²³ and prospectively registered on PROSPERO (CRD42018086297).

6.3.3.1 Study eligibility criteria

Studies were selected for inclusion based upon the pre-specified PICOS (population, intervention, comparator, outcomes, study design) criteria (**Table 6-1**). Only randomized controlled trials (RCTs) were included, to minimize potential confounding effects from less robust study designs. Published conference abstracts were eligible for inclusion. Eligible studies included adult participants where at least one subjective measure of PN, one composite measure or one objective measure of peripheral nerve structure or function was performed. Studies where the intervention was administered as a dietary manipulation, or where the intervention was administered in combination with another intervention were excluded, unless the co-intervention was given in the same dose and frequency in the comparator group.

Table 6-1. Prespecified primary and secondary outcome measures

Parameter	Study selection criteria
Population	Adult participants, aged >18 years, recruited from within any study setting, where the structure and/or function of peripheral nerves was assessed.
Intervention	Long or short chain omega-3 oral supplements, in any form or dosage.
Comparator	Placebo or no intervention.
Outcomes	Primary and secondary outcomes including both objective and subjective measures of peripheral nerve structure and/or function (full details of pre-specified outcomes are described in Table S1 of the supporting information [Appendix E])
Study design	Randomized controlled clinical trials, published in any year or language.

6.3.3.2 Search methods

Comprehensive searches were performed in Ovid MEDLINE, Embase, the Cochrane Central Register of Controlled Trials (CENTRAL), US National Institutes of Health Clinical Trials Registry and the WHO International Clinical Trials Registry Platform. Searches are up to date as at 21 February 2019, using the published strategies (Appendix S1).²³

6.3.3.3 Data extraction and management

Two review authors independently extracted key study data (Table S2; **Appendix E**) in Covidence²⁴ and resolved any discrepancies by consensus. Collected data included: study design, methodology, participants, interventions, outcomes and other relevant information (e.g., funding sources). Quantitative data were extracted for pre-specified outcomes. Any discrepancies were resolved by discussion and consensus. Study authors were contacted when further information was required. Extracted data were exported into the Cochrane Review Manager software (REVMAN)²⁵ by one review author (A.C.Z.) and independently verified by a second review author (L.E.D.).

6.3.3.4 Assessment of risk of bias in included studies

Risk of bias was evaluated using the Cochrane Handbook for Systematic Reviews of Interventions guidelines.²² Two review authors independently assessed risk of bias in each study as low, unclear or high in the following domains: (1) selection bias; (2) performance bias; (3) detection bias; (4) attrition bias; (5) reporting bias and (6) other sources of bias. Disagreements were resolved by consensus.

6.3.3.5 Measures of treatment effect

Data were extracted, for primary and secondary outcomes (Table S1; **Appendix E**), as the mean change from baseline, and standard deviation (SD) of the change, for the intervention and comparator groups. Where change from baseline was not reported, the mean and SD of the outcome were extracted at

the specified followed-up period, in each group. The unit of analysis was the participant. In one study where outcomes were measured in ocular tissues, the unit of analysis was the enrolled study eye of the participant.

6.3.3.6 Data synthesis

Meta-analyses were performed when: (1) at least two studies reported data in a consistent format (e.g., endpoint data), (2) there was an absence of significant statistical heterogeneity ($I^2 < 60\%$ and Chi-squared test p-value > 0.10),²³ and (3) when combining the datasets was considered clinically meaningful (e.g., same disease state). Clinical heterogeneity was assessed by considering differences in the intervention (e.g., type, dose and form) and baseline participant characteristics. A fixed-effect model was used for all meta-analyses including fewer than three studies, otherwise a random-effects model was used.²⁵ For all adverse events, tabulated results summaries were generated.

The risk of reporting bias (due to selective outcome reporting) was assessed by comparing the outcomes described in trial registries with those in the publication(s). There were seven studies that had been prospectively registered in a clinical trials registry; for these studies, the outcomes defined in the trial registry entries were compared with those reported in the publications. Sub-group analyses for prognostic factors (e.g., type of disease, baseline severity of PN and age) and potential intervention modifiers (e.g., omega-3 dose, duration and type of supplement) could not be performed as there was an insufficient number of trials (considered two studies per subgroup or more) to perform these analyses. A funnel plot was intended to be used to assess for any potential publication bias, considering relevant factors such as sample size, to interpret any asymmetries in the funnel plot in association with the trial characteristics. However, as fewer than 10 studies were included in the meta-analyses, a sensitivity analysis could not be performed.

6.3.3.7 Summary of findings table

A 'Summary of findings' table (**Table 6-2**²⁶⁻²⁹) was produced for: (1) change in peripheral neuropathy impairment composite score; (2) pain; (3) corneal nerve fiber length (CNFL); (4) sensory nerve action potential (SNAP) amplitudes of the sural nerve; (5) motor nerve conduction velocity (NCV) of the peroneal nerve. The Grading of Recommendations, Assessment, Development and Evaluation (GRADE) approach was used to assess the certainty of evidence.³⁰

Table 6-2. Summary of findings of table

Outcome	No. of participants (studies)	Certainty of the evidence (GRADE) ^a	Relative effect (95% CI)	Anticipated absolute effects (95% CI) ^b	
				Placebo oral supplements	Omega-3 oral supplements
PN impairments, assessed with a validated, composite neuropathy impairment score(s), with a follow-up range of 16 to 25 weeks	128 (2 RCTs)	Low ^c	RR 0.58 (0.43 to 0.77)	Risk: 758 per 1,000	Risk difference: 318 fewer per 1,000 (432 fewer to 174 fewer)
Symptoms of PN	-	-	-	-	-
Pain, assessed using a validated, patient-assessed pain scale(s) at 35-days of follow-up	41 (1 RCT)	Very low ^d	-	The mean pain score was 47.2	MD relative to placebo 4.0 units higher (10.18 lower to 18.18 higher)
Change in CNFL, defined as the total length of nerves in a given area (mm/mm ²), assessed with laser-scanning IVCN at 90-days of follow-up	12 (1 RCT)	Low ^e	-	The mean change in CNFL was -2.7 mm/mm ²	MD relative to placebo 5.6 mm/mm ² higher (2.31 higher to 8.89 higher)

IENFD	-	-	-	-	-
SNAP amplitudes of the sural nerve (μV), with a follow-up range of 16 to 25 weeks	116 (2 RCTs)	Low ^c	-	The mean SNAP amplitudes of the sural nerve was 6.02 μV	MD 4.19 μV higher (2.19 higher to 6.19 higher)
Motor NCV of the peroneal nerve (ms^{-1}), with a follow-up range of 16 to 25 weeks	116 (2 RCTs)	Low ^c	-	The mean motor NCV of the peroneal nerve was -43.0 ms^{-1}	MD 1.99 ms^{-1} higher (0.51 lower to 4.49 higher)

Abbreviations: CI, confidence interval; CNFL, corneal nerve fiber length; IENFD, Intraepidermal nerve fiber density; IVCN, *in-vivo* confocal microscope; MD, mean difference; NCV, nerve conduction velocity; PN, peripheral neuropathy; RR, risk ratio; SNAP, sensory nerve action potential.

^aGRADE Working Group grades of evidence are: 'high' certainty: we are very confident that the true effect lies close to that of the estimate of the effect; 'moderate' certainty: we are moderately confident in the effect estimate: the true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different; 'low' certainty: our confidence in the effect estimate is limited: the true effect may be substantially different from the estimate of the effect; 'very low' certainty: we have very little confidence in the effect estimate: the true effect is likely to be substantially different from the estimate of effect.

^bThe risk in the intervention group (and its 95% confidence interval) is based on the assumed risk in the comparison group and the relative effect of the intervention (and its 95% CI).

^cData derived from two studies (Esfahani et al.,²⁶ n=71; Ghoreishi et al.,²⁷ n=57); low number of events does not meet optimal information size (OIS), as defined in the GRADE handbook. Both studies were appraised as having a low risk of bias in most domains, however ref 20 was funded by industry; this was considered a crucial limitation for one criterion, for one of two included studies, sufficient to lower the confidence in the certainty of the effect.

^dData derived from one study (Fontani et al.,²⁸ n=46), which was industry funded. Study involved a small number of participants, where the pain outcome was not measured in the context of peripheral neuropathy.

^eData derived from one study (Chinnery et al.,²⁹ n=12) involving a very small number of participants.

6.3.4 Results

6.3.4.1 Characteristics of included studies

The electronic searches yielded 933 non-duplicate citations (**Figure 6-1**). Full-texts were obtained for 45 studies, judged to meet or potentially meet the eligibility criteria, including three articles that were translated into English (two in Russian,^{31,32} one in Chinese³³). In total, 15 RCTs met the eligibility criteria and were included; Table S3 (**Appendix E**) provides a list of studies excluded at the full-text stage, and the primary reason for their exclusion.

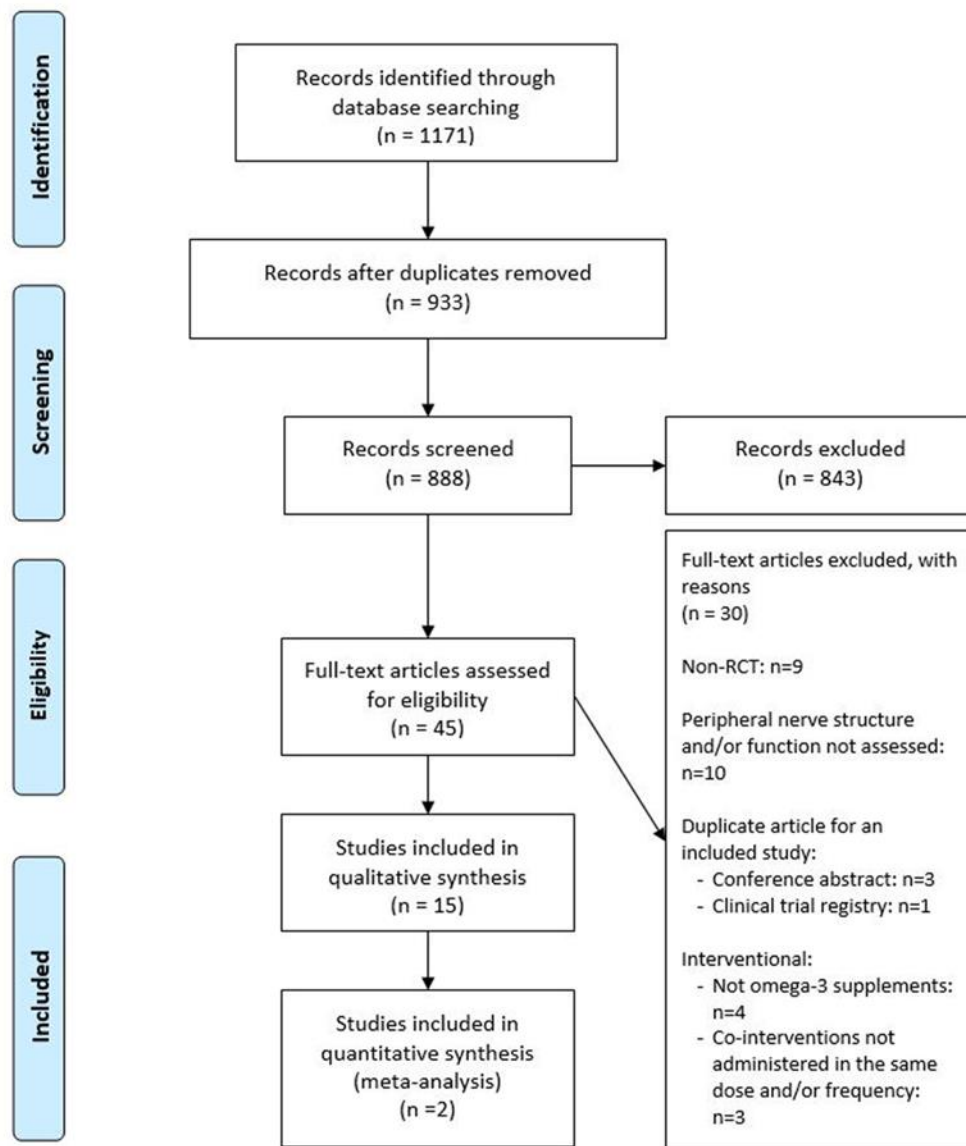


Figure 6-1. PRISMA flow chart of the literature search process

Table 6-3^{26-29,34-44} summarizes the main characteristics of the included studies. Detailed study information is provided in Table S2 (**Appendix E**). Of the 14 RCTs, 12 were full-text articles,^{26-29,34-37,39,40,42,44} two were conference abstracts^{38,43} and one was a trial registry entry.⁴¹ Four of the studies were considered to be from the same trial (Wessex Evaluation of fatty Liver and Cardiovascular markers in non-alcoholic fatty liver disease (NAFLD) with OMacor thErapy trial (WELCOME) trial), including two full-text articles^{37,39} and two conference abstracts.^{38,43} The trials were conducted in seven countries: one in Australia,²⁹ three in Iran,^{26,27,34} one in Italy,²⁸ one in Japan,⁴² one in Spain,⁴⁴ five in the United Kingdom^{37-40,43} and three in the United States.^{35,36,41}

Study sample sizes ranged from 12 to 101 participants and examined the effects of omega-3 PUFA oral supplementation in clinical populations undergoing chemotherapy,^{26,27,34} with non-alcoholic fatty liver disease,^{37-39,43} mental or physiological stress,^{36,40,42} diabetes mellitus^{41,44} dry eye disease,²⁹ chronic inflammatory pain²⁸ and pre-hypertension.³⁵ The follow-up periods of included studies ranged from one to 18 months. Thirteen of the included studies assessed peripheral nerve structure or function in the upper or lower extremities (i.e., arm or leg).^{26-28,35-42,44} One study assessed peripheral nerve structure in the cornea²⁹ and one conference abstract did not report the location of the peripheral nerve assessment.⁴³ The unit of analysis for all studies was per participant, except for the one study where peripheral nerve assessment was conducted in the eye,²⁹ for which the unit of analysis was the enrolled eye of the participant.

Table 6-3. Main characteristics of included studies

Study (year)	Article type	Type of participants	Intervention (dosage/day)	Comparator (dosage/day)	Follow-up period
Anoushirvani et al (2018) ³⁴	Full text	63 adults undergoing taxol treatment (omega-3 group: 21; vitamin E group: 21; control group: 21)	Omega-3 640 mg three times a day (total dosage: 1920 mg/day); form not specified	Placebo (type and dosage not reported)	3 months
Carter et al. (2012) ³⁵	Full text	67 adults with normo-tension (treatment group: 19, control group: 19) or pre-hypertension (treatment group: 15, control group: 14)	Fish oil pills (9000 mg) containing DHA (1100 mg) and EPA (1600 mg); form not specified	Olive oil (9000 mg)	8 weeks
Carter et al. (2013) ³⁶	Full text	67 adults with normo-tension being put under mental stress (treatment group: 34, control group: 33)	Fish oil pills (9000 mg) containing DHA (1100 mg) and EPA (1600 mg); form not specified	Olive oil (9000 mg)	8 weeks
Chinnery et al. (2017) ²⁹	Full text	12 adults with moderate dry eye disease (treatment group: 8, control group: 4)	Omega-3 capsules containing DHA (~500 mg) and EPA (~1000 mg) in either re-esterified triacylglyceride or phospholipid forms	Olive oil (1500 mg)	90 days
Clough et al. (2016) ^{37a}	Full text	90 adults with non-alcoholic fatty liver disease, with and without type-2 diabetes, without diabetic neuropathy or retinopathy (treatment group: 44, control group: 46)	Omacor omega-3 capsules (combined 4000 mg/day) containing DHA + EPA; in ethyl ester form	Olive oil (4000 mg), reported in clinical trials registry	15-18 months

Esfahani et al. (2016) ²⁶	Full text	71 adults with Metastatic colon cancer (stage III), undergoing treatment with oxaliplatin (130 mg/m ² IV) and oral capecitabine (1000 mg/m ² , b.i.d) for eight cycles (treatment group: 36, control group: 35)	Omega-3 pearls (1920 mg) containing DHA (1037 mg) + EPA (192 mg); form not specified	Sunflower oil (dosage not reported)	25 weeks
Fontani et al. (2010) ²⁸	Full text	46 female adults with fibromyalgia or widespread musculoskeletal pain (treatment group: 23, control group 23)	Omega-3 capsules (4000 mg) containing DHA (800 mg), EPA (1600 mg) and other types of omega-PUFAs: alpha linolenic, stearidonic, eicosatetraenoic and ocosapentaenoic acid (400 mg); form not specified	Sunflower oil containing oleic acid (4000 mg)	35 days
Ghoreishi et al. (2012) ²⁷	Full text	69 ^b female adults with breast cancer, undergoing treatment with 175 mg/m ² paclitaxel for four courses (treatment group: 30, control group 27)	Omega-3 capsules (1920 mg) containing DHA (1037 mg) and EPA (192 mg); form not specified	Sunflower oil (dosage not reported)	16 weeks
McCormick et al. (2015) ^{38a}	Conference abstract	101 adults with non-alcoholic fatty liver disease, with and without type-2 diabetes, without diabetic neuropathy (assignment not reported).	Omega-3 (4000 mg) in ethyl ester form	Placebo, type not reported	15-18 months
McCormick et al. (2015a) ^{39a}	Full text	100 adults with non-alcoholic fatty liver disease, with and without type-2 diabetes, without overt neuropathy or retinopathy (treatment group: 51, control group: 49)	Omacor omega-3 capsules (4000 mg) containing DHA (1520 mg) and EPA (1840 mg) in ethyl ester form	Olive oil (4000 mg) containing ~67% oleic acid, ~15% linoleic acid, ~15% palmitic acid. ~2% stearic acid and ~1% α -linolenic acid	15-18 months

Monahan et al. (2004) ⁴⁰	Full text	18 healthy adults being put under physiological stress (treatment group: 9, control group: 9)	Omega-3 capsules (10000mg) containing DHA (2000mg) and EPA (3000mg), form not specified	Olive oil (10000mg)	1 month
NCT00931879 (2009) ⁴¹	Clinical trials registry	38 adults with type-2 diabetes mellitus (treatment group: 19, control group: 19)	Lovaza omega-3 capsules (combined 4000mg/day) containing DHA + EPA, in ethyl ester form	Not reported	12 months
Ochi et al. (2017) ⁴²	Full text	21 normal adults undergoing eccentric contraction exercises (treatment group: 10, control group: 11)	Fish oil capsules (2400mg) containing DHA (260mg) and EPA (600mg), form not specified	Corn oil (2400mg)	62 days
Palmer et al. (2014) ^{43a}	Conference abstract	86 adults with non-alcoholic fatty liver disease (assignment not reported)	High dose purified omega-3 fatty acids (4000mg)	Placebo, type not reported	15-18 months
Stiefel et al. (1999) ⁴³	Full text	18 adults with type-1 diabetes (treatment group: 8, control group: 10)	Omega-3 capsules containing DHA (330mg) and EPA (630mg), form not specified	“Usual diet”, no supplements	90 days

Abbreviations: CI, confidence interval; CNFL, corneal nerve fiber length; d, days; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; IVCN, in-vivo confocal microscope; IENFD, Intraepidermal nerve fiber density; MD, mean difference; mo, months; NCV, nerve conduction velocity; PN, peripheral neuropathy; RR, risk ratio; SNAP, sensory nerve action potential; wk, weeks.

^aAs these studies are derived from the same trial (the WELCOME study), data may derive from the same cohort of participants.

^bn=12 participants were lost to follow up, including 5 in the treatment group and 7 in the placebo group

The daily dose of omega-3 supplements ranged from 640 mg/day to 5000 mg/day (combined DHA and EPA). The DHA component ranged from 260 mg/day to 2000 mg/day and the EPA component ranged from 192 mg/day to 3000 mg/day. None of the omega-3 interventions were administered with any other co-interventions. One study included an additional intervention arm, involving a Vitamin E treatment.³⁴ For the comparator (control) intervention, six studies used olive oil supplements,^{29,35-37,39,40} three studies used sunflower oil supplements,²⁶⁻²⁸ one study used corn oil supplements,⁴² one study used usual diet (i.e., no supplement intervention),⁴⁴ and four studies did not specify the form or concentration of the placebo.^{34,38,41,43}

6.3.4.2 Risk of bias in included studies

Figure 6-2^{26-29,34-44} summarizes the risk of bias assessments. No study was judged as having a low risk of bias in all seven domains (Figure S1; **Appendix E**). The most well conducted risk of bias categories were attrition bias (10/15 studies judged as low risk) and blinding of participants and personnel (9/15 studies judged as low risk). None of the studies were considered to have a high risk of selection bias. The least well-performing domains were for reporting bias, as related to incomplete outcome reporting (3/15 studies high risk) and other risk of bias (4/15 high risk, due to industry funding).

	Random sequence generation (selection bias)	Allocation concealment (selection bias)	Blinding of participants and personnel (performance bias)	Blinding of outcome assessment (detection bias)	Incomplete outcome data (attrition bias)	Selective reporting (reporting bias)	Other bias
Anoushirvani et al. (2018) ³⁴	+	?	+	?	+	?	+
Carter et al. (2012) ³⁵	?	?	?	?	?	?	+
Carter et al. (2013) ³⁶	?	?	?	?	+	?	+
Chinnery et al. (2017) ²⁹	+	+	+	+	+	?	+
Clough et al. (2016) ³⁷	+	?	+	+	+	?	?
Esfahani et al. (2016) ²⁶	+	+	+	+	+	-	-
Fontani et al. (2010) ²⁸	+	+	+	+	-	?	-
Ghoreishi et al. (2012) ²⁷	+	+	+	+	+	-	+
McCormick et al. (2015) ³⁸	?	?	-	-	?	?	?
McCormick et al. (2015a) ³⁹	+	+	+	+	+	+	?
Monahan et al. (2004) ⁴⁰	?	?	?	?	?	?	+
NCT00931879 (2009) ⁴¹	?	?	+	+	+	+	-
Ochi et al. (2017) ⁴²	+	?	+	?	+	-	-
Palmer et al. (2014) ⁴³	?	?	?	?	?	?	+
Stiefel et al. (1999) ⁴⁴	?	?	-	-	+	?	+

Figure 6-2. Risk of bias summary. Review authors' judgments about each risk of bias item for each included study. RCT, randomized controlled trial.

6.3.4.3 Effects of interventions

Table 6-2 summarizes findings for the pre-specified outcomes. There were no extractable data for the following outcomes: peripheral neuropathy symptoms; patient-reported disability; intraepidermal nerve fiber density (IENFD); minimum F-wave latency of the peroneal, tibial, median or ulnar nerves; corneal sensory function; or warm detection thresholds in the skin.

6.3.4.3.1 Primary outcome

Two studies reported data on the incidence of PN, quantified using a validated, composite score.^{26,27} Both studies considered populations at risk of chemotherapy-induced peripheral neuropathy (CIPN), where none of the participants had PN at baseline, and the incidence of neuropathy was quantified at the study endpoint, using the reduced Total Neuropathy Score (rTNS). Pooled data (**Figure 6-3**^{26,27}) showed a significant reduction in the relative risk of developing PN with omega-3 supplementation (RR 0.58, 95% CI: 0.43 to 0.77, $p=0.0002$, $n=128$, studies: 2) relative to placebo, at the end of the follow-up period (ranging from 16 to 25 weeks). The level of heterogeneity was negligible ($I^2 = 0\%$). One additional study reported the incidence of PN in a population at risk of CIPN.³⁴ Results from this study could not be pooled because the type of scoring system was not reported by the authors, and a response was not received from the authors four weeks after requesting this information via email. In this study,³⁴ the incidence of CIPN in the omega-3 group was reported to be 28.6% (6/21 participants), compared to 71.4% (15/21 participants) in the placebo group. The certainty of evidence, judged using GRADE, was low (downgraded due to imprecision and risk of bias).

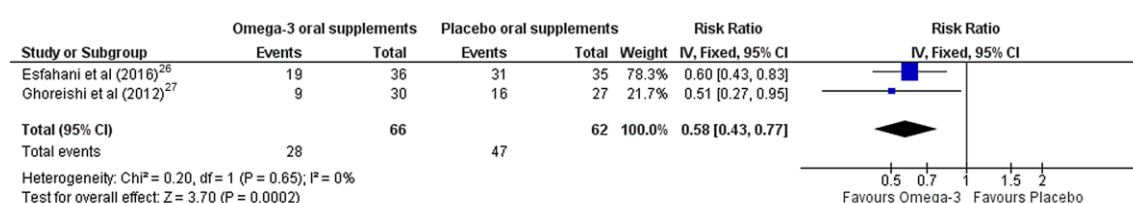


Figure 6-3. Forest plot of comparison for incidence of peripheral neuropathy in chemotherapy-induced peripheral neuropathy. Omega-3 vs placebo oral supplements for peripheral neuropathy impairments (quantified by reduced total neuropathy score). Data are reported as the incidence of peripheral neuropathy at the end of the follow-up period (ranging from 16 weeks (Ghoreishi et al.²⁷) to 25 weeks (Esfahani et al.²⁶)).

6.3.4.3.2 Secondary outcome: pain

One study reported data relating to pain intensity, measured using visual analogue scores (VAS) ranging from 0 to 100, in individuals with chronic inflammatory disorders.²⁸ No significant difference was observed between the

omega-3 and placebo supplement groups after 35 days (mean $\pm$ SEM: omega-3: 47.2 ± 4.7 , $n=23$ versus placebo: -43.2 ± 5.5 , $n=23$; mean difference (MD) calculated using values in the paper: 4.0 units, 95% CI: -10.18 to 18.18, $p=0.58$). The certainty of evidence, judged using GRADE, was very low (downgraded for risk of bias, indirectness and imprecision, as data were from one small study ($n=46$), which was industry funded, and pain outcomes were not measured in the context of PN).

6.3.4.3.3 Secondary outcome: anatomical markers

Data relating to the change, from baseline, in central corneal nerve fiber length (CNFL), defined as the total length of all nerve fibers in the image capture frame (mm/mm^2) using *in vivo* confocal microscopy, were obtained from one study.²⁹ In this RCT, a significant increase in CNFL was reported after 90 days of omega-3 PUFA oral supplementation, compared with placebo (mean $\pm$ SEM: omega-3: 2.90 ± 1.6 , $n=8$ versus placebo: -2.7 ± 1.6 mm/mm^2 , $n=4$; $p=0.01$), in people with dry eye disease. The certainty of evidence for this outcome, judged using GRADE, was low (downgraded for imprecision, as data were from a single study involving a very small number of participants, $n=12$).

6.3.4.3.4 Secondary outcome: Nerve conduction studies (NCS)

For NCS, a quantitative data synthesis was performed to combine two studies,^{26,27} where the clinical questions were similar, and the study outcomes were reported as mean (SD) or median (IQR) at the end of follow-up (summarized in **Table 6-4**^{26,27,41,44} and **Table 6-5**^{26,27,41,44}). Both of these trials investigated the effects of omega-3 supplementation on the development of CIPN. One additional study reported on the effects of omega-3 supplementation on the development of CIPN³⁴; however, this study could not be included in the meta-analyses as the results reported in the article could not be interpreted, and a response was not received from the authors further to requesting missing outcome data. Results as reported in the publication are supplied in Table S2 (**Appendix E**).

Table 6-4. Sensory nerve conduction studies results

		Pooled data from two studies (Esfahani et al. (2016) ²⁶ ; Ghoreishi et al. (2012) ²⁷) ^a	Unpublished data extracted from one study (NCT00931879 (2009) ⁴¹) ^b	Data extracted from one study (Stiefel et al. (1999) ⁴⁴) ^c
Study population		CIPN	Type-2 diabetes	Type-1 diabetes
SNAP amplitudes (μV)	Sural nerve	MD: 4.19, 95% CI: 2.19 - 6.19, p<0.0001, favoring omega-3 supplements	No significant inter-group difference in endpoint values (MD: 0.32, 95% CI: -4.38 to 5.02, p=0.89)	No significant inter-group difference in change from baseline (MD: -4.20, 95% CI: -8.72 to 0.32, p=0.07)
	Median nerve	No data available	No significant inter-group difference in endpoint values (MD: 3.6, 95% CI: -8.02 to 15.22, p=0.54)	No significant inter-group difference in change from baseline between groups (MD: -3.6, 95% CI: -11.54 to 4.34, p=0.37)
	Ulnar nerve	MD: 5.57, 95% CI: 0.42 to 10.72, p=0.03, favoring omega-3 supplements	No significant inter-group difference in endpoint values (MD: -5.28, 95% CI: -17.33 to 6.77, p=0.37)	No data available
SNAP latencies (ms)	Sural nerve	No data available	No significant inter-group difference in endpoint values (MD: 0.02, 95% CI: -0.38 to 0.42, p=0.92)	No data available
	Median nerve	No data available	No significant inter-group difference in endpoint values (MD: -0.47, 95% CI: -1.19 to 0.25, p=0.20)	No data available

SNAP latencies (ms) (cont'd)	Ulnar nerve	No data available	No significant inter-group difference in endpoint values (MD: 0.11, 95% CI: -0.22 to 0.44, p=0.52)	No data available
Sensory NCV (ms⁻¹)	Sural nerve	Due to considerable statistical heterogeneity ($I^2=87\%$), a pooled estimate is not presented. Individual study findings were: Esfahani et al. (2016) ²⁶ : significant difference in endpoint values favoring the omega-3 group (mean $\pm$ SD: omega-3: 42.29 ± 15.51 versus placebo: 29.67 ± 13.01 ms ⁻¹ , p=0.018). Ghoreishi et al. (2012) ²⁷ : no significant inter-group difference in endpoint values (mean $\pm$ SD: omega-3: 54.79 ± 8.35 versus placebo: 52.52 ± 8.05 ms ⁻¹ , p=0.514).	No significant inter-group difference in endpoint values (MD: -0.42, 95% CI: -4.84 to 3.98, p=0.85)	No significant inter-group difference in change from baseline (MD: -2.87, 95% CI: -6.78 to 1.04, p=0.15)
	Median nerve	No data available	No significant inter-group difference in endpoint values (MD: 2.83, 95% CI: -4.23 to 9.89, p=0.43)	No significant inter-group difference in change from baseline (MD: -0.48, 95% CI: -2.09 to 1.13, p=0.56)
	Ulnar nerve	No significant effect: MD: 2.21, 95% CI: -0.64 to 5.06, p=0.13	No significant inter-group difference in endpoint values (MD: -1.72, 95% CI: -6.54 to 3.10, p=0.48)	No data available

Abbreviations: CI, confidence interval; CIPN, chemotherapy-induced peripheral neuropathy; MD, mean difference; NCV, nerve conduction velocity; SNAP, sensory nerve action potential

^aData are reported as the mean difference in endpoint values between omega-3 supplementation group and placebo groups, at the end of the follow-up period (at 6 months follow-up, with acceptable follow-up ranges between 3 and 9 months from baseline).

^bMD and p-values are calculated based upon endpoint data provided (mean $\pm$ SEM) in the clinical trial registry, for the omega-3 supplementation group and the placebo group, at 12 months follow-up.

^cMD and p-values are calculated based upon values provided, in change from baseline, for the omega-3 supplementation group compared to the placebo group, at 12 months follow up.

Table 6-5. Motor nerve conduction studies results

		Pooled data from two studies (Esfahani et al. (2016)²⁶; Ghoreishi et al. (2012)²⁷)^a	Unpublished data extracted from one study (NCT00931879 (2009)⁴¹)^b	Data extracted from one study (Stiefel et al. (1999)⁴⁴)^c
Study population		CIPN	Type-2 diabetes	Type-1 diabetes
Distal CMAP amplitudes (mV)	Peroneal nerve	MD: 1.08, 95% CI: 0.11 to 2.05, p=0.03, favoring omega-3 group	MD: -0.53, 95% CI: -0.78 to -0.28, p<0.00001 favouring control group	No significant inter-group difference in change from baseline (MD: -4.03, 95% CI: -11.92 to 3.86, p=0.32)
	Tibial nerve	MD: 2.36, 95% CI: 0.40 to 4.32, p=0.02, favoring omega-3 group	No significant inter-group difference in endpoint values (MD: -0.28, 95% CI: -0.70 to 0.14, p=0.19)	No data available
	Median nerve	No data available	MD: 0.85, 95% CI: 0.49 to 1.21, p<0.00001, favoring omega-3 group	No significant inter-group difference in change from baseline (MD: 0.85, 95% CI: -2.66 to 4.36, p=0.64)
	Ulnar nerve	No significant inter-group difference in change from baseline (MD: 1.16, 95% CI: -0.19 to 2.52, p=0.09)	No data available	No data available
Distal CMAP latencies (ms)	Peroneal nerve	No significant inter-group difference in change from baseline (MD: -0.59, 95% CI: -1.28 to 0.09, p=0.09)	No significant inter-group difference in endpoint values (MD: -0.38, 95% CI: -0.77 to 0.01, p=0.06)	No data available

Distal CMAP latencies (ms) (cont'd)	Tibial nerve	MD: -1.02, 95% CI: -1.45 to -0.59, $p < 0.00001$, favoring omega-3 group	MD: 1.76, 95% CI: 1.38 to 2.14, $p < 0.00001$, favoring control group	No data available
	Median nerve	No data available	MD: -0.37, 95% CI: -0.62 to -0.12, $p = 0.004$, favouring omega-3 group	No data available
	Ulnar nerve	MD: -0.27, 95% CI: -0.53 to -0.01, $p = 0.04$, favoring omega-3 group	No data available	No data available
Motor NCV (ms⁻¹)	Peroneal nerve	No significant inter-group difference (MD: 1.99, 95% CI: -0.51 to 4.49, $p = 0.12$)	No significant inter-group difference in endpoint values (MD: -0.40, 95% CI: -1.25 to 0.45, $p = 0.35$)	No significant inter-group difference in change from baseline (MD: 0.85, 95% CI: -1.63 to 3.33, $p = 0.50$)
	Tibial nerve	Due to considerable statistical heterogeneity ($I^2 = 74\%$), a pooled estimate is not presented. Both Esfahani et al. ²⁶ and Ghoreishi et al. ²⁷ reported no significant inter-group difference in endpoint values (Esfahani: mean $\pm$ SD: omega-3: 45.67 ± 6.18 versus placebo: 42.34 ± 4.68 ms ⁻¹ , $p = 0.123$; Ghoreishi: mean $\pm$ SD: omega-3: 45.16 ± 4.24 versus placebo: 46.03 ± 6.65 ms ⁻¹ , $p = 0.359$).	MD: -3.55, 95% CI: -6.59 to -0.51, $p = 0.02$, favoring control group	No data available

Motor NCV (ms⁻¹) (cont'd)	Median nerve	No data available	No significant inter-group difference in endpoint values (MD: 0.85, 95% CI -0.12 to 1.82, p=0.08)	Significant change relative to baseline in the omega-3 group (+2.12 ± 1.35) versus placebo group (-0.80 ± 2.34; p<0.01). Calculated MD: 2.92, 95% CI: 1.19 to 4.65, p=0.0009
	Ulnar nerve	No significant inter-group difference (MD: 1.92, 95% CI: -1.19 to 5.02, p=0.23)	No data available	No data available
F-wave latencies (s)	Peroneal nerve	No data available	No data available	No data available
	Tibial nerve	No data available	No data available	No data available
	Median nerve	No data available	No data available	No data available
	Ulnar nerve	No data available	No data available	No data available

Abbreviations: CI, confidence interval; CIPN, chemotherapy-induced peripheral neuropathy; CMAP, compound muscle action potential; MD, mean difference; NCV, nerve conduction velocity

^aData are reported as the mean difference in endpoint values between the omega-3 and placebo supplement groups, at the end of the follow-up period (at 6 months follow-up, with acceptable follow-up ranges between 3 and 9 months from baseline).

^bMD and p-values are calculated based upon endpoint data provided (mean ± SEM) in the clinical trial registry, for the omega-3 supplementation group and the placebo group, at 12 months follow-up.

^cMD and p-values are calculated based upon values provided, in change from baseline, for the omega-3 supplementation group compared to the placebo group, at 12 months follow up.

Considering the other two studies that reported on this outcome, one RCT⁴⁴ reported the change from baseline of sensory NCS in the sural and median nerves, and of motor NCS in the median and peroneal nerves at 90 days of follow-up, in individuals with type-1 diabetes. One unpublished study⁴¹ provided end-point data on sensory NCS in the sural, median and ulnar, nerves, and motor NCS in the median, tibial and peroneal nerves in a population with type-2 diabetes mellitus, at 12 months of follow-up. Data from these two studies^{41,44} could not be pooled due to clinical heterogeneity, or because data was reported by the authors in different formats that could not be combined.

Sensory NCS

As summarized in **Table 6-4**, pooled data for SNAP amplitudes, from the two CIPN trials, showed a significant difference in mean values between the intervention and control groups, favoring omega-3 PUFA supplementation, in both the sural nerve (MD: 4.19 μ V, 95% CI: 2.19 to 6.19, $p < 0.0001$, $n = 116$, studies: 2) (**Figure 6-4A**^{26,27}) and ulnar nerve (MD: 5.57 μ V, 95% CI: 0.42 to 10.72, $p = 0.03$, $n = 116$, studies: 2) (**Figure 6-4B**^{26,27}), at the study endpoint (ranging from 16 to 25 weeks). For both these analyses, the level of heterogeneity was negligible ($I^2 < 2\%$).

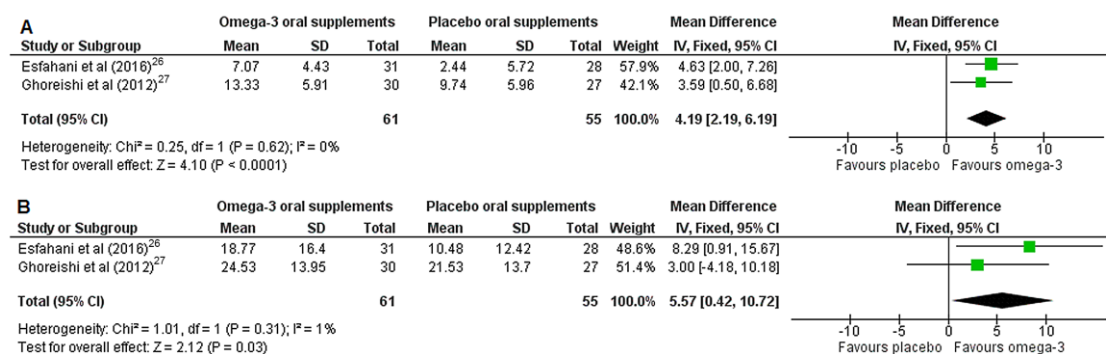


Figure 6-4. Forest plots of comparison for SNAP amplitudes. Omega-3 vs placebo oral supplements for the effect on sensory nerve action potential (SNAP) amplitudes of the (A) sural nerve and (B) ulnar nerve (μ V) in chemotherapy-induced peripheral neuropathy. Data are reported at the end of the follow-up period (ranging from 16 weeks (Ghoreishi et al.²⁷) to 25 weeks (Esfahani et al.,²⁶ data calculated from median and interquartile range)).

One of these studies²⁶ had significant inter-group differences at baseline, with the omega-3 PUFA supplement group having higher baseline SNAP amplitudes (sural nerve: median (interquartile range (IQR)): omega-3: 9.95 μ V (5.52-16.40) *versus* placebo: 5.50 μ V (3.40-14.10), $p=0.033$; ulnar nerve: median (IQR): omega-3: 27.90 μ V (16.82-42.77) *versus* placebo: 17.50 μ V (12.40-35.50), $p=0.144$). In this study, the authors also reported the percentage change from baseline for SNAP amplitudes in both nerves, with a greater relative reduction in the placebo group (sural nerve: omega-3: -36.68% *versus* placebo: -100.00%, p -value not reported; ulnar nerve: omega-3: -18.28% *versus* placebo: -42.06%; p -value not reported). The certainty of evidence, for the effect of omega-3 PUFA supplementation on sural SNAP amplitudes, was considered low, on the basis of imprecision and risk of bias.

Pooling data from the same two trials,^{26,27} NCV was not significantly different between groups in the ulnar nerve (MD: 2.21 ms^{-1} , 95% CI: -0.64 to 5.06, $p=0.13$, $n=116$, studies: 2) (Figure S2; **Appendix E**), at the end of the follow-up period (ranging from 16 to 25 weeks). For sural NCV, a pooled estimate is not presented as considerable statistical heterogeneity was evident ($I^2 = 87\%$). In the study by Esfahani et al.,²⁶ a significant difference was found, favoring the omega-3 intervention compared to placebo for sural NCV at the end of 25 weeks of follow up (mean $\pm$ SD: omega-3: 42.29 $\pm$ 15.51 *versus* placebo: 29.67 $\pm$ 13.01 ms^{-1} , $p=0.018$). Ghoreishi et al.²⁷ reported no significant inter-group difference at 16 weeks (mean $\pm$ SD: omega-3: 54.79 $\pm$ 8.35 *versus* placebo: 52.52 $\pm$ 8.05 ms^{-1} , $p=0.514$).

Motor NCS

Table 6-5 summarizes data relating to motor NCS. Pooling data from the same two CIPN trials,^{26,27} there was a significant inter-group difference in distal compound motor action potential (CMAP) amplitudes, favoring the omega-3 PUFA intervention, in both the peroneal nerve (MD 1.08 mV, 95% CI: 0.11 to 2.05, $p=0.03$, $n=116$, studies: 2) (**Figure 6-5A**^{26,27}) and tibial nerve (MD: 2.36 mV, 95% CI: 0.40 to 4.32, $p=0.02$, $n=116$, studies: 2) (**Figure 6-5B**^{26,27}), at

the end of the follow-up period (ranging from 16 to 25 weeks). For both of these analyses, statistical heterogeneity was below the pre-defined significance threshold ($I^2 < 60\%$).

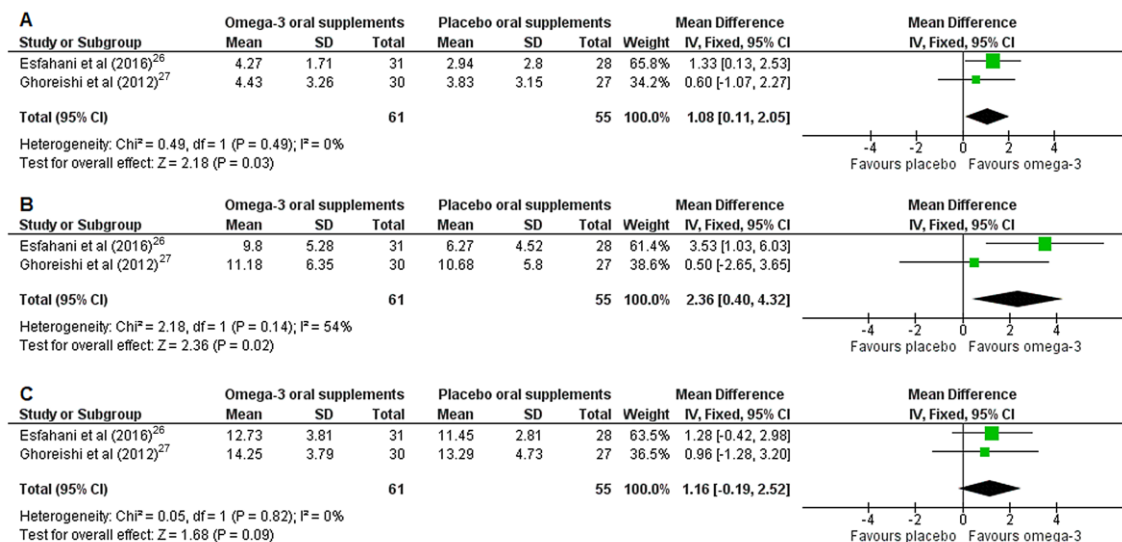


Figure 6-5. Forest plots of comparison for CMAP amplitudes. Omega-3 vs placebo oral supplements for distal compound motor action potential (CMAP) amplitude of the (A) peroneal nerve, (B) tibial nerve and (C) ulnar nerve (mV) in chemotherapy-induced peripheral neuropathy. Data are reported at the end of the follow-up period (ranging from 16 weeks (Ghoreishi et al.²⁷ to 25 weeks (Esfahani et al.,²⁶ data calculated from median and interquartile range)).

No significant inter-group difference was evident for distal CMAP amplitude of the ulnar nerve (MD: 1.16 mV, 95% CI: -0.19 to 2.52, $p=0.09$, $n=116$, studies: 2) (Figure 6-5C^{26,27}). There was a significant difference at the study endpoint in distal CMAP latency, favoring the omega-3 intervention, in both the tibial nerve (MD: -1.02 ms, 95% CI: -1.45 to -0.59, $p<0.00001$, $n=116$, studies: 2) (Figure S3A; **Appendix E**) and ulnar nerve (MD: -0.27 ms, 95% CI: -0.53 to -0.01, $p=0.04$, $n=116$, studies: 2) (Figure S3B; **Appendix E**). No significant inter-group differences were found in the distal CMAP latency of the peroneal nerve (MD: -0.59 ms, 95% CI: -1.28 to 0.09, $p=0.09$, $n=116$, studies: 2) (Figure S3C; **Appendix E**). For motor NCV, there were no significant difference between treatment groups in the peroneal (MD: 1.99 ms^{-1} , 95% CI: -0.51 to 4.49, $p=0.12$, $n=116$, studies: 2) (Figure S4A; **Appendix E**) or ulnar nerves (MD: 1.92 ms^{-1} , 95% CI: -1.19 to 5.02, $p=0.23$, $n=116$, studies: 2) (Figure S4B; **Appendix E**) at the end of the follow-up period. For each of these analyses,

statistical heterogeneity was below the pre-defined significance threshold. For motor NCV in the tibial nerve, a pooled estimate is not presented as considerable statistical heterogeneity was evident ($I^2 = 74\%$). Both studies that reported no significant inter-group difference in tibial NCV at the study endpoints (see **Table 6-5**). The certainty of evidence for the effect of omega-3 PUFA supplementation on peroneal motor NCV was considered low, downgraded on the basis of imprecision and risk of bias.

6.3.4.3.5 Secondary outcome: Small fiber function

One study²⁸ reported data relating to small nerve fiber function, measured using Quantitative Sensory Testing (QST), in a population with musculoskeletal pain and fibromyalgia. Although there was a reduction in the number of positive tender points clinically, the authors reported no significant difference between the omega-3 (n=23) and placebo (n=22) groups for mechanical thresholds (measured using von Frey hair esthesiometry: mean $\pm$ SEM: omega-3: 432.0 ± 47.0 versus placebo: 422 ± 55.0 g; p-values not reported), heat pain thresholds (mean $\pm$ SEM: omega-3: 41.7 ± 0.5 versus placebo: 43.2 ± 0.6 °C), cold detection thresholds (mean $\pm$ SEM: omega-3: 7.7 ± 1.1 versus group: 6.9 ± 1.3 sec), or cold pain thresholds (mean $\pm$ SEM: omega-3: 0.2 ± 1.8 versus placebo: -0.3 ± 2.1 °C), at 35 days of follow-up.

Another unpublished study⁴¹ reported the change from baseline, in cold detection thresholds and cold pain thresholds (measured from the big toe) in people with type-2 diabetes, at 12 months of follow-up. Inter-group differences were calculated using values provided in the clinical trial registry. Cold detection thresholds were not significantly different between the omega-3 (n=19) and placebo (n=19) groups (mean $\pm$ SEM: omega-3: -3.76 ± 1.54 versus placebo: -1.17 ± 1.18 °C; calculated MD -2.59 °C, 95% CI: -6.57 to 1.38 , p=0.20). Omega-3 supplementation was calculated to preserve cold pain thresholds as compared to placebo (omega-3: -1.41 ± 2.16 versus placebo: 5.66 ± 1.91 °C, 95% CI: -12.71 to -1.42 , p=0.01) at 12 months of follow-up.

6.3.4.3.6 Secondary outcome: Adverse events

As summarized in Table S4 (**Appendix E**), five trials reported data relating to adverse events.^{27,28,37,39,41} Together, these studies involved 343 participants (omega-3: n=172; placebo: n=171). One study,²⁸ involving 46 participants, reported no adverse events over a 35-day period. In two trials (Clough et al.³⁷, n=90; McCormick et al.³⁹ n=100) it was reported that there were no important adverse events over the course of each study (ranging from 450 to 540 days). In Ghoreishi et al.,²⁷ four out of 69 participants discontinued the study due to "critical conditions" (omega-3: n=3 *versus* placebo: n=1; calculated risk ratio (RR): 2.91, 95% CI: 0.32 to 26.66). Although there was no significant inter-group difference in the risk of developing a critical condition, this finding should be viewed with caution due to the low number of events and wide confidence intervals. In NCT00931879,⁴¹ involving 69 participants over a 180-day intervention period, a total of 10 adverse events (with three judged to be "serious") occurred in individuals supplemented with omega-3 PUFAs, and 11 adverse events (with four judged to be "serious") were reported in the placebo group.

With respect to the risk of developing an adverse event, data were pooled from three studies.^{27,28,41} There was no significant difference in the relative risk of developing an adverse event (Figure S5A; **Appendix E**) with an omega-3 or placebo oral supplement (RR 0.98, 95% CI: 0.56 to 1.70, p=0.94, n=148, studies: 3). Considering the specific risk of a serious adverse event (defined as an event requiring hospitalization or prolonged admission, a life-threatening event or death), data were combined from four trials,^{28,37,39,41} with no significant difference (RR 0.50, 95% CI: 0.10 to 2.41, p=0.39, n=439, studies: 4) between interventions (Figure S5B; **Appendix E**).

6.3.5 Discussion

6.3.5.1 Summary of main results

This is the first systematic review to consider the effects of omega-3 PUFA oral supplementation on peripheral nerve health. Fifteen relevant RCTs, involving a total of more than 560 adult participants, were identified and reported data relating to peripheral nerve structure and/or function in a variety of acute and chronic conditions, including CIPN and diabetes. Based upon data from two trials, this review finds with low certainty (as judged using the GRADE approach), that omega-3 fatty acid supplementation is beneficial for reducing the incidence of PN secondary to neurotoxicity caused by the chemotherapy agents oxaliplatin and paclitaxel. Furthermore, there was low certainty that omega-3 supplementation may assist with preventing chemotherapy-induced neurotoxic sensory loss, as demonstrated by the relative preservation of SNAP amplitudes. Based upon data from four trials, there were no significant concerns regarding the safety of omega-3 supplementation, with the relative risk of adverse events and serious adverse events being similar to a placebo intervention. There is currently limited RCT data to confidently draw conclusions regarding the effects of omega-3 PUFA oral supplementation in systemic conditions other than CIPN, where peripheral nerves are also vulnerable to damage (e.g., diabetes mellitus).

The methodological rigor of the trials included in this systematic review varied considerably, with the most variable bias domain being for ‘other sources of bias,’ relating to commercial funding. In a recent systematic review, industry sponsorship of interventional studies was shown to significantly bias results towards more favourable conclusions regarding the sponsor’s products, compared to studies not funded by industry.⁴⁵ This limitation reduces the overall certainty of the evidence, and the ability to draw definitive conclusions.

6.3.5.2 Overall completeness and applicability of evidence

i. Studies investigating omega-3 PUFA supplementation in chemotherapy-induced peripheral neuropathy (CIPN)

The PNS is particularly vulnerable to damage from neurotoxicity induced by chemotherapeutic agents.² CIPN produces a dose-limiting effect, affecting 30-70% of people undergoing chemotherapy; it can severely affect quality-of-life, as symptoms can persist after cessation of chemotherapy.⁴⁶ Two common chemotherapy compounds are paclitaxel, used to treat breast, ovarian and lung cancers, and oxaliplatin, typically used to treat colorectal cancer.⁶ Paclitaxel, a taxane-derived agent, has been proposed to interfere with microtubule-based axonal transport in the peripheral nerves, affecting predominantly distal-sensory responses.⁴⁷ Oxaliplatin, a platinum-based compound, induces PN as two distinct clinical presentations. The acute form is characterised by altered sensory and motor symptoms on exposure to cold temperatures, caused by chelating effects of the oxalate group to Ca^{2+} and Mg^{2+} ions, interrupting Na^+ currents and channel kinetics.⁴⁸ In contrast, platinum binding to neuronal DNA induces a cumulative, sensory-predominant form of neuropathy, leading to axonal degradation, neuronal degeneration and dorsal root ganglion apoptosis.⁴⁹ Although paclitaxel and oxaliplatin are proposed to have different underlying mechanisms of neurotoxicity leading to axonal dysfunction, both compounds induce PN that share similarities in their pathophysiology, involving interrupted axonal transport, neuronal injury and inflammation, oxidative stress and mitochondrial damage.^{50,51} Furthermore, both compounds can induce PN, which, in its chronic form, manifests as cumulative sensory neuropathy/ neuronopathy, affecting sensory cell bodies of the dorsal root ganglion; these neurons are particularly vulnerable as they are not protected by the blood-nerve barrier.^{46,49}

Pooled data from two studies (n=128 participants), which considered the effects of omega-3 PUFA supplementation in oxaliplatin- or paclitaxel-induced neuropathies, showed that omega-3 supplements in doses <2000 mg/day (combined EPA and DHA), taken over the course of chemotherapy and for one

month after cessation, significantly reduced the incidence of PN (RR 0.58, 95% CI: 0.43 to 0.77, $p=0.0002$). In both trials,^{26,27} the scale for measuring the incidence of PN was the rTNS score, a reliable, validated psychometric tool for assessing CIPN that considers multiple domains: subjective sensory symptoms, pin sensibility, vibration sensibility, muscle strength, deep tendon reflexes, and sural and peroneal NCS amplitudes.⁵²

Oxaliplatin- and paclitaxel-induced neuropathies frequently present as a progressive reduction of SNAP amplitudes, with relatively smaller changes to motor NCS or needle electromyography.^{50,53} A decrease in sural SNAP amplitudes of more than 50% from baseline is used as a criterion for diagnosing sensory neuropathy associated with chemotherapy.^{50,53} The presented data suggest that omega-3 PUFA supplements reduce sensory axonal loss induced by neurotoxic chemotherapy, relative to placebo, as indicated by the pooled results for sural SNAP amplitudes (MD: 4.19 μ V, 95% CI: 2.19 to 6.19, $p<0.0001$). However, the capacity for this analysis to derive clear estimates of overall treatment effects may be affected by inter-group baseline differences in one trial (i.e., the placebo group had lower baseline SNAP amplitudes).²⁶ Notwithstanding this difference, in both RCTs, the omega-3 PUFA supplementation group had a lower relative change from baseline in SNAP amplitude, compared with the placebo group (36.68% versus 100% with oxaliplatin)²⁶; no change versus 28.9% with paclitaxel.²⁷ Regarding the motor NCS, the omega-3 intervention group showed more favourable peroneal and tibial CMAP amplitudes, and tibial distal motor latency at the end of the follow-up period compared to the placebo group. However, in one of the two studies,²⁶ the baseline distal motor latencies throughout the study appeared very prolonged; these values are considered outside of the normal limits of any known techniques or laboratories. Additionally, the temperature of patient extremities was not mentioned, which can significantly impact recorded parameters, and relative differences between groups could potentially cause spurious results.

Currently, there are no therapeutic agents recommended for CIPN prophylaxis. Chemotherapy dose-modification and/or cessation are considered the most successful approaches for preventing functional compromise.^{6,46} International clinical guidelines for the 'Prevention and management of chemotherapy-induced peripheral neuropathy in survivors of adult cancers', published by the American Society of Clinical Oncology Clinical Practice (2014),⁵⁴ reported that on the basis of a dearth of high-quality, consistent evidence, further research was essential to identify agents for preventing CIPN. This guideline referenced the results of one of the RCTs included in the present review, involving 57 participants with breast cancer,²⁷ but did not recommend prescribing omega-3 fatty acid supplements for CIPN prevention at that time, given that the "promising-appearing result (had) not been replicated."⁵⁴ A recently-published RCT, involving 71 participants undergoing chemotherapy for metastatic colon cancer, by the same investigator group,²⁶ lends further support to the potential benefit of omega-3 supplementation in reducing sensory nerve damage, although larger-scale, multicentre trials are still lacking.

ii. Studies investigating omega-3 supplementation for other causes of PN

There was no capacity to quantitatively synthesize data from RCTs that investigated omega-3 PUFA oral supplementation for other causes of PN, as studies considered different disease states, used alternate outcome measures, did not report data quantitatively and/or reported data in different formats (e.g., change from baseline versus endpoint).

Two trials^{41,44} assessed an omega-3 intervention in people with diabetes mellitus. Of these studies, one trial⁴¹ included individuals with type-2 diabetes, and reported no significant difference in any routine NCS parameter between the omega-3 and placebo supplement groups. However, baseline data for these participants were not provided. The other trial⁴⁴ included people with type-1 diabetes. In this study, the only statistically significant difference was a relatively preserved median NCV in the omega-3 intervention arm. However, all lower

limb sensory and motor parameters, including sural SNAP amplitudes and conduction velocities were comparable between groups. Given that diabetes-related neuropathy typically presents in a length-dependent manner, with sensory symptoms developing before motor signs of weakness, the clinical significance of an isolated difference in a forearm motor parameter on NCS is questioned. Also, in these studies, temperature was not reported, and baseline data were not available, which significantly limits the interpretation of these results.

Notably, few of the included trials quantified small nerve fiber structure or function. One study quantified small nerve fiber structure in the cornea²⁹ and two trials reported on small nerve fiber function in the skin, measured using QST.^{28,41} While routine diagnostic procedures, such as NCS, are validated, reliable methods for quantifying large nerve fiber function,⁵⁵ these techniques have limited capacity to detect changes in small nerve fiber integrity, which may precede large nerve fiber involvement in PN.⁵⁶ Monitoring changes to small nerve fibers, which have regenerative capacity, may also provide a more accurate reflection of the efficacy of therapeutic interventions.^{57,58} In this regard, corneal confocal microscopy allows for the visualisation of small nerve fibers *in vivo* and is a sensitive, non-invasive marker of PN.⁵⁹ Moreover, increased CNFL is a marker for nerve regeneration.⁶⁰ In type-1 diabetes, CNFL was shown to improve after kidney and pancreas transplantation, suggesting it could be an indicator of early nerve repair that may be undetected by conventional neuropathy assessments.⁶¹

Whilst data from RCTs relating to effect of omega-3 fatty acid supplementation in a diabetes population could not be pooled, another open-label study,⁶² involving 40 participants, investigated the effect of 12 months of omega-3 supplementation in the form of seal oil. These authors reported a 29% improvement, from baseline, in CNFL (change from baseline: 8.3 ± 2.9 mm/mm² to 10.1 ± 3.7 mm/mm²; $p = 0.002$), using 10 mL/day of seal oil containing 2330 mg of long-chain omega-3 fatty acids (750 mg EPA, 560 mg DPA and

1020 mg/day DHA). This study was not included in the current systematic review as it is a single-arm, open-label trial, with no control or placebo group. Peripheral nerve function was also comprehensively assessed in this study using NCS, QST and autonomic testing; each of these parameters showed no significant change from baseline over 12 months.

The two studies included in this review that tested QST^{28,41} adopted this technique as the only measure of small fiber function. One of these studies²⁸ assessed patients with musculoskeletal pain and fibromyalgia. Although there is some evidence that fibromyalgia patients have abnormal C-fiber nociceptors with spontaneous activity and increased mechanosensitivity,⁶³ as well as abnormal IVCN morphology,⁶⁴ the role of small fiber neuropathy in this condition remains poorly understood. Furthermore, caution needs to be taken with using QST alone as a measure of small fiber function, as abnormalities with this psychophysical test can be due to central sensitisation, rather than peripheral nerve dysfunction.

Finally, only three studies included patient-reported outcomes,²⁶⁻²⁸ two of which were incorporated into a composite score.^{26,27} The use of patient-reported outcomes may not only reflect early-onset changes in PN,⁶ but might additionally provide valuable information on how treatments affect an individual and/or impact quality-of-life,^{6,56} and thus should be considered in future research in the field.

6.3.5.3 Dosage of the omega-3 PUFA intervention

The daily dose of long-chain omega-3 fatty acids (i.e., combined EPA and DHA) in the included studies was highly heterogeneous. Three studies adopted <1000 mg/day,^{34,42,44} six studies used dosages of 1000 to 3000 mg/day,^{26-29,35,36} and six studies used ≥ 3000 mg/day.^{37-41,43} No studies exceeded a daily dose of 5000 mg/day (combined EPA and DHA), considered the upper safety limit for adults by the European Food Safety Authority.⁶⁵ Dosage is likely an important consideration for the potential therapeutic effects of omega-3 supplements on

peripheral nerves. For example, systematic reviews show that doses <3000 mg/day are largely ineffective for improving cardiovascular outcomes.^{66,67} The currently recommended dosing regimen of omega-3 ethyl esters for hypertriglyceridemia is 4000 mg/day.⁶⁸ In contrast, doses in the range of ~1500 mg/day have been shown to modulate ocular inflammation.⁶⁹ Thus, further research is essential to clarify the optimal dose for the specific applications of preventing and/or treating PN.

Another consideration relates to the form of omega-3 supplement. Of the six articles using the highest doses, four specified that the omega-3 fatty acids were supplemented in ethyl ester form (Omacor or Lovaza).^{37-39,41} Only one other study specified the form of omega-3 supplements as triglyceride and/or phospholipid subtypes.²⁹ The incorporation of DHA and EPA into plasma, and their subsequent bioavailability, may vary depending on the preparation and formulation.⁷⁰ Intestinal absorption of fatty acids is further influenced by the concentration of fatty acids in the diet; this is particularly true for ethyl esters forms, which have enhanced absorption in the presence of high-fat diets.⁷¹ Diet may not only influence the bioavailability of fatty acids,⁷⁰ but can also play a role in determining the overall effects of the intervention, given therapeutic efficacy may differ in populations having omega-3 sufficient diets, compared to those who are omega-3 deficient.⁷² Capturing participant dietary information (including changes to foods rich in omega-3 PUFAs) and including information relating to the form of omega-3 supplement is therefore recommended for future RCTs.

Participant compliance was monitored in eight of the 15 studies.^{27-29,35,36,40,42,44} One study measured serum concentrations of EPA and DHA, before and after the supplementation period, which was reported by the authors to be significantly different between study groups at the end of the follow-up period.²⁷ One study reported in its clinical trial registry that compliance would be monitored by measuring the concentrations of omega-3 fatty acids in serum phospholipids, using gas chromatography; this outcome measure, however, was not reported in the published article.²⁶ Five studies did not report monitoring

compliance.^{34,37-39,43} Treatment adherence not only potentially influences treatment effect(s), but may also be an indicator of tolerability, particularly for higher doses.⁷³ None of the included studies reported concerns relating to the safety or tolerability of omega-3 PUFA supplementation in the adult study populations. Considering data from studies that reported adverse events, there was no significant difference in the incidence of adverse events or serious adverse events between intervention arms (RR 0.98, 95% CI: 0.56 to 1.70, $p=0.94$; and RR 0.50, 95% CI: 0.10 to 2.41, $p=0.39$ respectively).

6.3.5.4 Quality of the evidence

Overall, the certainty of the evidence relating to the pre-specified outcome measures was graded as 'low' or 'very low', using the GRADE approach.³⁰ The most frequent reason for downgrading, which was relevant to all of the pre-specified outcomes, was imprecision. The certainty of evidence was downgraded for the primary outcome, and secondary outcomes for the effects on SNAP amplitudes in the sural nerve, and motor NCV in the peroneal nerve for imprecision and risk of bias, acknowledging that data were pooled from two clinically-similar studies, conducted by the same research group, involving a small sample of participants ($n=57$) (as not meeting the optimal information size (OIS) as defined in the GRADE handbook,³⁰ and where one of the two studies²⁶ was funded by industry).

The certainty of evidence for effects on pain was downgraded to very low, for imprecision, as data was derived from one small study,²⁸ indirectness, as pain outcome was not measured in the context of peripheral neuropathy, and risk of bias due to industry funding. The certainty of evidence for effects of treatment on CNFL was downgraded to low, for imprecision, as data was derived from one very small study ($n=12$).²⁹ The certainty of evidence for the effects of omega-3 supplementation on symptoms of PN and on IENFD could not be reported, as no data were available for these outcomes.

6.3.6 Conclusions

In conclusion, clinical management of PN remains a challenge, with an established need for novel neuroprotective agents to prevent progressive nerve damage, to improve health outcomes for patients. To date, several RCTs have assessed the effects of systemic omega-3 supplementation on PN outcomes, in a range of acute and chronic conditions. This systematic review finds, with low certainty, that omega-3 oral supplementation (dose: <2000 mg/day combined EPA+DHA for 12 to 15 weeks), reduces the incidence of CIPN. Currently, there is insufficient evidence to ascertain with any certainty if omega-3 oral interventions can improve peripheral nerve structure or function in chronic diseases, such as diabetes mellitus.

Peripheral neuropathy (PN) is a potential complication of several systemic conditions, most commonly diabetes. Progressive PN is a leading cause of pain and disability, and can have a profound negative impact upon quality-of-life. While for some specific conditions, such as chronic inflammatory demyelinating polyneuropathy, Guillain-Barre syndrome and the vasculitic neuropathies, immunomodulatory therapies are available, for most other causes of PN there are no disease-modifying treatment for preventing progressing nerve damage. There is thus a strong clinical need for research to identify novel therapeutic strategies.

The findings from this review encourage a need for the conduct of appropriately-powered, well-designed RCTs to investigate the potential therapeutic effects of omega-3 PUFA oral supplements on peripheral nerve integrity. These trials should incorporate outcomes that are sensitive to detecting changes to both small and large peripheral nerve fibers. The design of future trials should also consider participants' baseline omega-3 PUFA intake, and patient-reported outcomes, to assess the impact of the intervention and disease on quality-of-life.

There are currently no universally-accepted outcome measures for assessing peripheral nerve integrity. The recommendations provided by the European Neuromuscular Centre (ENMC) International Workshop: Selection of Outcome Measures for Peripheral Neuropathy Clinical trials were considered in selecting outcome measures for this review.⁵⁶ The outcomes focussed on sensory and motor functional tests. The development of a 'core outcome set' would enable outcomes reported in different RCTs to be more readily compared (and meta-analysed to enable a comprehensive synthesis of the evidence), as recommended by the Core Outcome Measures in Effectiveness Trials (COMET) initiative.⁷⁴

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6.3.7 References

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7

Chapter 7. Oral omega-3 PUFA supplementation for peripheral nerve health in type 1 diabetes: a randomised controlled trial

7.1 Introduction

Our systematic review in **Chapter 6** identified only two RCTs that examined the effects of oral omega-3 PUFA supplements on outcomes relating to peripheral nerve structure and/or function in individuals with diabetes (NCT00931879, 2017; Stiefel et al., 1999). Furthermore, only one of these studies is published (Stiefel et al., 1999), and a meta-analysis of the data from the two RCTs was not feasible owing to data having been reported in different formats. Based on the evidence identified in the systematic review, it remains unclear whether oral omega-3 PUFAs supplements are useful for improving peripheral nerve health in individuals with diabetes.

Findings from this review support the rationale for a comprehensive investigation into the therapeutic potential of omega-3 fatty acids as a peripheral neuro-regenerative agent in diabetes, for which optimum care is challenged by a lack of treatments. This chapter describes a randomised, double-masked, placebo-controlled clinical trial, conducted to evaluate the effects of long-chain omega-3 PUFA supplements on peripheral nerve parameters in individuals with type 1 diabetes, over a six-month period.

7.2 Investigating the neuroPRotective effect of Oral Omega-3 Fatty acid Supplementation in type 1 diabetes (nPROOFS₁): a randomised, placebo-controlled trial.

The entire **Section 7.2** is the substantially unaltered manuscript that has been submitted for publication in *Lancet Diabetes and Endocrinology*. Supplementary materials for this manuscript include the clinical trial protocol (**Appendix F**), statistical analysis plan (**Appendix G**), and results of the exploratory analyses (**Appendix H**).

7.2.1 Abstract

Background: Omega-3 polyunsaturated fatty acids (PUFAs) have neuroprotective properties. This study evaluated the effects of oral omega-3 PUFA supplementation on peripheral nerve parameters in individuals with type 1 diabetes.

Methods: Single-site, randomised, double-masked, placebo-controlled trial. Eligible participants with type 1 diabetes were assigned (1:1) to either omega-3 PUFA (fish oil; 1800 mg/day) or placebo (olive oil; 600 mg/day) supplements for 180 days. Participants, investigators, and outcome assessors were masked. The primary outcome was change from baseline in central corneal nerve fibre length (CNFL; mm/mm²) at day 180. Secondary outcomes included change in corneal nerve fibre density (CNFD) and corneal nerve branch density (CNBD), corneal sensitivity, quantitative sudomotor axonal reflex testing, cutaneous silent periods, nerve conduction studies, axonal nerve excitability, and ocular surface measures. Efficacy was analysed following intention-to-treat (ITT). Safety assessments included diabetic retinopathy grade and adverse events. The trial was prospectively registered on the Australian and New Zealand Clinical Trials Registry (ACTRN12618000705280).

Results: Between July 6, 2017 and September 17, 2019, 43 participants received omega-3 PUFA (n=21) or placebo (n=22) supplements. All participants, except for two in the placebo group, completed the trial. At day 180, the estimated increase in CNFL in the omega-3 PUFA group (n=21), compared to placebo (n=22), was 2.70 mm/mm² (95%CI: 1.64 to 3.75; $p<0.001$). Treatment effects in CNFD and CNBD paralleled CNFL findings. The systemic Omega-3 Index, measuring erythrocyte eicosapentaenoic and docosahexaenoic acids, increased relative to placebo (3.3%; 95%CI: 2.4 to 4.2). There were no significant differences in most nerve excitability parameters, or small or large nerve fibre function. Adverse events were similar between groups.

Interpretation: Oral omega-3 PUFA supplements impart corneal neuroregenerative effects in type 1 diabetes, supporting their role in modulating peripheral nerve health.

Funding: University of Melbourne Neuroscience Interdisciplinary grant (LED, JTK, LJR, RJM); Rebecca Cooper Medical Research Foundation grant (LED).

7.2.2 Research in Context

Evidence before this study

A 2019 systematic review and meta-analysis by our team, conducted in accordance with Cochrane guidelines, evaluated the efficacy and safety of oral omega-3 polyunsaturated fatty acid (PUFA) supplements for improving peripheral nerve structure and function in randomised controlled trials (RCTs). This review, which involved searches in PubMed, Embase, Cochrane CENTRAL, and clinical trial registries from inception to February 2019, identified two RCTs examining the effects of oral omega-3 PUFA supplements on outcomes relating to peripheral nerve structure and/or function in individuals with diabetes. Only one of these studies is published; this study was unmasked and reported a relative difference in median nerve conduction velocity in the omega-3 PUFA supplemented group (960 mg/day combined EPA and DHA; n=8) compared with a non-supplemented (n=10) group that maintained their usual diet, after 90 days, in individuals with type 1 diabetes. The unpublished study, which involved 44 individuals with type 2 diabetes, considered the effects of omega-3 fatty acid ethyl esters (4000 mg/day, combined EPA and DHA) versus placebo, on motor and sensory nerve conduction studies and quantitative sensory testing thresholds. Data from the two RCTs could not be meta-analysed, however, as they were reported in different formats. Based on the evidence identified in this review, we concluded it was unclear whether oral omega-3 PUFAs supplements are useful for improving peripheral nerve health in people with diabetes.

A single arm, open-label trial, investigating the effect of 12-months oral omega-3 PUFA supplementation (2330 mg/day long-chain omega-3 PUFAs) in 40 individuals with type 1 diabetes also reported a 29% improvement in corneal nerve fibre length (CNFL) relative to baseline. Whilst these results are promising, the authors of this trial confirmed the need for adequately-powered RCTs, with a comparator group and masking, to definitively ascertain whether omega-3 PUFA supplements are beneficial for peripheral nerve health in individuals with type 1 diabetes.

Added value of this study

This study is, to our knowledge, the first randomised, double-masked, placebo-controlled trial to evaluate the efficacy of long-chain omega-3 PUFA supplements on peripheral nerve parameters in individuals with type 1 diabetes. This trial demonstrates that six months of oral long-chain omega-3 PUFA supplementation, dosed at 1800 mg/day (combined EPA and DHA), improved corneal sub-basal nerve plexus parameters, relative to placebo. In this trial, we also found that, compared to placebo, the omega-3 PUFA supplement group had improved recovery in the application of hyperpolarising currents and tear film function at six months. Using a robust study design and trial methodology, including double-masking, this trial provides high-quality clinical evidence that supports a role for omega-3 PUFA supplements in modulating corneal neuroregeneration in type 1 diabetes.

Implications of the available evidence

Given a lack of effective therapies for attenuating peripheral nerve damage in diabetic sensorimotor polyneuropathy (DSP), the positive findings from the current RCT indicate that oral omega-3 PUFA supplements could be a potential disease-modifying intervention for improving peripheral nerve health in type 1 diabetes. In DSP, it has been established that changes to corneal sub-basal nerve plexus parameters occur prior to the involvement of large nerve fibres. It is thus possible that improvements in corneal nerve density could be one of the earliest indicators of peripheral nerve recovery, potentially preceding improvements in sensory and/or motor nerve function. This RCT justifies the conduct of larger, long-term clinical trials in well-defined populations, to further evaluate the efficacy of oral omega-3 PUFA supplements on peripheral small and large nerve fibre function.

7.2.3 Introduction

Diabetic sensorimotor polyneuropathy (DSP) affects approximately 50% of individuals with diabetes (Boulton et al., 2004). Progressive nerve damage, which can lead to debilitating symptoms and disability, has profound quality of life and socioeconomic impacts (Mehra et al., 2014). Early-stage DSP is associated with loss of small sensory nerve fibres in the cornea (Breiner et al., 2014). Quantification of corneal sub-basal nerve plexus parameters, from *in vivo* confocal microscopy (IVCM) imaging, provides a non-invasive, sensitive and reliable marker for monitoring DSP progression (Lewis et al., 2020). IVCM has also been used to monitor corneal nerve regeneration following pancreas and kidney transplant in individuals with type 1 diabetes (Azmi et al., 2019).

Intensive glycaemic control is currently the only established method for slowing DSP progression in type 1 diabetes (Diabetes Control and Complications Trial Research Group, 1993). There is an unmet need for disease-modifying therapies to reduce progressive peripheral nerve damage. A promising intervention is omega-3 polyunsaturated fatty acids (PUFAs), derived from the diet or via supplementation. Long-chain omega-3 PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), impart a range of biological effects, including modulation of cellular signalling and production of anti-inflammatory and neuroprotective mediators (James et al., 2000). In rodent models, omega-3 PUFA supplementation improves sensory function recovery after peripheral nerve injury (Gladman et al., 2012), and reduces corneal nerve loss in experimental diabetes (Coppey et al., 2020).

In a pilot randomised controlled trial (RCT), long-chain omega-3 PUFA supplementation (1500 mg/day for three months) significantly increased corneal nerve density, relative to placebo, in dry eye disease (Chinnery et al., 2017). An open-label, single-arm trial found that long-chain omega-3 PUFA supplementation for 12 months (2330 mg/day) increased corneal nerve fibre length (CNFL) by 29% relative to pre-treatment levels, in type 1 diabetes. However, our recent systematic review identified a paucity of RCTs evaluating

the effects of oral omega-3 PUFA supplementation on peripheral nerve structure and/or function in individuals with diabetes (Zhang et al., 2020a).

To address this evidence gap, we conducted a double-masked RCT to investigate the effects of six-months of long-chain omega-3 PUFA supplementation on peripheral nerve parameters in type 1 diabetes.

7.2.4 Methods

7.2.4.1 Study design

This was a single-centre, double-masked, randomised, two-arm, parallel-group, superiority, placebo-controlled intervention trial. Procedures related to the trial were undertaken at the Department of Optometry and Vision Sciences, University of Melbourne, and Department of Neurology/Neurophysiology, St Vincent's Hospital Melbourne, Victoria, Australia.

This study was approved by St Vincent's Hospital Human Research Ethics Committee (HREC/17/SVHM/236), registered with University of Melbourne Human Research Ethics Committee (Ethics ID: 1851526), and undertaken in accordance with the Declaration of Helsinki. The trial was registered prospectively on the Australian and New Zealand Clinical Trials Registry (ACTRN12618000705280) and conducted according to an *a priori* protocol (**Appendix F**). Written informed consent was obtained from all participants prior to undertaking any study-related procedures. Trial reporting conforms with the 2010 CONSORT Statement (Schulz et al., 2010).

7.2.4.2 Participants

A full list of participant eligibility criteria is provided in the study protocol (**Appendix F**.) Key inclusion criteria were: adults with type 1 diabetes and a score of >2 on the Michigan Neuropathy Screening Instrument (MNSI) (Feldman et al., 1994). Key exclusion criteria were: neuropathy secondary to causes other than diabetes; scheduled or planned systemic or ocular surgery

over the study duration; known bleeding disorder; use of systemic anticoagulants other than aspirin; females currently pregnant, breastfeeding, or planning a pregnancy over the study duration; known hypersensitivity to any component of the study supplements or agents used for the study; history of gas permeable contact lens wear; diabetic retinopathy worse than moderate in either eye according to the simplified Early Treatment Diabetic Retinopathy Study (ETDRS) grading (ETDRS Research Group, 1991); presence of active ocular infection or inflammation, or ocular conditions that could disrupt normal corneal nerve morphology; and consumption of any oral omega-3 PUFA supplements >3 times/week in the past three months.

7.2.4.3 Randomisation and masking

Participants were randomised (1:1) to receive either oral omega-3 PUFA or placebo (olive oil) supplements, according to a computer-generated, randomisation list in block sizes of four. To ensure allocation concealment, the randomisation code was developed by an independent data manager, who held the code until unmasking. The randomisation sequence was provided by the independent data manager to a pharmacist (Dartnell's Pharmacy, Surrey Hills, Victoria, Australia), who packaged the supplements into identical opaque containers that were consecutively numbered to administer to participants. Participants and personnel, including outcome assessors and data analysts, were masked from treatment allocations.

Participants were advised to consume two supplement capsules (1800 mg) per day, for 180±24 days. The omega-3 PUFA (active) group received long-chain triglyceride omega-3 PUFA supplements (Caruso's Natural Health Triple Strength fish oil concentrate, New South Wales, Australia), totalling 1080 mg EPA and 720 mg DHA per day. The placebo group received olive oil supplements (BJP Laboratories Pty Ltd, Queensland, Australia) (Deinema et al., 2017), equivalent to 600 mg/day. Olive oil is an appropriate, inert control for omega-3 PUFA investigations; its major component (oleic acid, an omega-9

monounsaturated fatty acid) does not affect systemic PUFA levels (Lorenz et al., 1989).

Counting of unused supplement capsules, returned at the final visit, was performed by an independent technician. Investigators involved in the collection and analysis of study data were not involved in reconciling returned investigational product.

7.2.4.4 Study procedures

Participants were enrolled at the University of Melbourne, where they underwent eligibility assessment and ocular examinations (see protocol in **Appendix F**). Eligible participants attended St Vincent's Hospital Melbourne, within 35 days, for baseline neurophysiology examinations. Participants were instructed to begin taking their assigned supplements on the day they attended for neurophysiological examination (day 0). Participants attended follow-up visits at days 30 ± 14 and 90 ± 14 . The study endpoint was at day 180 ± 24 from initiation of treatment.

7.2.4.5 Outcome measures

The primary outcome measure was change in central CNFL (mm/mm^2), measured from IVCM images, collected at day 180. Image analysis was performed using automated software (ACCMetrics) (Petropoulos et al., 2014). For each participant, CNFL was recorded as the average measure derived from 12 randomly-selected images of the central cornea (see protocol in **Appendix F**).

Key secondary efficacy outcome measures were change from baseline at day 180 for: central corneal nerve branch density (CNBD, $\text{branches}/\text{mm}^2$) and corneal nerve fibre density (CNFD, $\text{nerves}/\text{mm}^2$), quantified from IVCM images; and central corneal sensitivity thresholds (mbar) to room-temperature and cooled stimuli, measured using non-contact corneal aesthesiometry (SDZ Electronics, Auckland, New Zealand).

Secondary outcome measures were change from baseline at day 180, for: patient-reported quality of life, using the EuroQol five-dimensional five-level descriptive system (EQ-5D-5L) questionnaire (Herdman et al., 2011); cutaneous silent period (CSP) onset latency and duration in the upper and lower limbs; sweat volume and latency in the foot, using quantitative sudomotor axonal reflex testing (QSART); MNSI and the Michigan Diabetic Neuropathy Scores (MDNS) (Feldman et al., 1994); nerve conduction studies (NCS) parameters for sural sensory amplitude, peroneal motor conduction velocity, and tibial minimum F-wave latency; and median nerve axonal excitability, using the TROND protocol (Kiernan et al., 2020), for the following parameters: depolarising threshold electrotonus (TEd) peak, TEd (10-20 ms), TEd (90-100 ms), hyperpolarising threshold electrotonus (TEh) 90-100 ms, superexcitability, subexcitability, resting current-voltage (I/V) slope, strength duration time constant (SDTC).

Safety outcomes were the incidence of adverse events and change from baseline at day 180 in habitual distance visual acuity, intraocular pressure, diabetic retinopathy grading, and blood pathology parameters.

Compliance was assessed using returned capsule counts; participants with compliance <75% or >125% were considered non-compliant. Change in erythrocyte fatty acid concentrations were quantified using dried blood spot analysis (PUFAcoat™ technology, Xerion Pty Ltd, Victoria, Australia), analysed by an independent laboratory (Wait Lipid Analytical Service, Australia) using validated protocols (Liu et al., 2014). Erythrocyte PUFA parameters were analysed for: Omega-3 Index (%), a metric of erythrocyte EPA and DHA concentrations, EPA (%), DHA (%), total omega-6 (%), total omega-9 (%), and the omega-6 to omega-3 ratio.

Exploratory outcomes included change from baseline at day 180 for: sweat volume and latency at the forearm, proximal leg, and distal leg, using QSART; motor NCS of the median, peroneal, and tibial nerves; sensory NCS of the

median, sural, and ulnar nerves; axonal nerve excitability of the median nerve; tear film and ocular surface parameters.

7.2.4.6 Statistical analysis

A total of 21 participants per treatment group was estimated to give 80% power at a two-sided 5% level of significance, assuming a difference between intervention arms in CNFL at Day 180 of 2.9 mm/mm², a standard deviation of 3.2 mm/mm², equal in both groups, and no correlation between baseline and post-baseline measurements (Chinnery et al., 2017).

Statistical analyses were performed according to the intention-to-treat principle, as defined in a statistical analysis plan (see **Appendix G**), finalized prior to unmasking. The primary outcome is presented as the absolute difference in change from baseline, obtained using a constrained longitudinal data analysis model that included visit, treatment, and visit by treatment interaction while assuming a common baseline mean across the two arms and an unstructured variance-covariance. Other continuous outcomes were analysed using the same model, with outcomes log base e transformed prior to model fitting, where applicable, presenting the geometric mean ratio.

For binary and categorical variables, Fischer's exact test was used to compare the proportion of participants from each treatment group regarding the change from baseline during the study.

Analyses of continuous outcomes provide valid inference when the missing data are at most missing at random. Binary and categorical variables were analysed using complete cases. Multiple imputation was not applied, given the low proportion of missing data and the sample size.

Two adjusted models were pre-specified for primary, key secondary, and secondary outcomes, adjusting for age, diabetes duration, HbA1c, and potential imbalances in baseline CNFL and Omega-3 Index. Primary and key secondary

outcomes were also analysed excluding participants who were lost to follow-up or did not return remaining study supplements for compliance assessment. For outcomes with baseline imbalance, a sensitivity analysis was performed by removing the constraint of balance at baseline. Subgroup analyses were planned for the primary outcome for subgroups with CNFL >12.5 mm/mm² and ≤12.5 mm/mm² (Perkins et al., 2018), and the presence/absence of small fibre neuropathy (SFN) at baseline (from QSART and CSP; defined in **Appendix F**) (Kamel et al., 2015). All 95% confidence intervals and p-values were reported two-sided and multiple testing was accounted for using the Benjamini-Hochberg stepwise method with a false discovery rate of 5% for key secondary outcomes.

Masking efficacy was assessed at the final visit using a forced-choice guess (omega-3 PUFA or placebo) by the participant and outcome assessor. Cohen's Kappa (κ) was used to assess the success of masking by calculating the level of agreement between the actual and supposed treatment assignments relative to the guesses made by the participant and outcome assessor, respectively.

Statistical analyses were performed in Stata 16 (StataCorp LP. 2019. College Station, TX).

7.2.4.7 Role of funding source.

The study funder had no role in the study design, data collection, data analysis, data interpretation or report writing. The first and corresponding authors had full access to the study data and take final responsibility for the decision to submit this paper for publication.

7.2.5 Results

Forty-five participants were recruited between July 6, 2017 and September 17, 2019. Forty-three participants were deemed eligible and randomly assigned to receive either omega-3 PUFA (n=21) or placebo (n=22) supplements. All participants in the omega-3 PUFA group and 20 participants in the placebo group completed the study (**Figure 7-1**); two participants were lost to follow-up.

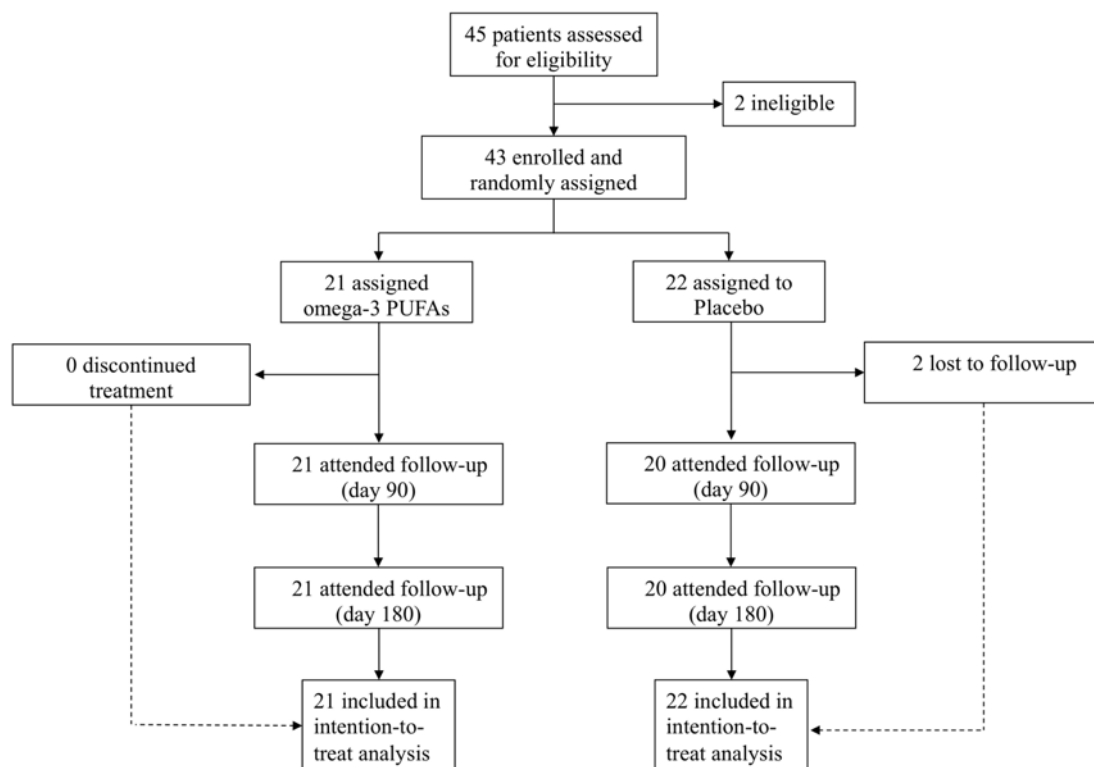


Figure 7-1. CONSORT flow diagram. PUFA=polyunsaturated fatty acid.

*One participant in the omega-3 PUFA group completed the study but did not undergo procedures relating to small or large nerve fibre function in the limbs.

Participants' baseline demographic and clinical characteristics are summarised in **Table 7-1**. Participants in each study group were similar for all key parameters; although, those in the omega-3 PUFA group were, on average, eight years older than those in the placebo group. Age was adjusted for in the pre-planned sensitivity analysis (see **Appendix G**). Concomitant medications are summarised in **Appendix H**.

Table 7-1. Baseline participant characteristics (intention-to-treat sample)

	Omega-3 PUFAs	Placebo
	(n=21)	(n=22)
Male sex, n [%]	9 [43%]	13 [59%]
Age, years	48.1 (19.2)	40.5 (19.6)
Body mass index, kg/m ²	25.1 (3.5)	26.1 (5.2)
Duration of type-1 diabetes, years, median (IQR)	14.0 (6.0-25.0)	16.5 (7.0-26.0)
HbA _{1c}		
Percentage, %	7.34 (0.81)	7.82 (1.11)
Value, mmol/mol	56.7 (8.9)	61.9 (12.2)
Mode of insulin delivery		
Multiple daily injections, n [%]	11 [52%]	10 [46%]
Pump, n [%]	10 [48%]	12 [55%]
Current soft contact lens wear, n [%]	0 [0%]	1 [5%]
History of (any) ophthalmic surgery, n [%]	6 [29%]	5 [23%]
Presence of small fibre neuropathy*, n [%]	6 [30%]	7 [32%]

Data are mean and standard deviation, unless stated otherwise.

*Abnormal small fibre function defined as having abnormal quantitative sudomotor axonal reflex testing parameters or abnormal cutaneous silent period (Kamel et al., 2015).

Data for the primary outcome were analysed for all randomised participants. The estimated between-group change from baseline in CNFL was 1.80 mm/mm² (95% confidence interval (CI): 0.83 to 2.78 mm/mm²) at day 90 (Appendix 3), and 2.70 mm/mm² (95% CI: 1.64 to 3.75 mm/mm²; p<0.001; **Table 7-2**; **Figure 7-2**) at day 180, favouring the omega-3 PUFA group.

Table 7-2. Primary and key secondary central corneal nerve efficacy outcomes (intention-to-treat sample)

	Omega-3 PUFAs			Placebo			Omega-3 PUFAs vs Placebo	
	Baseline (n=21)	Day 180 (n=21)	Change from baseline (n=21)	Baseline (n=22)	Day 180 (n=19)	Change from baseline (n=19)	Estimate (95% CI) (n=43)	p-value*
Primary outcome								
CNFL (mm/mm²)	11.49 (3.34)	13.55 (3.58)	2.06 (1.73)	12.38 (3.21)	11.41 (3.66)	-0.72 (1.68)	2.70 (1.64, 3.75)	<0.001
Key secondary outcomes								
CNFD (nerves/ mm²)	19.93 (7.72)	23.41 (8.08)	3.48 (4.15)	20.27 (7.36)	18.45 (7.57)	-1.57 (4.02)	4.98 (2.51, 7.44)	0.0040
CNBD (branches/ mm²)	19.69 (10.76)	27.06 (13.59)	7.37 (6.34)	23.48 (10.27)	19.54 (12.64)	-3.68 (7.35)	11.23 (7.01, 15.45)	0.0020
Central corneal sensitivity threshold (mbar), median (IQR)								
Room- tempera- ture stimulus [†]	0.47 (0.38 - 0.75)	0.38 (0.25 - 0.60)	-0.12 (-0.20 - -0.00)	0.30 (0.28 - 0.45)	0.30 (0.22 - 0.62)	0.12 (-0.03 - 0.20)	0.84 (0.60, 1.16) ^{†‡}	0.39 [‡]
Cooled stimulus [†]	0.43 (0.30 - 0.70)	0.35 (0.22 - 0.60)	-0.07 (-0.20 - 0.03)	0.32 (0.15 - 0.47)	0.40 (0.20 - 0.52)	0.05 (-0.00 - 0.20)	0.81 (0.54, 1.20) ^{†§}	0.29 [§]

Data are mean and standard deviation, unless stated otherwise. All values are derived from examination of the right eye of participants. CNBD=corneal nerve branch density. CNFD=corneal nerve fibre density. CNFL=corneal nerve fibre length. IQR, interquartile range. PUFAs=polyunsaturated fatty acids.

* Multiplicity-adjusted p-value for the key secondary outcomes.

[†] Descriptive data are presented as median and interquartile range; estimates are presented as geometric mean ratio of the change from baseline in Omega-3 PUFAs vs Placebo at day 180

[‡] In a sensitivity analysis adjusting for imbalance at baseline, the treatment effect is 0.68 (0.45, 1.02); adjusted p-value=0.081

[§] In a sensitivity analysis adjusting for imbalance at baseline, the treatment effect is 0.68 (0.45, 1.04); adjusted p-value=0.077

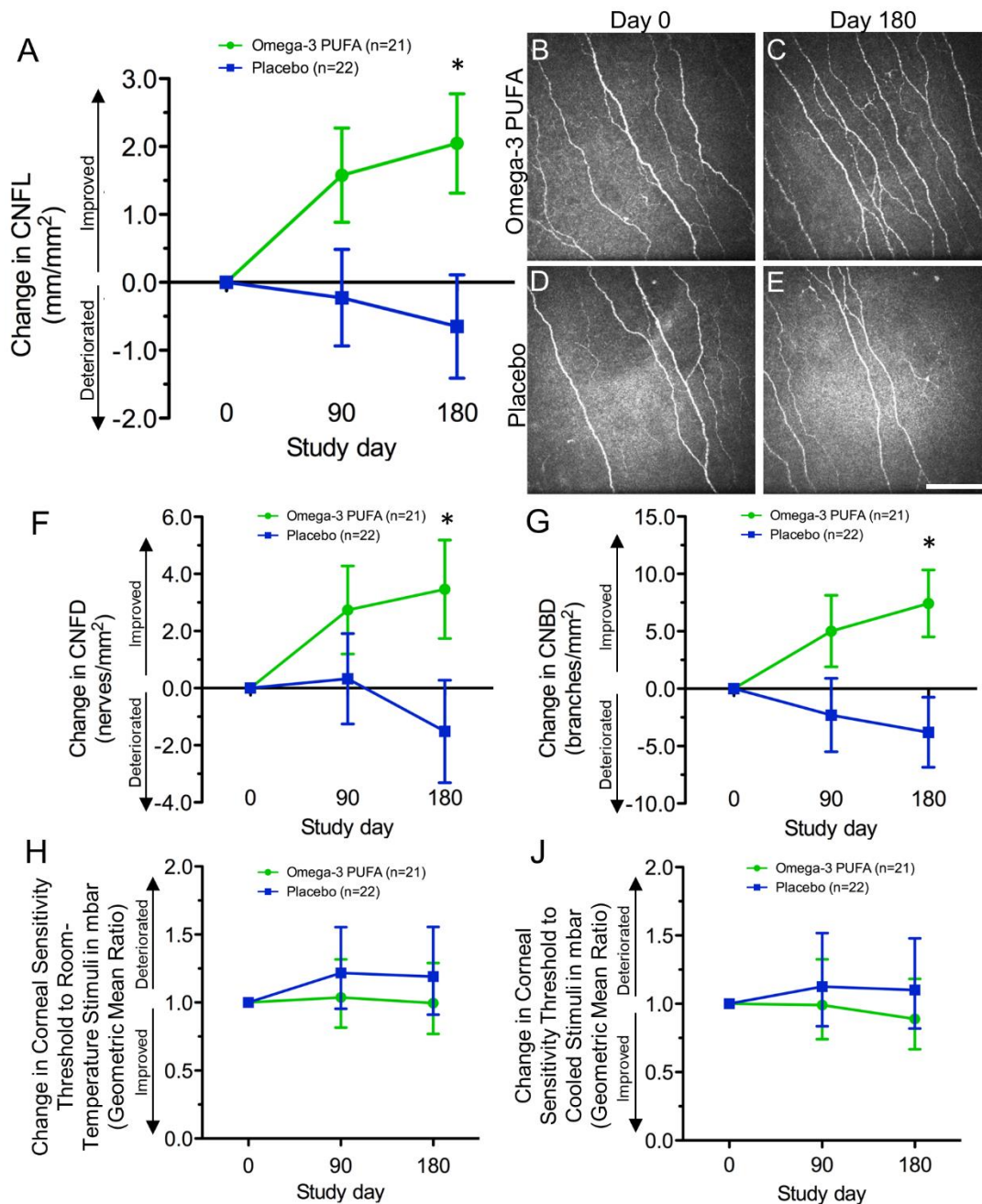


Figure 7-2. Efficacy plots for primary outcome and key secondary corneal parameters (intention-to-treat sample). (A) Estimated absolute change and 95%CI in CNFL over 180 days in the omega-3 PUFA and placebo groups. (B-E) Representative in vivo confocal microscopy images, acquired from the central cornea, showing changes in corneal sub-basal nerve plexus nerve density for two participants, one in the omega-3 PUFA group (B-C) and one in the placebo group (D-E). (F-J) Estimated absolute change and 95%CI over 180 days in the omega-3 PUFA and placebo groups for CNFD (F); CNBD (G); and in estimated relative change in geometric mean ratios and 95%CI for corneal sensitivities to room-temperature (H) and cooled (J) air-stimuli. CNBD=corneal nerve branch density. CNFD=corneal nerve fibre density. CNFL=corneal nerve fibre length. PUFA=polyunsaturated fatty acid.

The relative change in CNFD in the omega-3 PUFA group at day 180 compared to placebo was 4.98 nerves/mm² (95% CI: 2.51 to 7.44 nerves/mm²; adjusted p=0.0040; **Table 7-2**), and in CNBD was 11.23 branches/mm² (95% CI: 7.01 to 15.45 branches/mm²; adjusted p=0.0020; **Table 7-2**).

At day 180, there was no significant between-group difference in the relative change in geometric mean ratio for corneal sensitivity thresholds to a room-temperature (0.84; 95% CI: 0.60 to 1.16; adjusted p=0.39; **Table 7-2**) or a cooled (0.81; 95% CI: 0.54 to 1.20; adjusted p=0.29) stimulus.

At day 180, the estimated change in quality of life score, measured using the EQ-5D-5L questionnaire, in the omega-3 PUFA group relative to placebo was 0 (95% CI: -0.08 to 0.08) units for the EQ-5D-5L index score and -4.9 (-10.9 to 1.2) units for the EQ-5D-5L visual analogue scale score.

At day 180, there was no significant between-group difference in the relative change in geometric mean MNSI score for the omega-3 PUFA group compared to placebo (0.78; 95% CI: 0.55 to 1.10; **Table 7-3**). At baseline, most (86%) participants had no neuropathy, defined using the MDNS. At day 180, there was no significant between-group difference for change in MDNS (**Table 7-3**).

For small nerve fibre function at day 180, the estimated relative change from baseline in the omega-3 PUFA group did not significantly differ from the placebo group, for CSP onset latency and duration in the upper and lower limbs, or sweat volume and latency at the foot measured using QSART (**Table 7-3**). For NCS, there was no between-group difference in change from baseline for sural sensory conduction amplitude (-0.20, 95% CI: -2.26 to 1.86 μ V; **Table 7-3**), peroneal motor conduction velocity (0.01, 95% CI: -2.43 to 2.46 ms⁻¹; **Table 7-3**), or tibial minimal F-wave latency (0.04, 95% CI: -1.19 to 1.27 ms; **Table 7-3**).

Table 7-3. Secondary efficacy outcomes (intention-to-treat sample)

	Omega-3 PUFAs			Placebo			Omega-3 PUFAs vs Placebo	
	Baseline (n=21)	Day 180 (n=21)	Change from baseline (n=21)	Baseline (n=22)	Day 180 (n=19)	Change from baseline (n=19)	Estimate (95% CI) (n=43)	p-value*
Quality of life								
EQ-5D-5L Index	0.84 (0.09)	0.87 (0.10)	0.03 (0.13)	0.87 (0.12)	0.89 (0.16)	0.01 (0.14)	0.00 (-0.08, 0.08)	0.99
EQ-5D-5L VAS	82.9 (9.7)	82.5 (13.2)	-0.4 (9.9)	74.5 (7.2)	79.6 (11.0)	6.0 (9.5)	-4.9 (-10.9, 1.2)	0.11
Small nerve fibre function								
Cutaneous silent periods[‡]								
Upper limb latency onset (ms)	70.80 (10.33) (n=18)	70.62 (7.30) (n=19)	-0.13 (7.52) (n=18)	74.53 (13.22) (n=22)	72.60 (12.27) (n=19)	-2.13 (4.94) (n=19)	0.80 (-2.50, 4.10) (n=42) [¶]	0.64 [¶]
Upper limb duration (ms)	64.88 (18.65) (n=18)	64.88 (11.62) (n=19)	-0.58 (12.88) (n=18)	57.93 (11.42) (n=22)	58.26 (10.44) (n=19)	-0.07 (7.63) (n=19)	2.93 (-1.84, 7.71) (n=42) [#]	0.23 [#]
Lower limb latency onset (ms)	107.03 (9.43) (n=18)	103.79 (14.04) (n=19)	-3.25 (9.74) (n=18)	106.83 (17.76) (n=19)	111.24 (18.97) (n=18)	-0.08 (4.27) (n=16)	-3.72 (-8.87, 1.43) (n=42)	0.16
Lower limb duration (ms)	63.16 (13.38) (n=18)	64.68 (12.20) (n=19)	2.12 (14.40) (n=18)	64.38 (16.78) (n=19)	58.61 (15.75) (n=18)	-4.03 (17.95) (n=16)	6.28 (-2.19, 14.76) (n=42)	0.15

Quantitative Sudomotor Axonal Reflex Test at the foot [‡]								
Sweat Volume (μL)	1.30 (0.77) (n=20)	1.20 (0.73) (n=20)	-0.10 (0.87) (n=20)	1.29 (0.94) (n=22)	1.38 (1.04) (n=18)	0.25 (0.72) (n=18)	-0.28 (-0.74, 0.19) (n=42)	0.25
Response Latency (s)	124.20 (63.07) (n=20)	152.42 (59.97) (n=20)	28.22 (65.44) (n=20)	140.68 (65.48) (n=22)	134.89 (74.66) (n=18)	-9.94 (94.12) (n=18)	23.39 (-17.75, 64.53) (n=42) ^{**}	0.27 ^{**}
Large nerve fibre function								
MNSI score, median (IQR) ^{†‡}	5.0 (4.0 - 6.0)	3.0 (2.0 - 4.0)	-1.0 (-2.5 - -1.0)	5.0 (3.0 - 6.0)	4.0 (1.0 - 6.0)	-1.0 (-3.0 - 0.0)	0.78 (0.55, 1.10) [†]	0.15
MDNS neuropathy								
Class 0	17 [85%]	19 [95%]	-	19 [86%]	15 [79%]	-	-	0.66
Class 1	3 [15%]	1 [5%]	-	0 [0%]	1 [5%]	-	-	
Class 2	0 [0%]	0 [0%]	-	1 [5%]	2 [11%]	-	-	
Class 3	0 [0%]	0 [0%]	-	2 [9%]	1 [5%]	-	-	
Nerve conduction studies [‡]								
Sural sensory amplitude (μV)	11.93 (7.11) (n=20)	11.44 (6.70) (n=20)	-0.49 (3.88) (n=20)	12.26 (7.80) (n=22)	12.27 (8.25) (n=19)	-0.37 (2.84) (n=19)	-0.20 (-2.26, 1.86) (n=42)	0.85
Peroneal motor velocity (ms ⁻¹)	42.32 (3.86) (n=20)	42.04 (5.16) (n=20)	-0.28 (4.46) (n=20)	41.04 (5.16) (n=22)	41.14 (4.92) (n=19)	0.08 (3.73) (n=19)	0.01 (-2.43, 2.46) (n=42)	0.99
Tibial minimum F-wave latency (ms)	56.07 (5.97) (n=19)	55.49 (5.54) (n=19)	-0.25 (2.13) (n=18)	54.24 (6.10) (n=20)	54.10 (5.48) (n=16)	-0.09 (1.82) (n=16)	0.04 (-1.19, 1.27) (n=42)	0.95

Nerve excitability[§]								
TEd peak (%)	70.24 (4.24)	69.45 (4.69)	-0.80 (3.42)	67.58 (4.48)	69.39 (9.02)	1.37 (9.81)	-1.20 (-5.83, 3.44) ^{††}	0.61 ^{††}
TEd 90-100 (%)	45.08 (3.09)	44.94 (3.92)	-0.18 (4.21)	44.12 (4.89)	47.49 (13.85)	4.34 (15.21)	-2.90 (-9.87, 4.07)	0.42
TEh 90-100 (%)	-117.02 (17.34)	-126.35 (21.27)	-6.56 (12.56)	-117.77 (19.66)	-115.24 (19.16)	1.61 (14.66)	-9.33 (-18.41, -0.25)	0.044
TEd 10-20 (%)	70.52 (4.60)	70.27 (4.71)	-0.24 (3.78)	67.99 (4.30)	68.00 (4.71)	-0.74 (4.25)	1.09 (-1.42, 3.60)	0.40
Superexcitability (%)	-23.45 (5.11)	-23.48 (5.35)	0.15 (3.14)	-21.14 (6.79)	-21.60 (6.73)	-0.10 (4.70)	-0.19 (-2.73, 2.34)	0.88
Subexcitability (%)	13.61 (2.78)	13.78 (3.04)	0.08 (2.60)	12.75 (4.10)	12.51 (3.29)	-0.43 (4.07)	0.95 (-0.88, 2.78)	0.31
SDTC (ms)	0.49 (0.10)	0.48 (0.09)	-0.02 (0.08)	0.50 (0.11)	0.49 (0.09)	-0.01 (0.13)	-0.01 (-0.07, 0.05)	0.74
Resting I/V slope, median (IQR) [†]	0.61 (0.54 - 0.65)	0.58 (0.56 - 0.63)	0.01 (-0.03 - 0.04)	0.62 (0.56 - 0.71)	0.63 (0.56 - 0.76)	0.08 (-0.07 - 0.14)	0.96 (0.77, 1.19) [†]	0.73

Mean and standard deviation, unless stated otherwise. Numbers analysed for neurophysiology outcomes are listed below the outcome. EQ-5D-5L=EuroQol five-dimensional descriptive system in five levels. IQR=interquartile range. I/V=current/voltage. MDNS=Michigan Diabetic Neuropathy Score. MNSI=Michigan Neuropathy Screening Instrument. PUFAs=polyunsaturated fatty acids. SDTC=strength duration time constant, TEd=depolarising threshold electrotonus. TEh= hyperpolarising threshold electrotonus. VAS=visual analogue scale

* p-values are not adjusted for multiple testing.

[†] Descriptive data are presented as median and interquartile range; estimates are presented as geometric mean ratio of the change from baseline in Omega-3 PUFAs vs Placebo at day 180

[‡] Number of participants analysed for these variables are presented in parentheses rather than the 'n' indicated in the column heading.

[§] For nerve excitability, number of participants analysed in the treatment group was n=18 at baseline and n=16 at day 180; number analysed in the placebo group was n=22 at baseline and n=18 at day 180. Estimates for between-group change derived from n=40.

^{||} In a sensitivity analysis adjusting for imbalance at baseline, the treatment effect for EQ-5D-5L VAS is -6.0 (-12.0,0.1) units; unadjusted p-value=0.10

[¶] In a sensitivity analysis adjusting for imbalance at baseline, the treatment effect for cutaneous silent period upper limb latency onset is 1.95 (-2.03,5.92) ms; unadjusted p-value=0.96

[#] In a sensitivity analysis adjusting for imbalance at baseline, the treatment effect for cutaneous silent period upper limb duration is -0.71 (-2.27,5.85) ms; unadjusted p-value=0.83

^{**} In a sensitivity analysis adjusting for imbalance at baseline, the treatment effect for sweat latency at the foot is 35.19 (-14.51,84.90) s; unadjusted p-value=0.17

^{††} In a sensitivity analysis adjusting for imbalance at baseline, the treatment effect for TEd peak is -2.43 (-7.24,2.39) %; unadjusted p-value=0.32

For nerve excitability, there was a significant difference in the estimated relative change in TEh at 90-100 ms for the omega-3 PUFA group compared to placebo (-9.33 units, 95% CI: -18.41 to -0.25; **Figure 7-3**). No between-group differences were found in change from baseline at day 180 for the other nerve excitability variables (**Table 7-3**).

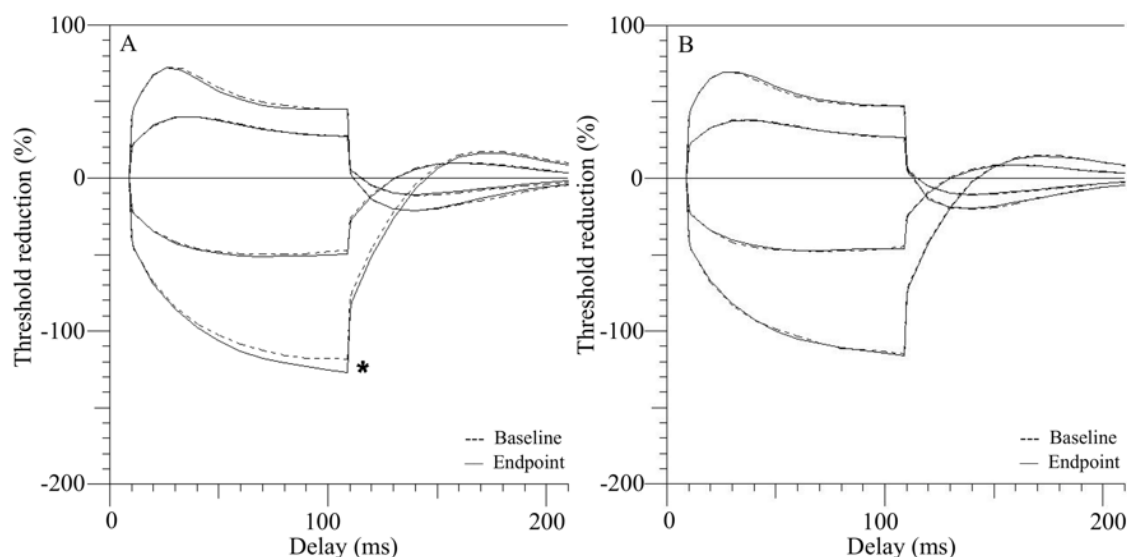


Figure 7-3. Axonal nerve excitability studies at baseline and endpoint (intention-to-treat sample). Threshold electrotonus at day 0 (dashed line) and day 180 (solid line) in (A) the omega-3 PUFA group and (B) the placebo group. *Asterisk indicates significant between-group difference for change in hyperpolarising threshold electrotonus at the 90-100ms interval (TEh 90-100 ms) during a -40% control threshold stimulus.

The estimated between-group change in Omega-3 Index at day 180 was 3.3%, favouring the omega-3 PUFA group (95% CI: 2.4 to 4.2%; **Table 7-4**). Omega-3 Index in the treatment group increased from 4.9% (standard deviation (SD): 0.8%) at baseline to 8.2% (1.7%) at day 180. In the placebo group, the Omega-3 Index was similar at both time-points (baseline: 4.6% (1.0%) versus day 180: 4.8% (1.3%). Using the omega-3 PUFA group as a reference, the estimated between-group difference in change from baseline at day 180 for omega-6 to omega-3 ratio was -3.5 (95% CI -4.3 to -2.6%; **Table 7-4**)

Table 7-4. Compliance outcomes using systemic fatty acid profiles (intention-to-treat sample)

	Omega-3 PUFAs			Placebo			Omega-3 PUFAs vs Placebo	
	Baseline (n=21)	Day 180 (n=21)	Change from baseline (n=21)	Baseline (n=22)	Day 180 (n=19)	Change from baseline (n=19)	Estimate (95% CI) (n=43)	p- value*
Omega-3 Index, %	4.9 (0.8)	8.2 (1.7)	3.3 (1.8)	4.6 (1.0)	4.8 (1.3)	0.12 (1.0)	3.3 (2.4, 4.2)	<0.001
EPA, %	0.6 (0.2)	2.11 (0.7)	1.5 (0.8)	0.6 (0.2)	0.7 (0.4)	0.08 (0.4)	1.4 (1.1, 1.8)	<0.001
DHA, %	1.9 (0.5)	3.0 (0.7)	1.1 (0.7)	1.8 (0.7)	1.8 (0.7)	0.01 (0.5)	1.1 (0.7, 1.5)	<0.001
Total omega-6, %	25.0 (4.7)	25.8 (3.4)	0.8 (4.1)	26.9 (2.8)	28.3 (2.6)	1.52 (4.0)	-2.1 (-3.9, -0.3) †	0.023 †
Total omega-9, %	20.9 (5.0)	21.1 (2.5)	0.3 (5.6)	21.0 (3.8)	20.5 (1.9)	-1.26 (3.5)	0.7 (-0.7, 2.0)	0.34
Omega-6: omega-3 ratio	6.1 (1.7)	3.7 (0.9)	-2.4 (2.0)	7.2 (1.8)	7.3 (1.8)	0.2 (1.9)	-3.5 (-4.3, -2.6) ‡	<0.001 ‡

Data are reported as mean and standard deviation. DHA=docosahexaenoic acid.
EPA=eicosapentaenoic acid. PUFA=polyunsaturated fatty acid

* p-values are not adjusted for multiple testing.

† In a sensitivity analysis adjusting for imbalance at baseline, the treatment effect for total omega-6 is -0.59 (-3.09, 1.92) %; unadjusted p-value=0.65

‡ In a sensitivity analysis adjusting for imbalance at baseline, the treatment effect for omega-6:omega-3 ratio is -2.54 (-3.77, 1.32) %; unadjusted p-value <0.001

There were 23 adverse events in total (see **Appendix H**), comprising 12 in the omega-3 PUFA group and 11 in the placebo group. Seventeen participants reported at least one AE (including SAE). A single SAE in the placebo group was deemed unrelated to the study treatment. Ocular and blood pathology safety endpoints are summarised in **Table 7-5**. At day 180, the relative change from baseline in the omega-3 PUFA group did not differ from the placebo group for habitual visual acuity, intraocular pressure, blood chemistry parameters, or diabetic retinopathy grading (**Table 7-5; Appendix H**).

Table 7-5. Safety outcomes and blood biochemistry (safety sample)

	Omega-3 PUFAs			Placebo			Omega-3 PUFAs vs Placebo	
	Baseline (n=21)	Day 180 (n=21)	Change from baseline (n=21)	Baseline (n=22)	Day 180 (n=19)	Change from baseline (n=19)	Estimate (95% CI) (n=43)	p-value*
Habitual distance visual acuity (LogMAR)								
Right eye	0.06 (0.08)	0.03 (0.09)	-0.03 (0.08)	0.02 (0.16)	-0.03 (0.10)	-0.03 (0.10)	0.02 (-0.02, 0.07)	0.31
Left eye	0.04 (0.13)	0.03 (0.09)	-0.01 (0.15)	0.06 (0.18)	-0.00 (0.13)	-0.05 (0.10)	0.03 (-0.02, 0.09)	0.24
Intraocular pressure (mmHg)								
Right eye	12.91 (2.70)	11.96 (2.66)	-0.92 (2.13)	12.20 (2.13)	12.39 (2.42)	0.19 (1.61)	-0.94 (-2.04, 0.17)	0.097
Left eye	13.15 (2.97)	12.33 (2.48)	-0.78 (1.95)	12.18 (2.28)	12.45 (2.51)	0.33 (1.73)	-0.81 (-1.83, 0.22)	0.12
Diabetic retinopathy in the worse eye, n [%]								
None	15 [71%]	15 [71%]	-	10 [45%]	11 [52%]	-	-	1.0
Minimal NPDR	2 [9.5%]	2 [10%]	-	2 [9%]	1 [5%]	-	-	
Mild NPDR	1 [5%]	2 [10%]	-	8 [36%]	7 [35%]	-	-	
Moderate NPDR	3 [14%]	2 [10%]	-	2 [9%]	1 [5%]	-	-	
Severe NPDR	0 [0%]	0 [0%]	-	0 [0%]	0 [0%]	-	-	
Proliferative diabetic retinopathy	0 [0%]	0 [0%]	-	0 [0%]	0 [0%]	-	-	
Diabetic macular oedema in the worse eye, n [%]								
None	21 [100%]	21 [100%]	-	20 [91%]	18 [9%]	-	-	No change
Macular oedema	0 [0%]	0 [0%]	-	1 [5%]	1 [5%]	-	-	
Clinically significant macular oedema	0 [0%]	0 [0%]	-	1 [5%]	1 [5%]	-	-	

Blood biochemistry ‡

HbA1c (mmol/mol)	56.70 (8.94)	57.90 (8.54)	1.20 (5.70)	61.95 (12.18)	61.50 (10.37)	1.00 (5.62)	-0.70 (-4.10, 2.70)	0.69
Cholesterol (mmol/L)	4.72 (0.83)	4.64 (1.03)	-0.12 (0.48)	4.59 (0.99)	4.63 (1.04)	-0.01 (0.63)	-0.10 (-0.47, 0.26)	0.58
HDL-C (mmol/L), median (IQR) [†]	1.64 (1.38 - 1.92)	1.52 (1.26 - 1.83)	-0.04 (-0.17 - 0.07)	1.52 (1.21 - 1.79)	1.50 (1.32 - 1.77)	0.07 (-0.14 - 0.12)	0.95 (0.89, 1.03) [†]	0.22
LDL-C (mmol/L), median (IQR) [†]	2.60 (2.45 - 3.00)	2.65 (2.15 - 3.15)	0.10 (- 0.40 - 0.40)	2.40 (2.10 - 3.30)	2.55 (2.00 - 3.35)	0.20 (-0.30 - 0.50)	0.99 (0.87, 1.13) [†]	0.87
Creatinine (µmol/L)	71.3 (13.0)	69.4 (12.1)	-2.0 (7.2)	70.3 (20.0)	77.3 (26.3)	2.9 (10.6)	-4.7 (-10.4, 1.0)	0.11
Vitamin B12 (pmol/L)	381.3 (103.7)	433.6 (147.9)	58.7 (108.9)	372.5 (143.2)	384.3 (156.6)	17.0 (56.3)	41.6 (-18.3, 101.6)	0.17
Folate (nmol/L)	30.90 (8.23)	30.75 (8.59)	0.69 (6.40)	29.19 (6.67)	33.97 (6.03)	2.85 (6.47)	-2.63 (-6.56, 1.30)	0.19

Data are presented as mean and standard deviation unless stated otherwise.

HbA1c=haemoglobin A1c. HDL=high-density lipoproteins-cholesterol. LDL=low density lipoproteins-cholesterol. NPDR=non-proliferative diabetic retinopathy.

* p-values are not adjusted for multiple testing.

† Descriptive data are presented as median and interquartile range; estimates are presented as geometric mean ratio of the change from baseline in Omega-3 PUFAs vs Placebo at day 180.

‡ For blood biochemistry, number of participants analysed in the treatment group was n=21 at baseline and n=20 at day 180; number analysed in the placebo group was n=19 at baseline and n=16 at day 180.

A pre-specified sub-group analysis of the primary outcome found a significant interaction effect for sub-groups with a baseline CNFL ≤ 12.5 mm/mm² at day 90, with the difference in estimated treatment effect being 3.11 mm/mm² (95% CI: 1.37 to 4.86; p<0.001; **Figure 7-4**) relative to the subgroup with baseline CNFL >12.5 mm/mm². At day 180, the interaction effect was 1.68 mm/mm² (95% CI: -0.45 to 3.82; p=0.12) between subgroups. For participants with clinically-defined SFN at baseline, the interaction effect at day 90 was -0.167 mm/mm² (95% CI: -2.29 to 1.96; p=0.88) and at day 180 was 1.67 mm/mm² (95% CI: -0.46 to 3.82; p=0.12), compared to those without SFN at baseline.

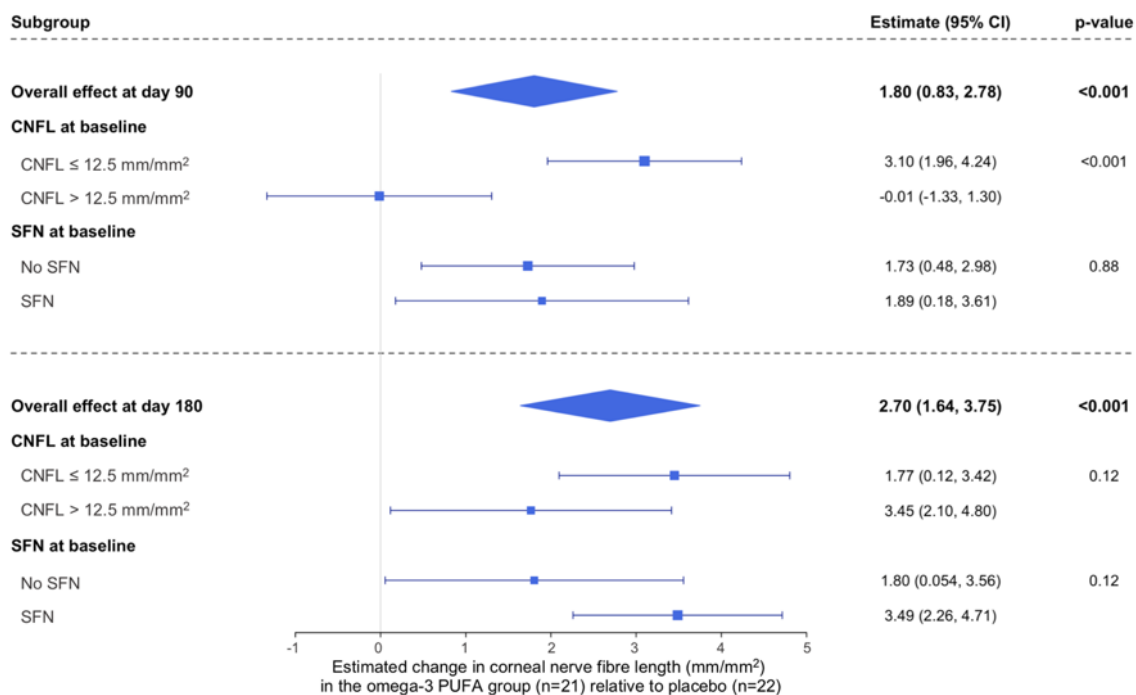


Figure 7-4. Forest plots for the primary outcome, corneal nerve fibre length, subgroup analysis (intention-to-treat sample). CNFL=corneal nerve fibre length. SFN=small fibre neuropathy.

Sensitivity analysis was performed for the primary and key secondary outcomes excluding individuals in the placebo group who were lost to follow-up or did not return remaining study supplements (n=3); exclusion of these participants did not alter the outcomes. The pre-specified analysis of primary, key secondary and secondary outcomes, adjusted for baseline variables (age, diabetes duration, HbA1c), also did not alter primary and key secondary outcomes. A second pre-specified model, additionally adjusting for potential baseline imbalances in CNFL and Omega-3 Index was not performed as these variables were not imbalanced. Sensitivity analyses accounting for baseline imbalances did not change the findings, with the exception of the omega-6 to omega-3 ratio (Table 7-4).

Exploratory outcomes are presented in **Appendix H**. There were no significant between-group differences in estimated mean change from baseline for sweat latency and volume (measured using QSART) at the distal leg, proximal leg, or forearm. For median motor latency, there was a between-group difference of -0.16 ms (95% CI: -0.32 to -0.01 ms); however, no other NCS parameters showed a between-group difference in change from baseline. For anterior ocular surface parameters, there was a 1.31-fold estimated relative change in the geometric mean score for tear break-up time in the omega-3 PUFA group, compared to placebo (95% CI: 1.01 to 1.71).

Confirming the integrity of the masking procedures, there was lack of agreement for the treatment assignment guess by the participant, $\kappa = -0.03$ (95% CI: -0.33 to 0.28), and outcome assessor, $\kappa = -0.18$ (95% CI: -0.48 to 0.12).

7.2.6 Discussion

This double-masked RCT has identified that, relative to placebo, triglyceride long-chain omega-3 PUFA supplements, dosed at 1800 mg/day for 180 days, promote corneal nerve regeneration (as indicated by an increase in the CNFL primary outcome measure and other corneal nerve parameters, CNFD and CNBD) in individuals with type 1 diabetes. Corneal sensitivity thresholds, to both room-temperature and cooled stimuli, did not differ significantly between study treatment groups at day 180. Compared with placebo, omega-3 PUFA supplementation did not alter small (measured using CSP and QSART), or large (measured using routine NCS) nerve fibre function.

The observed corneal neurotrophic effects with omega-3 PUFA supplementation likely relate to the effects of lipid metabolites, resolvins and protectins, which have anti-inflammatory and neuroprotective properties. Treatment with topical DHA and the DHA-derived lipid mediator, neuroprotectin-D1, improved corneal nerve density and sensitivity in rabbits following corneal nerve injury (Cortina et al., 2013). PUFAs are key components of membrane phospholipids, affecting membrane fluidity and regulating protein and receptor

function. Omega-3 PUFAs have widespread effects on gene expression; intracellular omega-3 PUFAs activate the transcription of peroxisome proliferator-activated receptor and retinoid X receptor genes, which modulate neuronal apoptosis and neuroinflammation (Zhao et al., 2011). The present findings are consistent with results from rodent studies that have shown oral omega-3 PUFA supplementation (using menhaden oil) can reverse CNFL loss and corneal sensitivity impairment in experimental diabetes (Coppey et al., 2020).

The natural history of change in CNFL in type 1 diabetes is estimated to be a mean of -0.8% per year (90% CI: -14.0 to 9.9%) (Lewis et al., 2020). Rapidly progressive CNFL loss, defined as $\geq 6\%$ per year, occurs in 17% of individuals and is associated with a higher risk of DSP development and progression. In this subpopulation, the mean annual reduction in CNFL has been estimated as -14.7% (SD: 11.5%) (Lewis et al., 2020). Consistent with the established heterogeneity of CNFL changes in type 1 diabetes populations, the mean effect evident in the placebo group in the current study fell within this reported range, with a mean change in CNFL of -5.8% (SD: 14.7%) over the six-month study duration.

The magnitude of improvement in CNFL in the omega-3 PUFA supplement group (mean (SD): 2.06 (1.73) mm/mm²) is similar to that described in an open-label study of seal oil supplements (750 mg EPA, 1020 mg DHA, and 560 mg docosapentaenoic acid per day) in a type 1 diabetes population (Lewis et al., 2017). Lewis *et al.* reported an increase in CNFL from 8.3 (2.9) mm/mm² to 10.1 (3.7) mm/mm² after 12 months of omega-3 PUFA supplementation. In this study (Lewis et al., 2017), compared to the reported mean treatment effect (29% increase in CNFL from baseline), a greater increase in CNFL (56%) was reported in individuals with baseline CNFL <15 mm/mm². In the present study, CNFL showed time-dependent changes that were influenced by baseline CNFL. Notably, the estimated change in CNFL at day 90 relative to placebo was predominantly driven by the effect in the subgroup with baseline

CNFL ≤ 12.5 mm/mm² (**Figure 7-4**). This finding suggests baseline CNFL may be a treatment predictor that identifies individuals who will derive more rapid benefits from omega-3 PUFA supplementation. At day 180, the heterogeneity in effect between subgroups was not significant. Thus, with a longer supplementation period, the relative CNFL increase is comparable between severity groups, potentially suggesting a ceiling effect that is achieved with temporal characteristics dependent on baseline corneal nerve integrity.

Nerve excitability abnormalities associated with diabetes are likely due to axonal membrane sodium/potassium pump dysfunction, as well as reduced nodal sodium channel conductance. Increased threshold change in TEh over time, as evident in the omega-3 PUFA group, could potentially be associated with reversal of these mechanisms (Kiernan et al., 2020). However, the clinical significance of this finding is unclear without an associated increase in threshold change in TEd or change in other nerve excitability parameters. Although nerve excitability abnormalities have been documented in type 1 diabetes in the absence of clinically-evident neuropathy, the mean baseline curves in our cohort appeared normal and, together with normal NCS, suggest unaffected large fibre axonal function. Thus, there may have been little capacity for improvement with intervention. Future studies are indicated in populations with abnormal nerve excitability at baseline, to investigate whether omega-3 PUFA supplementation can induce relative improvements, consistent with a neuroprotective response.

There were no between-group differences for change in small nerve fibre function, measured using CSP and QSART, at day 180. For NCS, although a relative reduction in median motor latency was observed in the omega-3 PUFA group compared with placebo, lack of differences in any other NCS parameters suggest that this isolated change should be interpreted with caution. The absence of detectable improvement in peripheral nerve function may relate to sample size, as the study was not powered to detect differences in clinical

neurophysiology outcomes for CSP, QSART, or NCS. Furthermore, both CSP and QSART can show inherent inter-test variability (Kamel et al., 2015).

In rodent models, improvements in corneal nerve structure and function have been associated with functional improvements in motor and sensory NCS and thermal nociception in the limbs (Coppey et al., 2020). However, this association has not been demonstrated in human studies where corneal nerve parameters improved. An open-label study of exenatide and pioglitazone, or insulin, in type 2 diabetes did not find a coincident change in sudomotor function despite an increase in CNFL relative to baseline after one year (Ponirakis et al., 2020). Similarly, Lewis *et al.* reported no improvement in quantitative sensory testing (QST) or NCS with omega-3 PUFA supplementation despite CNFL increasing from baseline (Lewis et al., 2017). DSP involves initial damage to peripheral small nerve fibres (i.e., unmyelinated C-fibres and thinly myelinated A δ -fibres, including those in the cornea) that precede large nerve fibre involvement (Breiner et al., 2014). Corneal sub-basal nerve plexus parameters are a valid surrogate endpoint for evaluating the efficacy of DSP interventions (Azmi et al., 2019). Considering that DSP progresses in a length-dependent pattern, it is possible that an improvement in CNFL is an early indicator of peripheral nerve recovery, preceding potential later improvements in sensory and/or motor function. In a population with type 1 diabetes, although corneal nerve regeneration was evident six months after simultaneous pancreas and kidney transplantation, an improvement in neurophysiology outcomes, relative to an untreated group, was not observed until 36 months post-transplantation (Azmi et al., 2019). A challenge with assessing neurophysiological outcomes is that most standard tests have poor diagnostic yield for early small nerve fibre damage (Mallik & Weir, 2005). These factors indicate a need to carefully consider the duration of therapeutic treatment, and selection of endpoints in future DSP trials.

The effect of oral omega-3 PUFA supplementation on improving corneal nerve density suggests this treatment may also be beneficial for tear function and

ocular surface health, in addition to their influence on tear film quality (Deinema et al., 2017). In diabetes, corneal sensory nerve loss disrupts corneal epithelial integrity and reduces basal tear production (Shih et al., 2017). These changes can impair corneal wound healing and lead to persistent epitheliopathy (Lockwood et al., 2006). In the current study, enhanced tear film stability was observed in the omega-3 PUFA group at day 180, compared with placebo. This improvement occurred in a study population without overt ocular surface disease, as indicated by baseline tear osmolarity and dry eye symptom scores within physiological ranges. These findings suggest that omega-3 PUFAs may have a role in limiting DSP-associated corneal keratopathy.

A moderate dose of long-chain omega-3 PUFAs was used in the current study (1800 mg/day). At the study endpoint, the systemic Omega-3 Index in the omega-3 PUFA group exceeded 8%, the therapeutic threshold in cardiovascular disease (Harris et al., 2017). With the prescribed dosing, there were no unexpected AEs deemed related to the study intervention, and none of the participants had a worsening in their diabetic retinopathy grade.

This is, to our knowledge, the first double-masked RCT to evaluate the potential efficacy of omega-3 PUFA supplementation for modulating peripheral nerve health in type 1 diabetes. All analyses were specified a priori in a statistical analysis plan, including an ITT protocol. Furthermore, there was high participant retention (95%); high participant compliance was confirmed using the Omega-3 Index. Treatment allocation was concealed and double-masking was objectively assessed as effective. A comprehensive suite of neurophysiology parameters was also evaluated.

Some potential considerations when interpreting the current findings include that additional assessments of small nerve fibre function, such as QST and skin biopsy for intraepidermal nerve fibre density, were not incorporated due to lack of availability. Multiplicity adjustment was not performed for variables outside of primary and key secondary analyses, increasing the risk of false positive

findings for secondary and exploratory outcomes; these should therefore be interpreted with caution. Our study population generally had no neuropathy or early stage DSP at baseline; while one-third of participants were diagnosed with SFN, most had no large fibre neuropathy (classified using the MDNS). The primary outcome measure, CNFL, was selected as it is a validated surrogate endpoint for small fibre structure (Malik et al., 2003; Tavakoli et al., 2013). However, as an objective, rather than patient-reported, outcome, it is yet to be established whether improvements in corneal small nerve fibres translate to meaningful improvements in sensory function.

In conclusion, a moderate daily dose of oral omega-3 PUFA supplements for six months improved corneal sub-basal nerve structure in people with type 1 diabetes, relative to placebo, consistent with a corneal neuroregenerative effect. There were no safety concerns with the intervention. Confirmatory trials in larger, well-defined populations, potentially over longer duration, are indicated to ascertain whether omega-3 PUFA supplements confer similar benefits in peripheral nerve function.

Contributors: LED created the study concept. ACZ, LED, RJM, LJR, and JTK developed the trial protocol. LED, JK, LJR, and RJM acquired the funding. ACZ, LED, LJR and JTK implemented the trial procedures. RJM, JTK, and LJR provided administrative and technical support. ACZ, LED, JTK, LJR acquired the data. ACZ, LED and SB developed the statistical analysis plan. ACZ and SB performed the statistical analysis. LED, JPC and RJM supervised the study. ACZ and LED have verified the underlying data. ACZ and SB have independently verified the data analysis. ACZ wrote the first draft of the report with input from LED. All authors revised the article.

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8

Chapter 8. Discussion & Conclusions

8.1 Overview of findings

This thesis encompasses a body of work examining the role of omega-3 polyunsaturated fatty acids (PUFAs) in corneal and peripheral nerve health. Corneal nerve parameters were selected as surrogate markers for evaluating peripheral nerve integrity. Outcomes included both corneal nerve morphology, evaluated using *in vivo* corneal confocal microscopy (IVCM), and corneal sensation, measured with a non-contact corneal aesthesiometer. This work was driven by the need for interventions that improve peripheral nerve outcomes in diabetes to be identified, as optimal clinical care in DSP is currently challenged by a lack of treatments. This thesis aimed to address this unmet need by evaluating the clinical utility of omega-3 PUFAs as a neuroprotective intervention for individuals with diabetes.

The clinical applications of omega-3 PUFAs were considered in five studies; three of these studies, as well as a systematic review protocol, are published (Zhang et al., 2020a; Zhang & Downie, 2019; Zhang et al., 2018; Zhang et al., 2020b). The work in the earlier chapters of this thesis offers insight into current clinical practices relating to omega-3 PUFAs and strategies to improve clinical implementation. Prospective clinical studies in subsequent chapters provide evidence for the role of omega-3 PUFAs in modulating corneal and peripheral nerve health in diabetes.

Chapter 3 describes a clinician's survey that assessed the knowledge and practice behaviours of optometrists relating to omega-3 PUFAs. Findings from this survey supported the hypotheses that Australian and New Zealand optometrists recommend both omega-3-rich foods and omega-3 supplements for improving eye health, and that there is a knowledge gap in the understanding of the differences between different forms (i.e., marine- vs plant-based) of omega-3 fatty acids. Driven by the need for a tool that can identify individuals who may preferentially benefit from dietary intervention, **Chapter 4** describes a clinically-applicable food-frequency questionnaire, which was validated against an objective systemic biomarker and a dietary profile survey.

Chapter 5 describes a cross-sectional study that investigated the association between systemic fatty acid levels and corneal nerve parameters in healthy individuals and those with diabetes without symptomatic neuropathy. A hypothesis for this study was that the systemic Omega-3 Index would be associated with corneal sub-basal nerve structure and corneal nerve function. Although this study found an association between the Omega-3 Index and corneal sub-basal nerve structure, a relationship between the Omega-3 Index and corneal sensitivity was not identified. Furthermore, this study identified a negative correlation between central corneal nerve fibre length (CNFL) and corneal sensitivity thresholds to a cooled stimulus in diabetes, but not in controls. This finding refutes the hypothesis that a similar corneal structure-function relationship would be evident in individuals with and without diabetes, and points to an altered corneal structure-function relationship in this condition.

Chapter 6 presents outcomes of a systematic review on the identification, appraisal, and synthesis of evidence from randomised controlled trials (RCTs) evaluating the efficacy and safety of oral omega-3 supplementation for peripheral nerve outcomes. The RCTs identified in this review reported data relating to peripheral nerve structure and function in a variety of acute and chronic conditions, including chemotherapy-induced peripheral neuropathy and diabetes. However, based on this data, there was insufficient evidence to

ascertain if omega-3 interventions can improve peripheral nerve outcomes in chronic conditions, such as diabetes. Finally, **Chapter 7** describes an RCT that evaluated the effects of oral omega-3 PUFA supplementation on peripheral nerve parameters in individuals with type 1 diabetes. Accepting the hypothesis for this study, this RCT found that relative to placebo, omega-3 supplementation for six months improved corneal sub-basal nerve structure in type 1 diabetes, supporting their role in modulating peripheral nerve health.

8.2 Overall summary and applicability of the evidence

Omega-3 fatty acids for corneal and peripheral nerve health

An important finding of the systematic review (**Chapter 6**) was that omega-3 supplementation attenuates sensory loss and reduces the incidence of chemotherapy-induced neuropathy relative to placebo. This finding indicates that omega-3 PUFAs may play a role in preventing sensory deficits secondary to neurotoxic agents. However, the certainty of this evidence was graded as low; therefore, the confidence in the effect estimate is limited. This finding demonstrates a need for further high-quality, consistent evidence to be able to confirm the applicability of oral omega-3 PUFA supplements as prophylactic agents for chemotherapy-induced peripheral neuropathy. Of the 15 RCTs identified in this review, only two included individuals with diabetes, and only one of these studies is currently published. This paucity of data highlights a need for further rigorous evaluation of omega-3 PUFA supplementation as a potential intervention for improving peripheral nerve outcomes in this condition.

Chapter 7 begins to address this need for high-quality evidence by describing the first RCT to evaluate whether oral omega-3 PUFA supplements promote corneal sub-basal nerve regeneration in type 1 diabetes. The finding that six-months of oral omega-3 supplementation increases CNFL is in agreement with that reported by Lewis *et al.*, in a single arm study of a similar type 1 diabetes population (Lewis *et al.*, 2017). This study found a 29% improvement in CNFL, relative to baseline, with oral long-chain omega-3 PUFA supplementation for 12

months. In our RCT, the magnitude of the CNFL increase with omega-3 supplementation relative to placebo is also comparable to that reported by Chinnery et al., in a double-masked RCT of individuals with dry eye disease (DED) and mild corneal neuropathy (Chinnery et al., 2017). Our findings corroborate established clinical evidence that long-chain omega-3 PUFA supplements promote neuro-regeneration within the corneal sub-basal nerve plexus.

Unlike the demonstrable improvement in corneal nerve structure, corneal sensitivity to either room-temperature or cooled stimuli failed to show significant change relative to placebo at six months (**Chapter 7**), although effects appeared to show a consistent direction. A similar lack of parallel improvement in corneal nerve structure and function has been reported in type 1 diabetes following simultaneous pancreas and kidney transplantation (Tavakoli et al., 2013). In this study, Tavakoli *et al.* found no significant changes in corneal sensitivity, despite corneal sub-basal nerve recovery at 12 months. These clinical findings are in contrast to animal studies that have demonstrated recovery of corneal structure and function to occur within the same time frame (Coppey et al., 2020; Cortina et al., 2013). The dissimilarity between structural and functional recovery could be indicative of differential mechanisms or temporal characteristics in corneal reinnervation. However, the consistent direction of the observed effects in our RCT suggests that this disparity could relate to the study sample size, which was powered based upon structural, and not functional, outcomes. Given that corneal sensitivity tests rely on subjective responses and, as such, tend to be prone to greater variability, a larger sample size may be required to detect a difference in corneal nerve function.

Furthermore, IVCN evaluates only the anatomical structure of the corneal sub-basal nerve plexus, whereas corneal sensitivity is a function of nerve receptors in both the corneal sub-basal nerve plexus and the superficial terminals within the epithelium. These differences may also explain the discrepancies between clinical studies and those conducted in animal models, where concurrent corneal structural and functional changes have been observed (Coppey et al.,

2020). Taken together, clinical findings from this thesis suggest that even though corneal sensitivity to cooled air stimuli precedes DSP symptoms in diabetes, change in corneal sub-basal nerve plexus structure is not necessarily a reliable predictor of corneal functional changes.

Another consideration is the underlying function of the regenerated corneal nerve fibres, which, to date, has been inadequately characterised. In **Chapter 5**, the finding of an altered structure-function relationship in individuals with diabetes without DSP symptoms points to potential disease-related effects that are specific to the sensory function of corneal cold thermoreceptors. If these effects exist, it is possible that corneal receptor subpopulations may also respond differentially to repair. **Chapter 7** examined for a temperature-modulated effect in corneal functional recovery by evaluating change in corneal sensitivity to both room-temperature and cooled stimuli with omega-3 PUFA intervention. However, neither improved significantly relative to placebo. Notably, although inferences can be made about corneal receptor subtypes based upon functional testing in clinical studies, details of the affected receptors cannot be confirmed without *ex vivo* histological examinations. Nonetheless, the recovery of corneal sensation to air-stimuli of different modalities (e.g., temperature, chemical) is deserving of further evaluation to understand the functional characteristics of the regenerated corneal nerve fibres.

The effects of omega-3 PUFA supplementation on systemic peripheral nerve function was explored in the RCT described in **Chapter 7**. This RCT found that omega-3 supplementation increased the threshold to electrotonus hyperpolarisation on axonal nerve excitability relative to placebo. However, it is unclear whether this change is of clinical significance, given that our study cohort had normal excitability curves at baseline and no relative improvement was observed in other nerve excitability variables. No clinically significant difference was found for other secondary outcomes measuring small or large nerve fibre function relative to placebo.

A lack of congruity between changes in corneal nerve morphology and systemic peripheral nerve function was also reported in the study by Lewis *et al.*, where no change was found in nerve conduction studies (NCS) or in quantitative sensory testing thresholds with 12-months of omega-3 PUFA supplementation, despite an improvement in CNFL (Lewis *et al.*, 2017). In DSP, effective disease-modifying treatments must have the capacity to improve distal limb function, because symptoms and functional impairments occur in a length-dependent pattern (Merkies & Lauria, 2006). Given that the corneal sub-basal nerve plexus presents one of the earliest sites for detecting DSP, corneal nerve regeneration may also be one of the earliest indicators for peripheral nerve recovery, potentially preceding improvements in sensory and/or motor function in the limbs. In a study of individuals with type 1 diabetes treated with simultaneous pancreas and kidney transplantation (Azmi *et al.*, 2019), neurophysiology parameters did not differ from those of an untreated group until 36 months after transplantation, even though corneal sub-basal nerve parameters showed significant improvement after 6 months.

Notably, our RCT found a significant reduction in median motor nerve conduction latency with omega-3 PUFA supplementation relative to placebo. However, median nerve function was an exploratory, and not a prespecified secondary, outcome. As no between-group difference was found in other NCS parameters, this finding was interpreted with caution. The finding of improved median nerve function does, however, corroborate with one other RCT identified in our systematic review (**Chapter 6**), which reported that omega-3 supplementation (960 mg/day combined EPA and DHA) for 90 days improved median nerve conduction velocity in type 1 diabetes, relative to a non-supplemented group (Stiefel *et al.*, 1999). However, this study was undertaken in a small population (n=18), without masking. Furthermore, neither of the RCTs was statistically powered based on median nerve parameters, nor did they specifically include individuals with median nerve dysfunction at baseline. Nonetheless, future investigations may be warranted to evaluate omega-3 PUFA

supplementation for populations with median nerve dysfunction, such as those with carpal tunnel syndrome.

From the findings of this thesis, it is clear that the effects of omega-3 PUFA supplementation on peripheral nerve function are deserving of further investigation. As our RCT was powered based upon CNFL, the resulting sample size, which is relatively conservative, may not have been adequately powered to detect a difference in clinical neurophysiology parameters. Further appropriately-powered RCTs, conducted in well-defined clinical populations and over longer study durations, are indicated to more unequivocally ascertain the effects of omega-3 PUFA supplements on peripheral nerve function in individuals with type 1 diabetes.

Omega-3 fatty acids for ocular surface disease in diabetes

As summarised in **Chapter 2**, dietary supplementation with omega-3 PUFAs is a recognised strategy for reducing ocular surface inflammation. A systematic review by Downie *et al.* identified a possible role for long-chain omega-3 supplementation in managing DED (although the evidence was graded as uncertain and inconsistent) (Downie *et al.*, 2019). In diabetes, tear abnormalities and corneal nerve loss predispose affected individuals to DED and neurotrophic keratopathy (Shih *et al.*, 2017). However, neither of the populations with diabetes that participated in studies contributing to this thesis exhibited overt signs of ocular surface disease. In the cross-sectional study (**Chapter 5**), most participants with diabetes without DSP symptoms did not fulfil the diagnostic criteria for DED according to the TFOS DEWS II (Wolffsohn *et al.*, 2017), although both corneal nerve structure and sensitivity were reduced compared to controls. In the RCT (**Chapter 7**), participants had tear osmolarity within the physiological range and sub-threshold dry eye symptom scores. The finding of improved tear film function with omega-3 PUFA supplementation, relative to placebo, suggests that treatment may be protective of progressive diabetes-associated ocular surface disease in diabetes. However, given that overt dry eye disease was not present in the study cohort, the clinical utility of omega-3

PUFAs for improving tear function in individuals with symptomatic diabetes-associated ocular surface disease requires further investigation.

Using *in vivo* confocal microscopy (IVCM), increased corneal dendritic cell (DC) density has been observed to be associated with immune-mediated corneal inflammation (Mastropasqua et al., 2006). This thesis found that in individuals with diabetes without DSP symptoms, DC density and morphology did not differ from those of healthy controls (**Chapter 5**). Furthermore, six-months of oral omega-3 supplementation did not significantly change DC density in type 1 diabetes relative to placebo (**Chapter 7; data are reported in exploratory outcomes in Appendix G**). Although IVCM studies have noted DC accumulation and maturation in diabetes populations (Lagali et al., 2018; Tavakoli et al., 2011a), the precise role of corneal DCs in the DSP disease process has not been elucidated. A limitation in the characterisation of DCs using IVCM is that it provides no information on DC surface markers. There is also little evidence to show how these cells respond to topical and/or systemic anti-inflammatory treatment. Given that dietary omega-3 PUFAs increase systemic anti-inflammatory mediators, it is possible that DC densities and/or morphologies may change in association with treatment-related effects in populations with overt ocular surface inflammation. Further research is warranted to understand the natural history of DC accumulation and morphological alterations with DSP progression, as well as the potential role of DCs as a marker of ocular surface inflammation in this condition.

8.3 Implications for clinical practice

Omega-3 PUFAs play a role in the management of a number of ocular conditions. The clinician's survey described in **Chapter 3** found that most (78%) Australian and New Zealand optometrists recommend omega-3 PUFA consumption, through diet and/or supplementation, to their patients for improving eye health. This study also highlighted knowledge gaps and perceived barriers that challenge the implementation of best-practice dietary

recommendations in eye care settings. These findings are beneficial for informing future education programs, which, if delivered to optometrists, could enhance practitioner knowledge and confidence in making nutritional and dietary recommendations. Future clinical education could address the differences in biological efficacies between omega-3 food sources (e.g., plant-based versus marine-based) and in the evidence for supplement versus whole-food sources of omega-3 PUFAs in age-related macular degeneration. From the sources of information used by clinicians to guide their decision-making, the optimal pathway to deliver clinical education is through continuing education conferences and articles.

The finding that almost no optometrists (1%) use quantitative tools to survey patients' existing dietary habits, despite almost all (99%) offering dietary advice, highlights an avenue for improving clinical practice in providing targeted nutritional advice. The reason(s) for clinicians not using a validated food survey could be due to a lack of awareness, perceived time-commitment, or current lack of accessible clinical tools. To overcome these barriers, a short food-frequency questionnaire was developed and validated (**Chapter 4**). This tool provides a rapid, non-invasive method for estimating dietary omega-3 PUFA intake. By providing information on the sufficiency of a patient's dietary omega-3 intake, the tool could assist clinicians in making appropriate recommendations surrounding dietary modification and/or supplementation. Although only preliminary validation of this tool was undertaken in this thesis, the intention is to evaluate the tool in larger, homogenous populations in future, and to be able to offer the tool via an accessible medium, such as a website, for increased clinical utility.

Optometrists play an essential role in the management of diabetes complications, including the detection and monitoring of diabetic retinopathy and management of ocular surface disease and diabetic keratopathy. Given the role that omega-3 PUFAs have been demonstrated to play in modulating corneal health and tear function, enhancing dietary omega-3 PUFAs may

become a strategy for managing corneal neuropathy in this population. However, as discussed earlier in this chapter, their long-term effects on peripheral nerve function are yet to be ascertained. Prior to clinical implementation, further research is indicated to confirm whether the clinical applicability of omega-3 PUFAs can be generalised to wider populations with diabetes. Potential populations to consider include those with clinically-evident diabetic neuropathy, painful diabetic neuropathy, established corneal keratopathy, and type 2 diabetes.

8.4 Implications for future research

Intervention considerations

Dosage is an important factor when considering the safety and potential efficacy of omega-3 PUFA interventions. In RCTs evaluating omega-3 supplementation for DED, a lack of consensus in the optimal intervention protocol concerning dosage, composition, and treatment duration potentially contributes to the conflicting outcomes observed. Given that no formal recommendations for an optimal dosage exist for ocular conditions, the 20-fold dosing range reported across RCTs may be a contributing factor towards the heterogeneity of clinicians' recommendations, which, for DED, varied from 250 to 6,000 mg/day in combined EPA and DHA (**Chapter 3**).

From the systematic review (**Chapter 6**), published RCTs that have evaluated omega-3 supplementation for peripheral nerve outcomes used dosages of between 640 to 5,000 mg/day (combined EPA and DHA). A supplementation dosage of 1800 mg/day was selected in our RCT (**Chapter 7**) based upon prior intervention studies that showed the ability of a similar dosage to confer benefit in corneal nerve outcomes (Chinnery et al., 2017; Lewis et al., 2017).

In cardiovascular disease, a treat-to-target approach, which adjusts dosage to reach a target erythrocyte EPA+DHA level, has been proposed as an alternative strategy to prescribing a fixed-dose (James et al., 2014). Using this approach, a

meta-analysis of ten cohort studies concluded that an Omega-3 Index of 8% is a reasonable therapeutic target (Harris et al., 2017). In our RCT, an average Omega-3 Index of 8% was achieved in the treatment group at six months. Further research into the dose-response effect is required to confirm whether the same therapeutic target of 8%, used in cardiovascular disease, is appropriate for improving peripheral nerve outcomes.

In addition to potential efficacy, the safety and tolerability of omega-3 PUFA supplementation is an additional consideration that can influence dosage selection. Due to their anticoagulant properties, high-dose omega-3 PUFAs can increase the risk of bleeding (Jeansen et al., 2017). This effect has not been demonstrated, however, in clinical trials using dosages of up to 10,000 mg/day, even with concomitant anticoagulant medications (Bays, 2007; Jeansen et al., 2017) or before major vascular surgery (Akintoye et al., 2018). Other potential adverse effects with high-dose (>5,000 mg/day) omega-3 PUFA supplements include impaired immune function, increased lipid peroxidation, and impaired lipid and glucose metabolism (EFSA, 2012). A higher prevalence of fishy/altered taste and skin rashes has also been reported with prescription omega-3 formulations (2,000–4,000 mg/day omega-3 ethyl esters) than with placebo (Chang et al., 2018).

Currently, the upper intake limit of long-chain omega-3 PUFAs, as recommended by the Australia and New Zealand National Health and Medical Research Council, is 3,000 mg/day (NHMRC, 2005) and, by the European Food and Safety Authority, is 5,000 mg/day (EFSA, 2012). The supplementation dose used in the RCT described in **Chapter 7** (1800 mg/day) is within these limits, and is less than half that of recommended for hypertriglyceridemia, which is 4,000 mg/day of omega-3 ethyl esters (Skulas-Ray et al., 2019). In our RCT, this treatment dosage did not raise any safety concerns, as the incidence of adverse events did not differ between treatment groups, and diabetic retinopathy grading did not change in either group over a six-month period.

Other considerations for the formulation are the ratio of EPA/DHA and the chemical form (i.e., triglycerides, phospholipids, or ethyl esters) of the supplements. The impact of EPA/DHA ratio on their conferred benefits in ocular or neurodegenerative conditions is not currently known. In **Chapter 5**, systemic DHA, but not EPA, was found to be associated with corneal sub-basal nerve structure. This supports the hypothesis from preclinical studies that DHA and its metabolites, neuroprotectins, are neuromodulators that promote peripheral nerve integrity (Cortina et al., 2013; Hong et al., 2014). In animal studies, oral DHA supplementation reduced neuropathy symptoms in experimental diabetes (Heng et al., 2015); topical DHA enhanced corneal nerve growth and improved corneal sensitivity after corneal nerve dissection (Cortina et al., 2013). However, in the clinical setting, only one single-arm interventional study has investigated EPA ethyl esters for alleviating clinical neuropathy symptoms in DSP (Okuda et al., 1996); DHA-only formulations have not been investigated.

The chemical form of the supplements (i.e., as triglycerides, phospholipids, or ethyl esters), as well as basal dietary PUFA levels, influences the bioavailability of omega-3 PUFA interventions (Dyerberg et al., 2010; Offman et al., 2013). In the systematic review (**Chapter 6**), only five of the fifteen existing RCTs evaluating omega-3 PUFA supplementation for peripheral nerve outcomes specified the chemical formulation of the investigational product. The preparation may not only affect absorption; phospholipid and triglyceride forms of omega-3 supplementation have been recognised to differentially modulate ocular surface inflammation (Deinema et al., 2017). Whether preparation-dependent differences impact efficacy in modulating neurological outcomes is currently unknown. Therefore, potential treatment differences conferred by different omega-3 supplement formulations, relating to its chemical form and the EPA/DHA ratio, may constitute the objective of future studies.

Population considerations

Beyond its relevance to clinical practice, baseline omega-3 PUFA intake is also a potential confounder in evaluating therapeutic efficacy in omega-3 fatty acid

intervention trials. Basal PUFA levels can be estimated using dietary records and food-frequency questionnaires, or quantified using biological markers. This information is valuable for identifying individuals who already obtain sufficient omega-3 PUFAs via their diet and who, therefore, may not demonstrate the same biological responses to treatment as those who were deficient.

Furthermore, capturing dietary habits in the comparator group is a potential strategy for minimising misclassification bias. In omega-3 RCTs, non-study-related intake of EPA and DHA cannot be feasibly eliminated in the control group as omega-3 PUFAs are readily available from food sources. To ascertain treatment efficacy, systemic omega-3 levels in the comparator group must remain below a threshold level that adequately distinguishes them from those in the treatment group. Quantification of basal omega-3 fatty acid intake is recommended in cardiovascular RCTs (Rice et al., 2016); however, no guidance exists for other indications. As summarised in **Chapter 2**, of the ten RCTs that have evaluated omega-3 PUFA supplementation for DED, only two quantified baseline omega-3 PUFA levels; both studies used biological markers. Where biological markers are unavailable, it may be possible to use dietary questionnaires to inform baseline omega-3 intake. A simple food-frequency questionnaire, such as the clinical omega-3 dietary survey (CODS), has utility in this respect. Unlike questionnaires designed for epidemiological studies, which are lengthy and relatively time-consuming, CODS can be rapidly administered and easily incorporated into most study protocols. Using this tool, an estimated long-chain omega-3 PUFA intake of 550 mg/day equates to an Omega-3 Index of 8%, the proposed therapeutic target for cardiovascular trials.

Baseline DSP status is a potential confounding factor in DSP intervention trials. Most participants with diabetes that participated in studies contributing to this thesis did not have clinically-evident diabetic neuropathy. In the cross-sectional study (**Chapter 5**), limiting study eligibility to those with minimal DSP was intentional. However, this relied upon patient-reported symptoms only and was not based on clinical outcomes, owing to logistical constraints, which is recognised as a limitation. Therefore, in the RCT described in **Chapter 7**, a

Michigan Neuropathy Screening Instrument (MNSI) cut-off score was implemented as an eligibility criterion to recruit individuals with early to moderate DSP. Contrary to these intentions, most (87%) participants, when diagnosed using the Michigan Diabetic Neuropathy Score (MDNS), failed to demonstrate clinically-evident DSP at baseline. Although one-third of the cohort had small fibre neuropathy, most of the study population had normal large fibre function, as evidenced by normal mean baseline axonal excitability and nerve conduction. The normal neurophysiology values could have been a factor that limited the capacity for improvement in these outcomes. Furthermore, the RCT excluded individuals with retinopathy grading of worse than 'moderate', using the simplified Early Treatment Diabetic Retinopathy Study (Wisconsin) classification, to mitigate the perceived risk of omega-3 PUFA supplementation on bleeding. However, given that retinopathy severity is associated with severity of neuropathy (Barr et al., 2006), this likely served to limit the ability to recruit individuals with more severe disease. Future trials might consider whether the same criteria are necessary in seeking populations with evident clinical neuropathy, or whether potential treatment benefits may outweigh the, as yet unfounded, theoretical risks. Furthermore, the inclusion of individuals with more severe DSP symptoms and/or clinical signs in future studies could further inform the potential effects of omega-3 PUFA intervention on disease impairment, disability and quality of life.

Outcome selection

As acknowledged in the literature review (**Chapter 2**), there are currently no universally-accepted clinical outcome measures for assessing peripheral nerve health. The systematic review (**Chapter 6**) further found the synthesis of reports of peripheral nerve outcomes in established RCTs to be challenging, owing to differences in the types and formats of outcomes reported. This finding highlights a need for development and consensus of a core outcome set to enable a comprehensive synthesis of data from clinical studies in future.

In addition to corneal nerve parameters, the RCT described in the thesis (**Chapter 7**) adopted clinical and neurophysiology outcomes to measure both small and large nerve fibre function. Small A δ - and C-fibre function were respectively evaluated with cutaneous silent period (CSP) and quantitative sudomotor axonal reflex testing (QSART). However, both tests are inherently variable (Kamel et al., 2015). In our RCT, neither parameter showed significant change relative to placebo. Therefore, the utility of either test as an endpoint in future neuropathy trials warrants further evaluation. For large nerve fibre function measured using NCS, the key parameters that were selected as secondary outcomes were consistent with those recommended by Merkies and Lauria, in the 131st Europe Neuromuscular Centre International Consensus Document (sural sensory amplitude, peroneal motor velocity, and minimal F-wave latency) (Merkies & Lauria, 2006). Although NCS is reliable and repeatable, a reported temporal delay the observed improvement, relative to corneal nerve recovery, indicates that longer treatment periods (minimum 36 months) may be required for evaluating this outcome (Azmi et al., 2019). Compared with NCS, which measures only impulse conduction, nerve excitability provides information on ion channel function and the axonal membrane properties (Kiernan et al., 2020). Prior studies have shown that nerve excitability testing can detect axonal dysfunction earlier in the DSP disease process than NCS (Sung et al., 2012); axonal excitability can also demonstrate recovery with treatment (Kamel et al., 2020). Findings from our RCT suggest that future studies should preferentially include individuals with abnormal baseline axonal function to ascertain improvement in this outcome measure.

For evaluating small nerve fibre structure, quantification of somatic intraepidermal nerve fibre density can be performed using skin biopsy, but the procedure is relatively invasive and not easily repeatable (Quattrini et al., 2007). In contrast, the non-invasive and repeatable nature of IVCM posits corneal nerve morphology as a more suitable endpoint in DSP trials. Corroborating prior findings (Lewis et al., 2017; Tavakoli et al., 2013), our results confirm that corneal nerves have the ability to regenerate and, thus, are sensitive surrogate

markers for demonstrating the potential neuro-regenerative capabilities of new therapeutic agents. Furthermore, as corneal nerve morphology is one of the earliest markers to show change, it can be used to inform, in early phase studies, the potential value of expansion to larger, more costly clinical trials conducted over a longer duration. There remains a need to standardise the method of IVCN analysis and interpretation in future clinical research, with consideration for how improvements in corneal small nerve fibres translate to meaningful improvements in sensory function. To improve the comparability of corneal IVCN measures in future research, there is also a need to establish a uniform approach to undertaking IVCN imaging procedures, as the existing variations in imaging methodologies across studies may lead to potential biases and affect the reliability of reported data. For example, selecting an insufficient number of corneal images, or images with excess overlap, as a representation of overall corneal nerve density could lead to sampling bias, and outcome assessors that are not masked to group allocations could lead to performance bias (De Silva et al., 2017). Another consideration for future studies employing corneal structural parameters as surrogate markers is whether a similar CNFL change is observed using automatic and manual analyses, as higher CNFL values have been reported with manual analyses (Dehghani et al., 2014a), and how this change might relate to other corneal structural features, such as corneal epithelial cell density (Rosenberg et al., 2000).

8.5 Conclusion

In conclusion, the work in this thesis adds to a growing body of research on the applications of omega-3 PUFAs for corneal and peripheral nerve health. Findings from a clinician's survey advance the understanding of clinical practices relating to omega-3 PUFAs in eye care settings. By identifying practice patterns and knowledge gaps, these findings highlight opportunities for future professional education to improve clinical recommendations relating to diet and omega-3 PUFAs in eye care practice. A dietary assessment tool was developed and validated. By providing a rapid and accessible method for

evaluating dietary omega-3 PUFA intake, this tool could assist clinicians in providing targeted dietary advice.

The included prospective clinical studies enhance the understanding of the use of anatomical and functional corneal parameters as markers of peripheral nerve integrity in diabetes. A systematic review, synthesising current best-available evidence on the efficacy and safety of oral omega-3 supplementation for improving peripheral nerve outcomes, provides insight into the potential utility of omega-3 PUFA supplementation in chemotherapy-induced peripheral neuropathy. Findings from a double-masked RCT demonstrate that oral omega-3 PUFA supplementation improves corneal nerve structure in individuals with type 1 diabetes, consistent with a corneal neuroregenerative effect. These findings also highlight opportunities for the conduct of longer-term RCTs to ascertain the effects of omega-3 PUFAs in populations with clinically-evident DSP and on outcomes of peripheral nerve function.

Peripheral neuropathy is a distressing condition that can lead to significant functional and psychosocial impairment and adversely impact daily living. There remains a need to find treatments that improve peripheral nerve outcomes in diabetes, to optimise clinical management. This thesis begins to address this need by providing clinical evidence for the role of omega-3 PUFAs in modulating peripheral nerve health. Given the availability of dietary omega-3 PUFAs, an extension of the positive findings described in this thesis could rapidly translate to clinical implementation and ultimately improve the clinical care for the millions of individuals with diabetes, who are at risk of developing, or are burdened with, peripheral neuropathy.

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<https://doi.org/10.1016/j.ajo.2018.01.004>

Appendix A. Chapter 3: Supplementary materials [1 of 1]

Supplementary materials for the corresponding publication

Supplementary materials for the published article:

Zhang, A. C., Singh, S., Craig, J. P., & Downie, L. E. (2020). Omega-3 Fatty Acids and Eye Health: Opinions and Self-Reported Practice Behaviors of Optometrists in Australia and New Zealand. *Nutrients*, 12(4), 1179.
<https://doi.org/10.3390/nu12041179>

Table A1. Knowledge questions surveyed and, in bold, answers considered as ‘correct’ for deriving an ‘omega-3 knowledge score’.

Table A1. Knowledge questions surveyed and, in bold, answers considered as ‘correct’ for deriving an ‘omega-3 knowledge score’.

Questions (Answer Considered ‘Correct’ in Bold)
There are two forms of omega-3 fatty acids, derived either from plant-based sources (e.g., chia seeds, walnuts, flaxseed) or marine-based foods (e.g., fish and seafood).
3.1 Which of the following do you consider to best describe the difference between plant-based and marine-based omega-3 fatty acids, if they are consumed at the same dose?
(a) Plant-based (short-chain) omega-3 fatty acids have <u>MORE</u> biological effect than marine-based (long-chain) omega-3 fatty acids
(b) Plant-based (short-chain) omega-3 fatty acids have LESS biological effect than marine-based (long-chain) omega-3 fatty acids
(c) Plant-based (short-chain) omega-3 fatty acids have the <u>SAME</u> biological effect as marine-based (long-chain) omega-3 fatty acids
(d) Don’t know
Omega-6s are the other main form of essential fatty acids. For the next two questions, consider which option best describes the biological roles of omega-3 and omega-6 fatty acids.
3.2 Omega-3 fatty acids are:
(a) Always anti-inflammatory
(b) Mostly anti-inflammatory
(c) Equally anti-inflammatory and pro-inflammatory
(d) Mostly pro-inflammatory
(e) Always pro-inflammatory
3.3 Omega-6 fatty acids are:
(a) Always anti-inflammatory
(b) Mostly anti-inflammatory
(c) Equally anti-inflammatory and pro-inflammatory
(d) Mostly pro-inflammatory
(e) Always pro-inflammatory
3.4 Based upon your current knowledge, what do you consider to be an ideal ratio of omega-6 to omega-3 in the human diet?
(a) 10:1
(b) 4:1
(c) 1:1
(d) 1:4
(e) 1:10
3.5 Based on your understanding, which of the following options would you consider to be closest to the ideal approximate adult dietary target of long-chain (marine-based) omega-3 fatty acids (from food and/or supplementation)?
(a) 100 mg/day
(b) 250 mg/day
(c) 500 mg/day
(d) 1000 mg/day
(e) 1500 mg/day

Appendix B. Chapter 4: Supplementary materials [1 of 1]

Supplementary materials for the corresponding publication

Supplementary materials for the published article:

Zhang, A. C., & Downie, L. E. (2019). Preliminary Validation of a Food Frequency Questionnaire to Assess Long-Chain Omega-3 Fatty Acid Intake in Eye Care Practice. *Nutrients*, 11(4). <https://doi.org/10.3390/nu11040817>

Figure S1: The Clinical Omega-3 Dietary Survey

Figure S2: Diagram adapted from Ocke & Kaaks, to describe the method of triads (Ocke & Kaaks, 1997).

Figure S1: Clinical Omega-3 Dietary Survey

Clinical Omega-3 Dietary Survey																																																																						
How many times have you eaten fish or shellfish in the past week? ☐ 0 times ☐ 1-3 times ☐ More than 3 times																																																																						
Over the past 3 months, about how often have you eaten fish or shellfish in any form? ☐ Never ☐ _____ times per (please circle) Day/Week/Month																																																																						
Each time you ate fish or shellfish, how much did you eat? ☐ Less than 50 g or half a fillet or less than 4 pieces/1-2 hand roll of sushi ☐ 100 g or about 1 small fillet or 8 pieces of sushi/3 hand rolls ☐ 150 g or 1 large fillet or 12 pieces of sushi/4 hand rolls ☐ 200 g+ or more than 2 fillets or 16 pieces of sushi/6 hand rolls																																																																						
Please mark the food(s) you eat regularly (i.e., at least once per month) over the last three (3) months and indicate: • your average portion for <u>EACH</u> serve • on average, how often you eat that type of food																																																																						
90 g = approximately the size of your palm or a deck of cards.																																																																						
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Other meats			
☐	Bacon	 g	 times a week/month
☐	Beef	 g	 times a week/month
☐	Kangaroo	 g	 times a week/month
☐	Lamb	 g	 times a week/month
☐	Salami	 g	 times a week/month
☐	Pork	 g	 times a week/month
☐	Egg (per egg)	 eggs	 times a week/month
Omega-3 Supplements			
☐	Omega-3/Fish oil (please specify Brand/type/dose)	 g/caps/TBS	
.....		 times a day/week	

Figure S1: Clinical Omega-3 Dietary Survey (CODS)

Figure S2: Method of triads

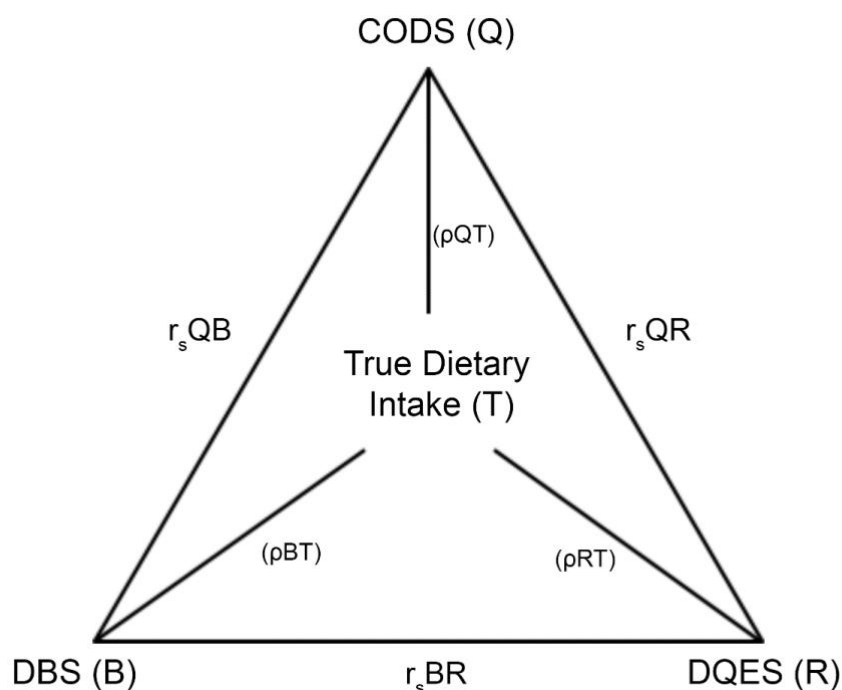


Figure S2: Diagram adapted from Ocke & Kaaks, to describe the 'method of triads' [43] used to estimate the validity coefficient (q) for long-chain omega-3 dietary intake estimated using the questionnaire (Q), Clinical Omega-3 Dietary Survey (CODS); a reference marker (R), the Dietary Questionnaire for Epidemiology Studies (DQES) v3.2; and a biomarker (B), the dried blood spot (DBS) test. Spearman's correlation coefficient (r_s) was used to estimate the correlation coefficient between each method. Validity coefficients (q) were calculated using methods as described by Ocke & Kraaks for each of the reference methods against an estimated true intake (T) [43].

Appendix C. Chapter 5: Supplementary materials [1 of 2]

Test-retest repeatability of corneal sensitivity thresholds measurements

C.1 Introduction

In this thesis, examination of corneal sensitivity thresholds was performed using a non-contact corneal aesthesiometer (NCCA), which evaluates corneal sensitivity to an air stimulus. The NCCA instrument used in this thesis, for studies described in **Chapters 5 and 7**, was modelled on the instrument designed and validated by Murphy, Patel, and Marshall in the mid-1990s (Murphy *et al.*, 1996).

Although similar NCCA instruments have been validated (Murphy *et al.*, 1998; Swanevelder *et al.*, 2019), the particular instrument used in the present studies had not been assessed for repeatability or reproducibility. Repeatability, describing the variations in repeat measurements performed on the same subjects under similar conditions, is examined in this Appendix. Reproducibility, which describes the agreement of tests for each individual under different conditions (such as environment, location, and setting) is beyond the scope of this evaluation.

The objective of this study was to assess the agreement between corneal sensitivity threshold measurements to room-temperature and cooled stimuli on the same individuals, for measurements taken on different days, at different times, or both.

C.2 Methods

This project was approved by the University of Melbourne Human Research Ethics Committee (HREC #1749830.2). All participants provided written informed consent to participate.

Examination procedure

Corneal sensitivity thresholds to both room-temperature and cooled stimuli were examined on four occasions: on two separate days, spaced one week apart, in the morning (9am-12pm) and in the afternoon (after 3pm-5pm), in the same temperature- and humidity-controlled environment (**Figure C1**).

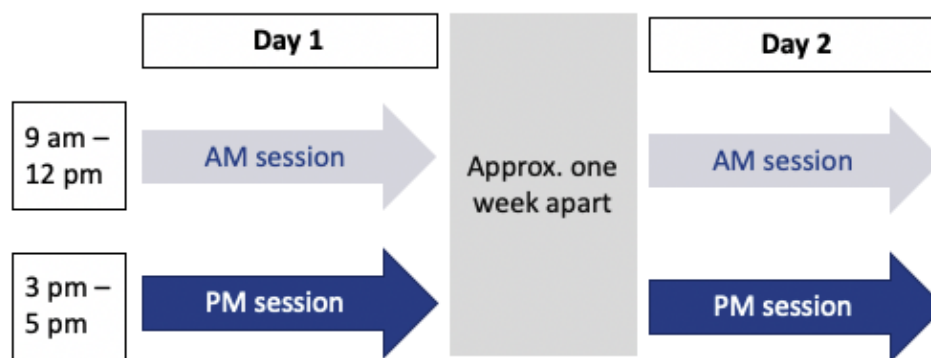


Figure C1. Diagrammatic representation of the four test-retest sessions.

Abbreviations: AM, morning; Approx., approximately; PM; afternoon.

The corneal sensitivity threshold examination method was the same as described in **Chapter 5**, using a double-staircase algorithm and calculated as the average threshold from two reversals (per staircase). For all examinations, the starting threshold in each staircase was randomly selected as 0.2, 0.3, or 0.4 mbar. During testing, if two consecutive responses were detected at 0.1 mbar

(the lowest detectable threshold), the algorithm would enter a decision tree to determine the final threshold (**Figure C2**).

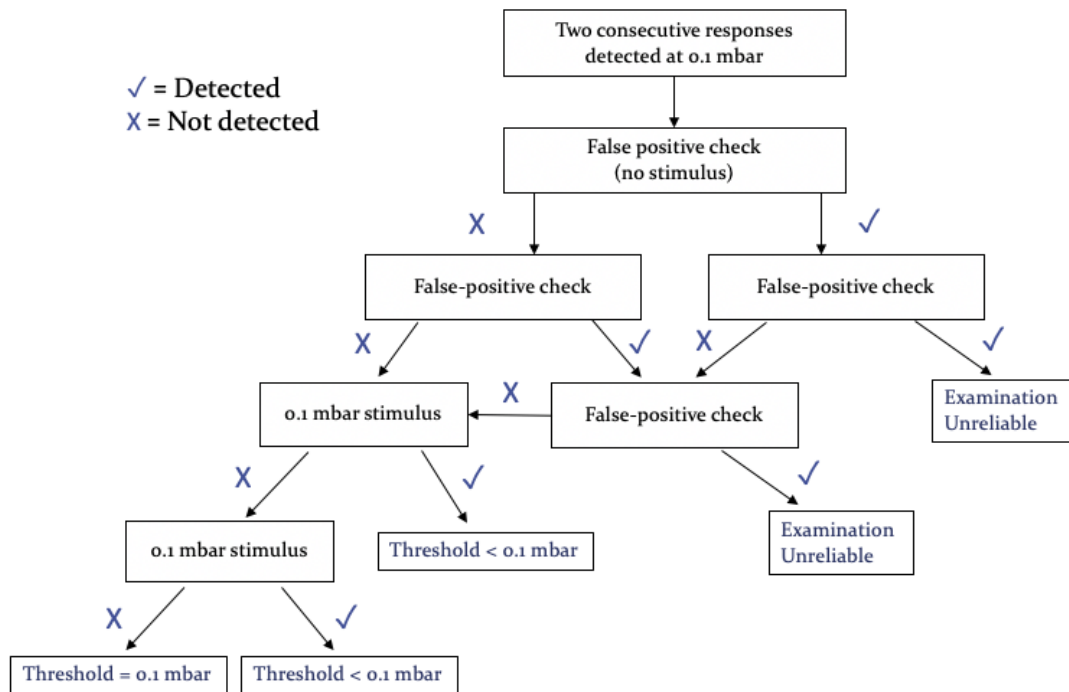


Figure C2. Non-contact corneal aesthesiometry protocol for if two consecutive responses were detected at 0.1 mbar.

Statistical analysis

In these preliminary studies for assessment of repeatability, corneal sensitivity thresholds deemed as being <0.1 mbar, from the flow chart in **Figure C2**, were analysed as 0.1 mbar. The minimum step-size of the instrument, representing the minimum level of precision that can be measured, is 0.1 mbar.

Bland-Altman plots were used to evaluate the agreement between the corneal sensitivity threshold measurements of the test-retest sessions.

Data relating to the difference between the test and re-test conditions were evaluated using the Shapiro-Wilk test for assessing normality. As recommended by Bland & Altman, a log transformation was applied if differences were not normally distributed (Bland & Altman, 1999).

Descriptive statistics for corneal sensitivity thresholds are summarised as the median and interquartile range. Corneal sensitivities from multiple sessions were compared using the Wilcoxon matched-pairs signed rank test to compare data from two sessions, and the Friedman test to compare data arising from more than two sessions.

C.3 Results

Participants

Ten participants were recruited (**Table C1**), five of whom self-reported being healthy with no ocular or systemic conditions. Two participants reported histories of corneal refractive surgery at least four years prior; three participants reported systemic conditions not affecting the eye.

Table C1. Participant characteristics and examination times

Partici- pant number	Sex	Age	Systemic/ocular conditions	Examination time			
				Day 1 AM	Day 1 PM	Day 2 AM	Day 2 PM
1	F	38	Healthy	9:15	15:35	8:37	15:43
2	M	27	Healthy	10:09	16:40	10:26	16:35
3	F	29	History of refractive surgery	10:36	15:15	9:55	5:40
4	F	27	History of refractive surgery	10:35	15:40	10:00	15:35
5	F	37	Psoriasis	10:20	15:25	10:15	15:00
6	F	25	Healthy	9:44	15:35	9:48	16:05
7	F	26	Hypothyroidism	10:15	15:50	9:35	15:00
8	F	40	Hypothyroidism	10:30	16:20	10:05	16:50
9	F	50	Healthy	10:35	15:00	11:45	15:00
10	M	27	Healthy	10:15	15:05	10:00	15:50

F, female; M, male, AM, morning; PM; afternoon

Table C2 shows corneal sensitivity thresholds on two examination days.

Table C2. Corneal sensitivity thresholds for room-temperature and cooled stimuli

Partici- pant number	Room-temperature stimulus (mbar)				Cooled stimulus (mbar)			
	Day 1 AM	Day 1 PM	Day2 AM	Day 2 PM	Day 1 AM	Day 1 PM	Day2 AM	Day 2 PM
1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
2	0.4	0.1	0.1	0.1	0.1	0.1	0.15	0.1
3	0.2	0.25	0.2	0.4	0.425	0.225	0.25	0.15
4	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
5	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
6	0.2	0.2	0.45	0.175	0.25	0.2	0.1	0.1
7	0.1	0.1	0.1	0.15	0.1	0.1	0.1	0.1
8	0.35	0.4	0.425	0.2	0.325	0.3	0.375	0.45
9	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
10	0.225	0.35	0.1	0.15	0.1	0.175	0.15	0.25

AM, morning; PM; afternoon

For room-temperature stimuli, median corneal sensitivity thresholds for all participants was 0.15 (IQR: 0.10 to 0.26) mbar in the morning and 0.10 (0.10 to 0.28) mbar in the afternoon on day one, and 0.10 (0.10 to 0.26) mbar in the morning and 0.13 (0.10 to 0.18) mbar in the afternoon on day two.

For cooled stimuli, median corneal sensitivity thresholds were 0.10 (IQR: 0.10 to 0.27) mbar in the morning and 0.10 (0.10 to 0.21) mbar in the afternoon on day one, and 0.10 (0.10 to 0.18) mbar in the morning and 0.10 (0.10 to 0.18) mbar on day two.

Comparison of mean corneal sensitivity threshold measurements from all four sessions, using the Friedman test, found no significant differences in mean thresholds for room-temperature stimuli ($p=0.94$) or cooled stimuli ($p=0.93$).

Repeatability analysis 1: Same time, different day

For corneal sensitivity thresholds to room-temperature stimuli, evaluated at the same time of the day, but on different days, the mean $\pm$ SD difference between measurements was 0.01 ± 0.14 mbar for the morning, and 0.03 ± 0.11 mbar for the afternoon. For cooled stimuli, the mean difference was 0.02 ± 0.01 mbar in the morning, and -0.01 ± 0.07 mbar in the afternoon.

Figure C3 shows Bland-Altman plots comparing corneal sensitivity thresholds (log-transformed) measured at the same time on different days, for both room-temperature and cooled stimuli.

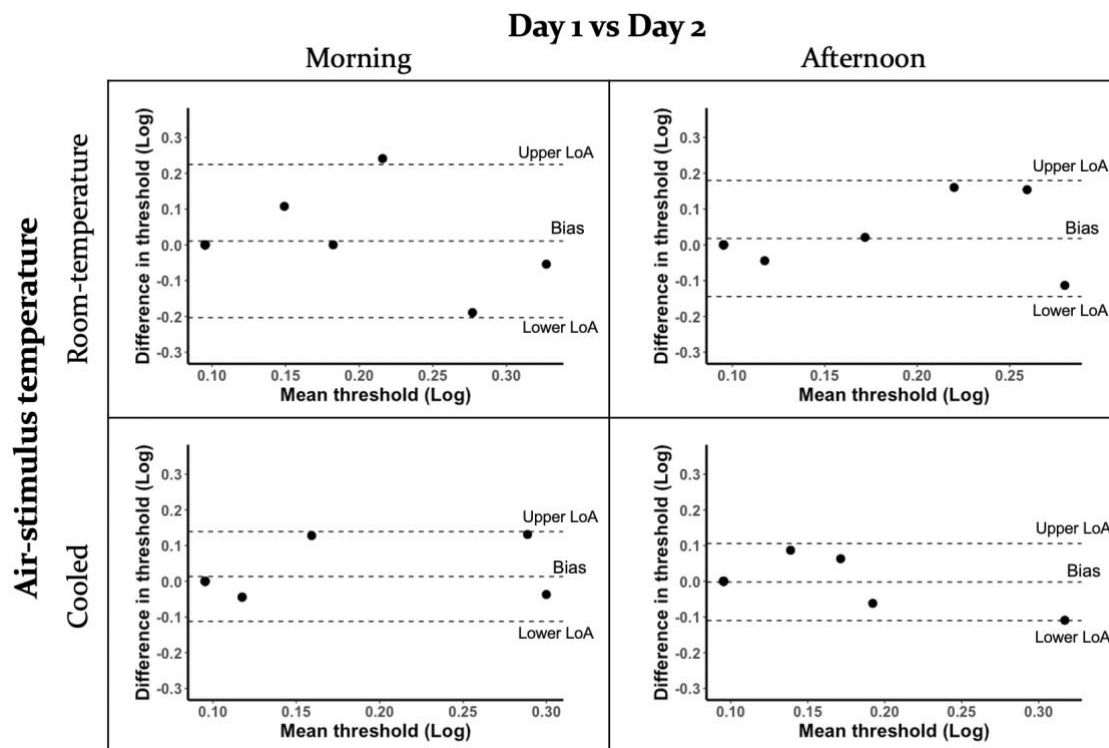


Figure C3. Bland-Altman plots for corneal sensitivity thresholds to room-temperature and cooled air stimuli comparing day one with day two ($n=10$). LoA, 95% limits of agreement. Fewer than 10 plots points are visible as some points are coincident.

For corneal sensitivity thresholds to room-temperature stimuli taken in the morning, the geometric mean ratio of thresholds, evaluated on day one compared with day two, was 1.01 (95% LoA: 0.82 to 1.25). This shows that the 95% limits of agreement for corneal sensitivity threshold measurements on day 1 are between 18% lower and 25% higher than measurements that are taken at

a similar time of day, on day 2 (Bland & Altman, 1999). For measurements in the afternoon, the geometric mean ratios value comparing day one and day two was 1.02 (95% LoA: 0.87 to 1.20).

For corneal sensitivity thresholds to cooled stimuli, the geometric mean ratio of values for inter-day measurements was 1.01 (95% LoA: 0.89 to 1.15) for the morning, and 1.00 (95% LoA: 0.90 to 1.11) for the afternoon.

Figure C4 shows Wilcoxon matched-pairs signed rank test plots comparing raw corneal sensitivity thresholds evaluated at the same time of day, on different days, for both room-temperature and cooled stimuli.

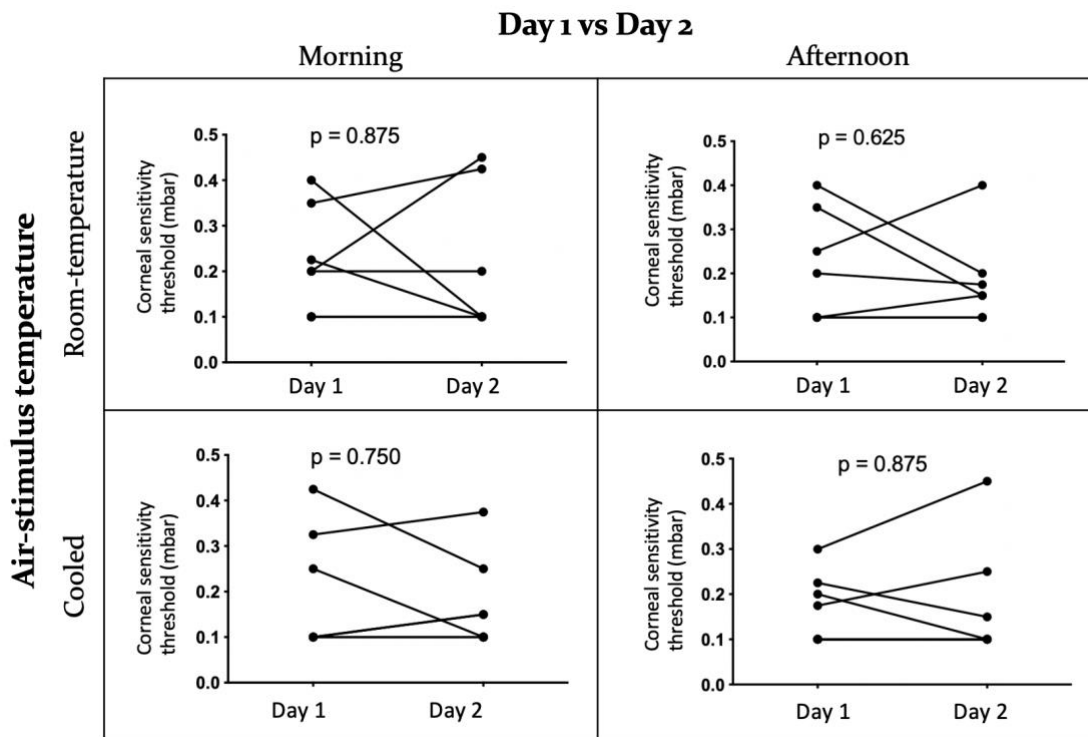


Figure C4. Wilcoxon matched-pairs signed tank test plots for corneal sensitivity thresholds to room-temperature and cooled air stimuli comparing day one with day two. Fewer than 10 plots points are visible as some points are coincident.

No statistically significant differences in corneal sensitivity thresholds were identified between room-temperature or cooled stimuli for morning or afternoon sessions when the test was repeated at a similar time of day, on different days ($p > 0.05$).

Repeatability analysis 2: Same day, different time

For corneal sensitivity thresholds evaluated on the same day but at different times, for room-temperature stimuli, the mean $\pm$ SD difference between measurements was 0.02 ± 0.13 mbar for the morning, and 0.02 ± 0.14 mbar for the afternoon. For corneal sensitivity to cooled stimuli, the mean inter-day difference was 0.02 ± 0.07 mbar in the morning, and -0.03 ± 0.06 mbar in the afternoon.

Figure C5 shows Bland-Altman plots comparing corneal sensitivity thresholds (log-transformed) evaluated on the same day, at different times, for both room-temperature and cooled stimuli.

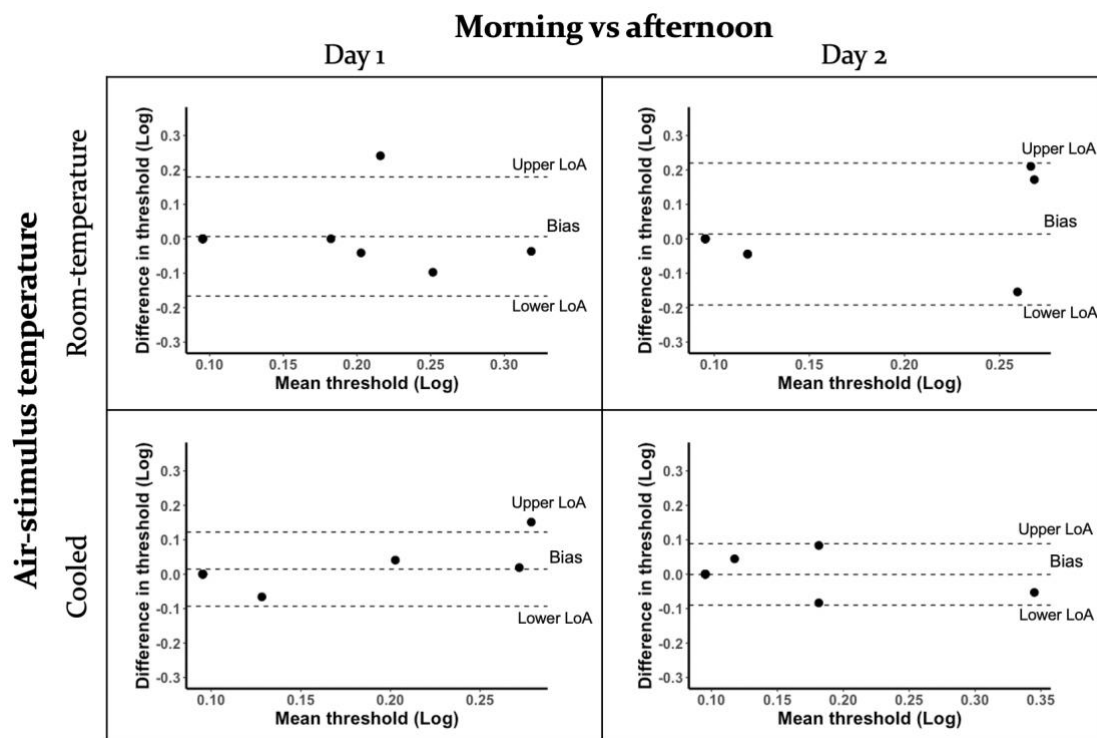


Figure C5. Bland-Altman plots for corneal sensitivity thresholds to room-temperature and cooled stimuli comparing morning to afternoon measurements. LoA, 95% limits of agreement. Fewer than 10 plots points are visible as some points are coincident.

For corneal sensitivity thresholds to room-temperature stimuli, the geometric mean ratios of values for measurements in the morning compared with the afternoon was 1.01 (95% LoA: 0.85 to 1.20) on day one, and 1.01 (95% LoA: 0.83 to 1.25) on day two.

For corneal sensitivity thresholds to cooled stimuli, the geometric mean ratios of values for the measurements was 1.01 (95% LoA: 0.91 to 1.13) on day one, and 1.00 (95% LoA: 0.91 to 1.10) on day two.

Figure C6 shows Wilcoxon matched-pairs signed rank test plots comparing raw corneal sensitivity thresholds evaluated at two different times on the same testing day, for both room-temperature and cooled stimuli.

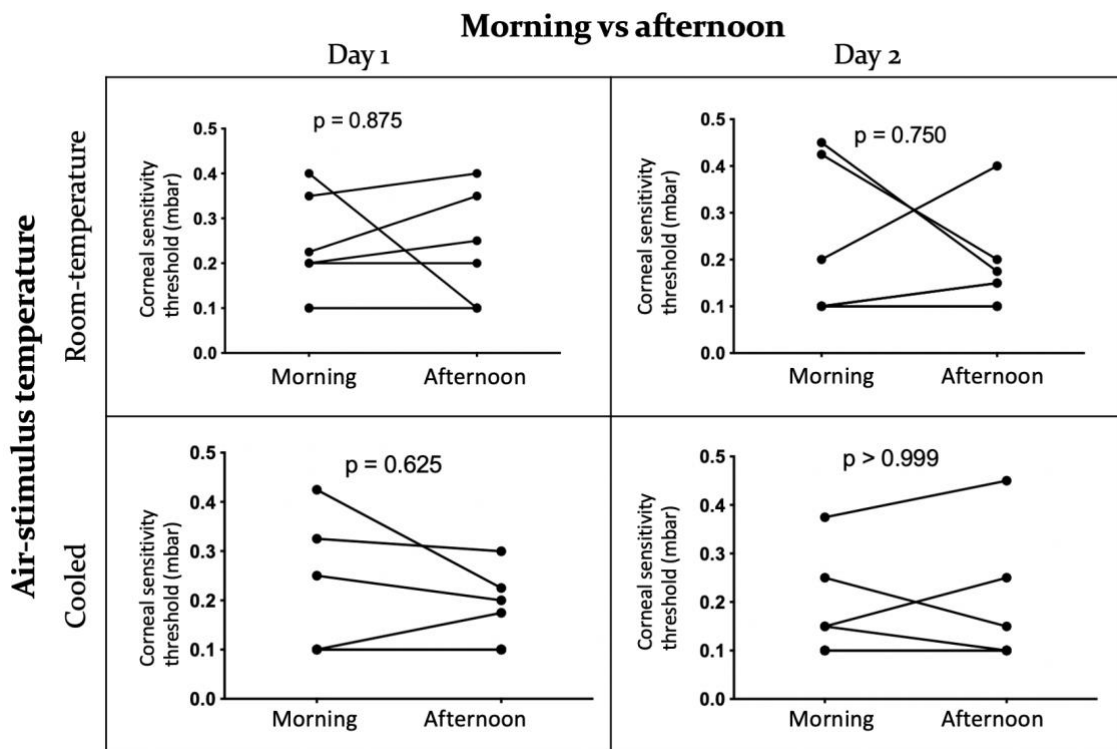


Figure C6. Wilcoxon matched-pairs signed rank test plots for corneal sensitivity thresholds to room-temperature and cooled air stimuli comparing morning to afternoon measurement times. Fewer than 10 plots points are visible as some points are coincident.

There were no statistically significant differences in corneal sensitivity thresholds measured with room-temperature or cooled stimuli on day one or day two when the test was repeated at a different time ($p > 0.05$).

Repeatability analysis 3: Different day, different time

Agreement between corneal sensitivity threshold measurements was further evaluated for measurements taken both on different days and at different times.

Comparing corneal sensitivity threshold measurements taken in the morning on day one with the afternoon on day two, the mean $\pm$ SD difference between measurements was 0.03 ± 0.13 mbar for room-temperature stimuli, and 0.02 ± 0.12 mbar for cooled stimuli. Comparing measurements taken in the afternoon on day one to the morning on day two, the mean $\pm$ SD difference was 0.03 ± 0.11 mbar for room-temperature stimuli, and 0.002 ± 0.46 for cooled stimuli.

Figure C7 shows Bland-Altman plots for comparing corneal sensitivity thresholds (log-transformed) repeated both on a different day and at a different time, for both room-temperature and cooled stimuli.

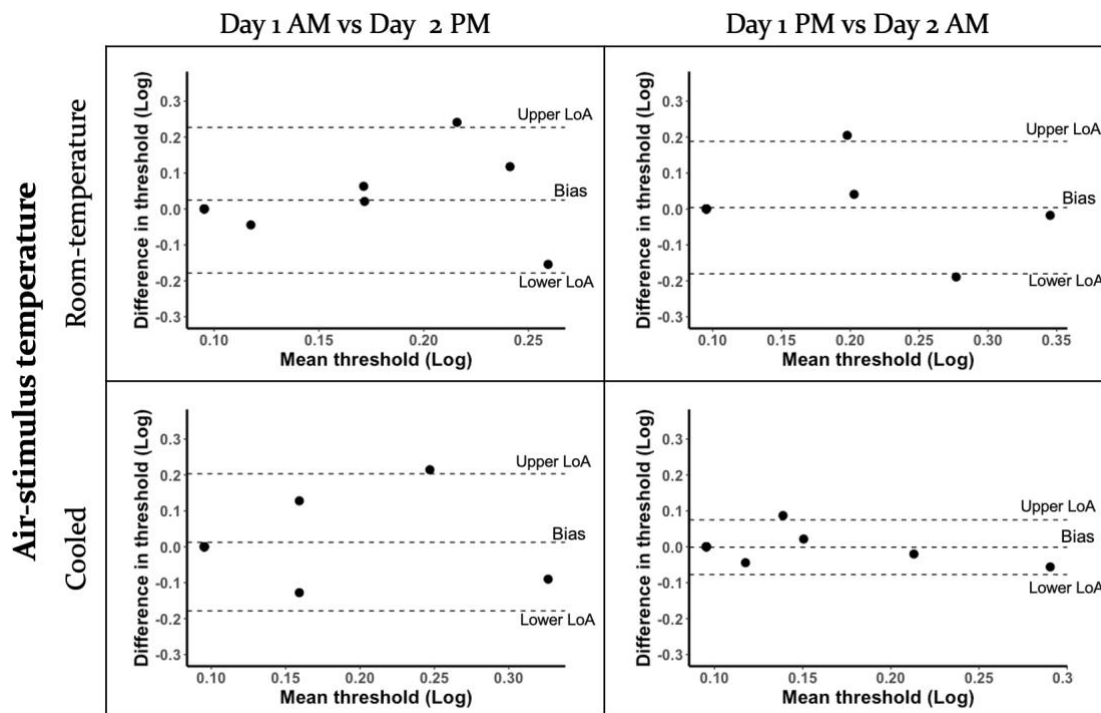


Figure C7. Bland-Altman plots for corneal sensitivity thresholds to room-temperature and cooled air stimuli comparing measurements taken on a different day and at a different time. LoA, 95% limits of agreement. Fewer than 10 plots points are visible as some points are coincident.

Comparing corneal sensitivity threshold measurements evaluated in the morning on day one with the afternoon on day two, the geometric mean ratios of values between measurements was 1.02 (95% LoA: 0.84 to 1.25) for room-temperature stimuli and 1.01 (95% LoA: 0.84 to 1.23) for cooled stimuli.

Comparing corneal sensitivity threshold measurements evaluated in the afternoon on day one with the morning on day two, the geometric mean ratios of values between measurements was 1.00 (95% LoA: 0.83 to 1.21) for room-temperature stimuli and 1.00 (95% LoA: 0.93 to 1.08) for cooled stimuli.

Wilcoxon matched-pairs signed rank tests (**Figure C8**) showed no significant differences in raw corneal sensitivity thresholds evaluated on a different day and at a different time, for both room-temperature and cooled stimuli ($p > 0.05$ for each comparison).

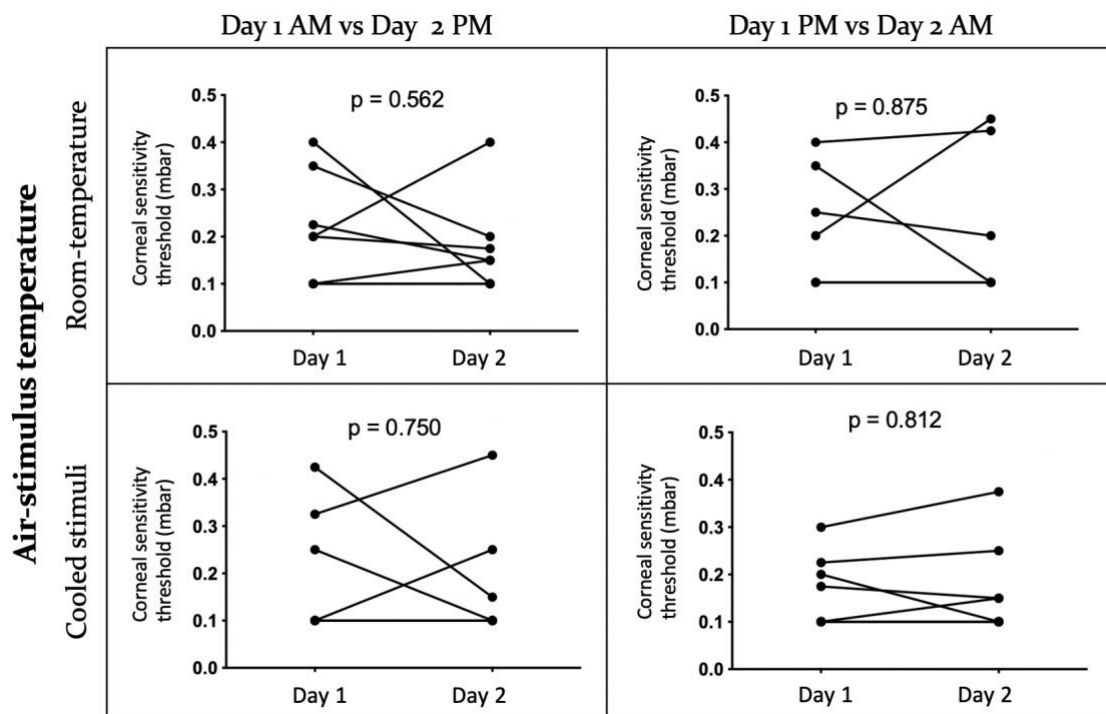


Figure C8. Wilcoxon matched-pairs signed rank test plots for corneal sensitivity thresholds to room-temperature and cooled air stimuli comparing measurements taken on a different day and at a different time. Fewer than 10 plots points are visible as some points are coincident.

C.4 Summary and discussion

This experiment, involving 10 participants, evaluated the agreement between corneal sensitivity threshold measurements, quantified using the NCCA instrument (SDZ Electronics, Auckland, New Zealand), over a range of test-retest conditions. The results indicate that corneal sensitivity threshold measurements are repeatable for assessments evaluated on a different day, at different times of day, or both.

The mean inter-condition difference between measurements for all comparisons was less than 0.05 mbar, which is half of the minimum step size of the instrument (of 0.10 mbar).

For the 95% limits of agreement (derived using the log-transformed data), the geometric mean ratios for all comparisons were ≥ 0.75 or ≤ 1.25 for both room-temperature and cooled stimuli. This indicates that any two measurements will lie within 25% of each other, approximately 95% of the time.

Corneal sensitivity thresholds to cooled stimuli generally had narrower limits of agreements, being within 20% of the comparison value for all estimates except for in one condition (comparing morning of day 1 to the afternoon of day 2). For room-temperature stimuli, 50% of the comparison had 95% limits of agreement of between 20 to 25%.

Other factors that can influence corneal sensitivity measures

Corneal sensitivity threshold measurements provide information on corneal nerve function. Potential factors that might affect the validity of measurements include diurnal variation, type of aesthesiometer, examination room temperature, and humidity (Swanevelder *et al.*, 2019).

All examinations were undertaken in the same quiet, ophthalmic examination room that is maintained at an average temperature of $20 \pm 3^{\circ}\text{C}$, and humidity of $50 \pm 5\%$. Within this room, all air conditioning vents were partitioned from the

testing area to eliminate detectable air flow. All examinations were undertaken by one of two trained examiners (ACZ and RS), using the same protocol. Examiners were trained to provide identical examination instructions. As the identical protocol was used by both examiners, and an objective method was used to derive the final sensitivity threshold (i.e., the double stair-case algorithm), intra- and inter-operator were not additionally assessed.

There are several factors to consider when interpreting the present findings. Repeatability assessment was performed in a small population, and included two participants with a history of ocular surgery. Although ocular surgery (over four years prior in the examined participants) may affect corneal sensitivity, there is no evidence that it affects the repeatability of measurements. However, assessments in a larger population that excludes these participants may be warranted to confirm. Most of our participants had mean corneal sensitivity thresholds of 0.1 mbar or below. Due to 0.1 mbar being the lowest measurable value, exact corneal sensitivity threshold values below 0.1 mbar could not be quantified. For this preliminary validation, values below 0.1 were analysed as 0.1 mbar, as the minimum step-size of the instrument. In subsequent clinical studies, sensitivity thresholds deemed as 0.1 mbar were analysed as 0.05 mbar, as an alternative approach (Beal, 2001).

Comparison with other studies

Golebiowski *et al.* showed that corneal sensitivity, measured using a non-contact jet aesthesiometer, did not vary over the course of a working day for stimuli delivered at ocular surface temperature (Golebiowski *et al.*, 2011).

Using a modified Belmonte gas aesthesiometer, Stapleton *et al.* found higher corneal sensitivity thresholds in the afternoons than in the mornings, in both the central and inferior cornea, for air stimuli delivered at ocular surface temperature (34 °C) (Stapleton *et al.*, 2004). However, measurements taken with 20 °C stimuli (defined by the authors as a cooled stimuli) did not demonstrate diurnal variations. No significant differences were found between

corneal sensitivity thresholds measured with either temperature stimulus measured on different days. Authors concluded from their findings that, in general, better repeatability was observed for afternoon measurements and for the lower stimulus temperature (Stapleton *et al.*, 2004). However, the NCCA instrument described from this study differs from our instrument in instrument design, stimulus type, and stimulus temperature range. These factors influence the interpretation of results and limit the ability to compare findings.

C.5 Conclusion

Corneal sensitivity thresholds to both room- and cool-temperature stimuli, measured using the custom-designed NCCA (SDZ Aesthesiometer, SDZ Electronics, Auckland, New Zealand), are repeatable when evaluated on different days, at different times during the day, or both.

C.6 Appendix C References

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Appendix D. Chapter 5: Supplementary materials [2 of 2]

Repeatability of corneal dendritic cell quantification

D.1 Introduction

For each individual who participated in the studies described in **Chapter 5** and **Chapter 7**, corneal sub-basal nerve plexus parameters were quantified from an average of 12 randomly-selected images from the central cornea and eight images from the inferior peripheral cornea, overlapping by less than 20%. In addition, corneal dendritic cell (DC) densities were quantified in each region.

The understanding of intra- and inter-observer variation is essential for evaluating research methodology. A repeatable method is critical for ensuring that results are interpreted accurately. As DC quantification is generally performed using manual cell count tools, the accuracy of DC identification relies on the subjective discrimination of the cell within the image. Significant differences in judgement by different observers, or on a different occasion by the same observer, introduces observer bias. Observer bias can lead to measurement errors and affect the accuracy of the results. In addition to the variation in the use of grading schemes reflected in the literature, measurement error is also a factor influencing the comparability of DC assessments across different studies.

The repeatability of DC quantification in the conjunctiva has been previously reported to be excellent, with intra-class correlation coefficients of 0.95 for total cell densities ranging between 0 to 28 cells/mm² (assessed using at least six non-overlapping fields) (Alzahrani, 2016). Classification of corneal DC morphological features, according to cell size and dendrite appearance, have previously demonstrated strong intra- and inter-observer agreement (weighted kappas >0.80) (Tajbakhsh *et al.*, 2019). However, to our knowledge, the repeatability of DC density quantification in the cornea has not been established.

The aim of this study was to evaluate the intra- and inter-observer repeatability in the quantification of putative DCs in the corneal sub-basal nerve plexus.

D.2 Methods

DC quantification was performed on corneal *in vivo* confocal microscopy (IVCM) images according to the methods described in **Chapter 5**. DC counting was first performed by examiner one (ACZ) in January 2018. This quantification involved manually counting the total number of DCs within each image frame. Each cell was putatively classified as one of three subtypes, based on morphological appearance described by Lagali *et al.* (Lagali *et al.*, 2018):

- i) mature dendritic cells (mDCs): slender bodies and extensions of long-arm-like processes (dendrites), >25 µm in total end-to-end length;
- ii) immature dendritic cells (imDCs): small, reflective bodies either with short-dendrites or without discernible dendrites, with end-to-end length of <25 µm, and
- iii) globular cells: larger than mDCs and imDCs, and with round or oval cell bodies, and no visible dendrites.

Counting was performed by marking, using the cell count plugin (<https://imagej.net/plugins/cell-counter.html>) on FIJI (ImageJ; US National Institutes of Health, Bethesda, Maryland, USA) (Schindelin *et al.*, 2012). Each

cell was tagged with a different colour according to its morphological subtype (**Figure D1**). For cells near the edge of the image, the entire cellular structure had to be visible for the cell to be included as a presumed DC. The total number of DCs per image was calculated as the sum of cells of the three subtypes. A scale measuring 25 μm , generated using the ruler tool on FIJI, was used as a reference for assessing total cell length.

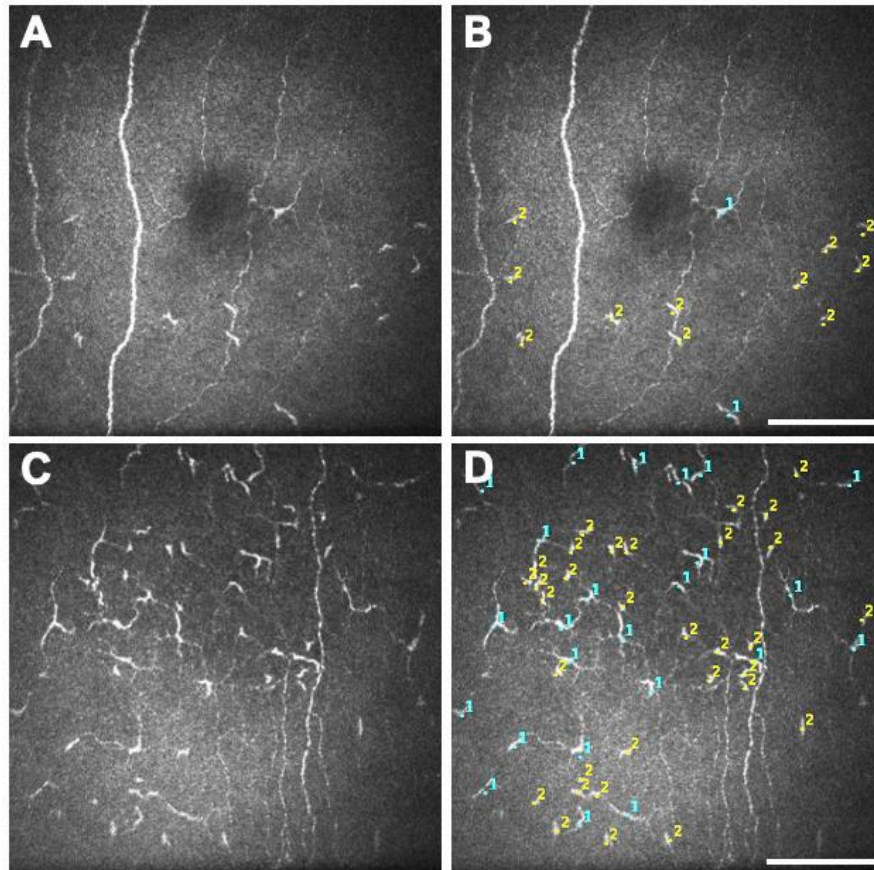


Figure D1. Dendritic cells were quantified using the cell count plugin on FIJI (Schindelin *et al.*, 2012). In vivo confocal microscopy images and markers showing DC identification for an image from the central cornea, showing less than 20 cells (A-B), and from the peripheral cornea, showing more than 20 cells (C-D). Scale bar=100 μm .

Subsequent repeatability assessments were performed in June 2020. For these assessments, 120 images from the central cornea and 64 from the peripheral cornea were randomly selected for re-analysis, representing 20% of the total number of images analysed in **Chapter 5**.

A second clinical observer (RS) was trained by ACZ to undertake corneal DC quantifications. Training entailed DC counting and classification using a different set of (approximately 20) images, independently by both examiners, who then compared and discussed their results. Discrepancies relating to either DC presence or sub-classification were resolved by consensus. Following training, the order of images in the repeatability image set was randomised and DC quantification was undertaken independently by both examiners.

Statistical analysis

Three sets of outcomes for each set of images were obtained, comprising two sets from observer one (ACZ), and one set from observer two (RS). Intra-observer repeatability was assessed by comparing both sets of measurements by observer one. Inter-observer variability was assessed by comparing measurements by observer two with measurements from the initial assessment by observer one (trial one).

The unit of analysis is a single image. To facilitate the appreciation of the direct differences within/between observers and to provide a direct interpretation, analyses were performed for the number of cells within the image (i.e., counts of cells/image), and not for cellular densities (i.e., converted to cells/mm² by dividing by 0.16 mm²).

Statistical analyses were performed in R: A language and environment for statistical computing (R Core Team (2013). R Foundation for Statistical Computing, Vienna, Austria). To assess reliability, intraclass correlation coefficients (ICC) and their 95% confidence intervals were calculated. The ICC was calculated using a single-rating, absolute-agreement, two-way random effects model. ICC values are interpreted using the approach of Koo *et al.*, with ICC <0.50 as indicative of poor reliability; $0.50 \leq \text{ICC} < 0.75$ indicative of moderate reliability, $0.75 \leq \text{ICC} < 0.90$ indicative of good reliability, and ICC >0.90 indicative of excellent reliability (Koo & Li, 2016).

Bland-Altman plots were generated to assess the agreement between cell counts derived from two sets of counts (Bland & Altman, 1999). This method, by plotting the difference between the two methods against the average of the two methods, was used to estimate the intra- and inter-observer mean bias and 95% limits of agreement (LoA), where we would expect 95% of difference between the pair of measurements to lie.

Prior to Bland-Altman analysis, a Shapiro-Wilk test was performed to evaluate whether differences between the measurements were normally distributed. A linear regression model was applied to evaluate the relationship between the differences and the means to check whether the differences were related to the magnitude of the quantity being measured (i.e., for proportional bias). Using the methodology described by Bland and Altman, if the differences of the means were not normally distributed, a logarithmic transformation was applied to the data prior to analysis. As stated by Bland & Altman, although other transformations could, in principal, be applied, a logarithmic transformation is the only transformation that is appropriate for this analysis as it is the only one that allows the results to be interpreted in relation to the original data (Bland & Altman, 1999). For log-transformed data, a back transformation (anti-log) was applied to derive values relating to the geometric mean ratio between the two methods for interpretation.

If a significant relationship between the mean difference and the magnitude of the measurement was evident after a log-transformation was applied, the regression approach for a nonuniform difference was undertaken on the non-transformed data, following the recommendations by Bland & Altman (Bland & Altman, 1999).

Using this approach, first, the difference between the methods was regressed against the average in a linear model to consider the level of variation of the difference for the magnitude of the mean, using the equation:

$$\hat{D} = b_0 + b_1 A$$

Where $\hat{D}$ is the difference between measurements and A is the average of the measurements. If the slope was not significant, the difference between the measurements was evaluated as the mean difference.

Second, the variation around the line of best agreement was considered by modelling the scatter of the residuals as a function of the size of measurement, using this equation:

$$\hat{R} = c_0 + c_1 A$$

Where $\hat{R}$ is the absolute residual and A is the average of the measurements. If no significant relationship was found to exist between R and A , the estimated standard deviation was taken as the standard deviation of the adjusted differences. If a significant relationship was present, the 95% limits of agreement were obtained by combining the regression equation, determined around the line of best fit.

$$\hat{D} \pm 1.96 \sqrt{\frac{\pi}{2}} R$$

D.3 Results

Table D1 shows the median (IQR) number of DCs per image quantified by both observers, for across the whole cornea and by corneal regions.

Table D1. Total number of dendritic cells per IVCN image quantified by the two observers

	Observer 1 (ACZ)		Observer 2 (RS)
	Trial 1	Trial 2	
All corneal regions (cells/image)	7 (2-19)	5 (2-14)	6 (2-13)
Central cornea (n=120)	5 (2-11)	5 (2-10)	5 (2-8)
Peripheral cornea (n=64)	14 (5-29)	10 (3-21)	12 (3-24)

Data are shown as median (IQR).

D.3.1 Intra-class correlation coefficients

Table D2 shows the ICCs for intra- and inter-observer agreement.

Table 2. Intra-class correlation coefficients for intra- and inter-observer assessment

	Intra-observer ICC (95% CI)	Inter-observer ICC (95% CI)
Total DCs	0.95 (0.89 to 0.97)	0.94 (0.90 to 0.97)
mDCs	0.63 (0.35 to 0.77)	0.51 (0.23 to 0.68)
imDCs	0.96 (0.94 to 0.97)	0.92 (0.90 to 0.94)
Globular cells	0.94 (0.92 to 0.95)	0.48 (0.36 to 0.59)

Intra-class correlation coefficients and their 95% confidence intervals (CIs) were calculated using the two-way random effects, single-rater, absolute agreement model. **Abbreviations:** CI, confidence interval; mDCs, mature dendritic cells; imDCs, immature dendritic cells.

For analyses of total DC counts (cells/image), there was excellent intra- and inter-observer reliability (ICC >0.9 for both comparisons) (Koo & Li, 2016). For quantification of the DC numbers according to morphological subtype, intra- and inter-observer assessment of mature DCs demonstrated moderate reliability (ICC between 0.5 and 0.75 for both comparisons) and there was excellent reliability for immature DCs (ICC >0.9 for both comparisons). For globular cells, intra-observer assessment showed excellent reliability (ICC >0.9), but inter-observer assessment demonstrated poor reliability (ICC <0.5).

D.3.2 Bland-Altman analysis

Differences between the DC counts for both intra- and inter-observer sets were not normally distributed, therefore, data were log-transformed prior to Bland-Altman analysis (Bland & Altman, 1999). For data interpretation, a back-transformation was performed on the estimated mean difference and 95% limits of agreement to obtain the geometric mean ratios between the intra- and inter-observer estimates.

Intra-observer agreement

Figure D2 (A–D) shows Bland Altman plots (of log-transformed data) demonstrating the levels of intra-observer agreement for total DC counts and each morphological subtype, in the central and peripheral cornea.

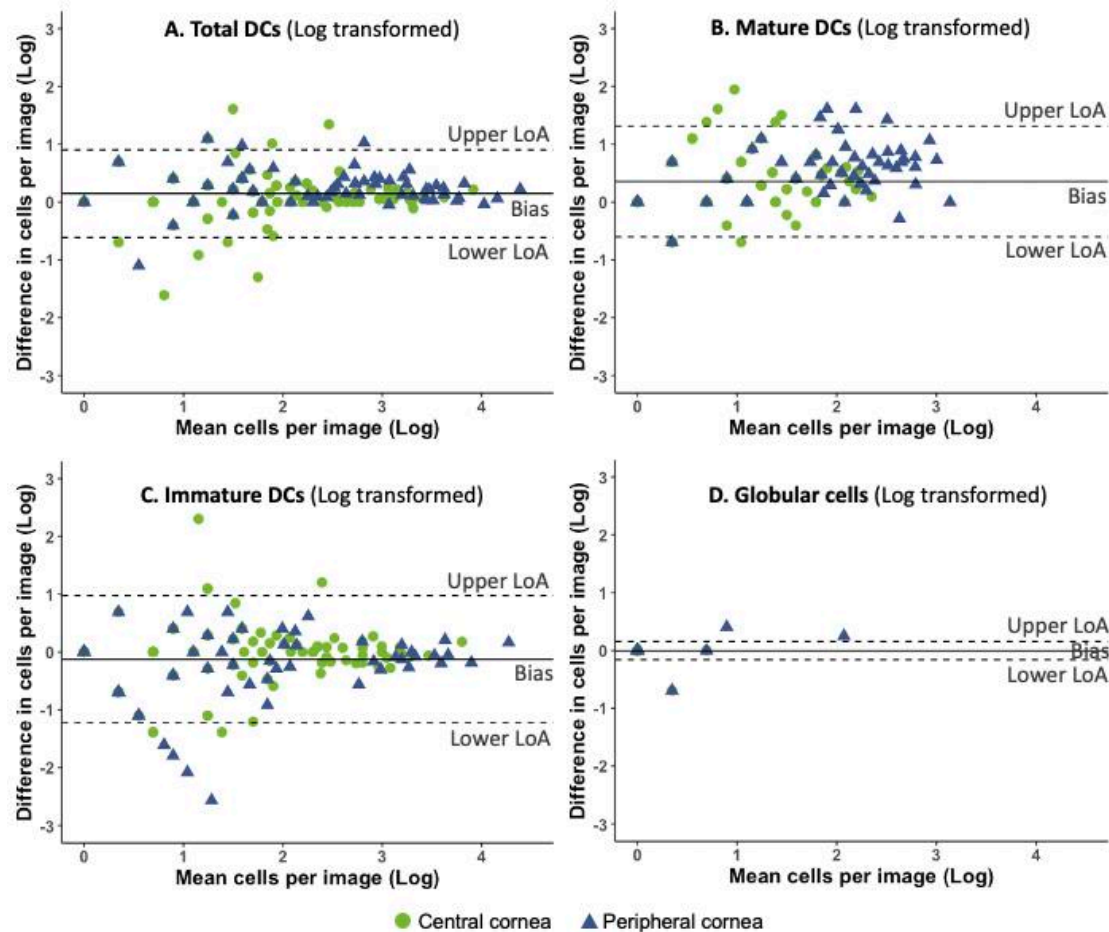


Figure D2. Bland-Altman plots of intra-observer agreement for log-transformed values, for differences in dendritic cell (DC) counts vs mean DC counts for (A) total DCs; (B) mature DCs; (C) immature DCs and (D) globular cells. Geometric mean ratios are derived as the antilog of mean bias (solid line) and 95% limits of agreement (LoA) values (dashed line).

For intra-observer agreement, the geometric mean ratio of total DC counts was 1.16 (95% LoA: 0.54 to 2.47). For the morphological subtypes, the geometric mean ratio for counts of mature cells was 1.43 (95% LoA: 0.55 to 3.72), of immature cells was 0.88 (95% LoA: 0.29 to 2.65), and of globular cells was 1.00 (95% LoA: 0.85 to 1.17).

The Bland-Altman plots (**Figure D2**) show that the bias and variability were not uniform over the measurement range, despite having applied a log-transformation. Therefore, to allow better fit of the data, a regression approach for non-uniform difference was undertaken (on the non-transformed data) to demonstrate the progressive increase in bias and limits of agreement with the magnitude, in consideration of heteroscedasticity (Bland & Altman, 1999).

Figure D3 shows Bland-Altman regression plots to facilitate an appreciation of the extent of variation in intra-observer discrepancies and widening limits of agreement with increasing DC number.

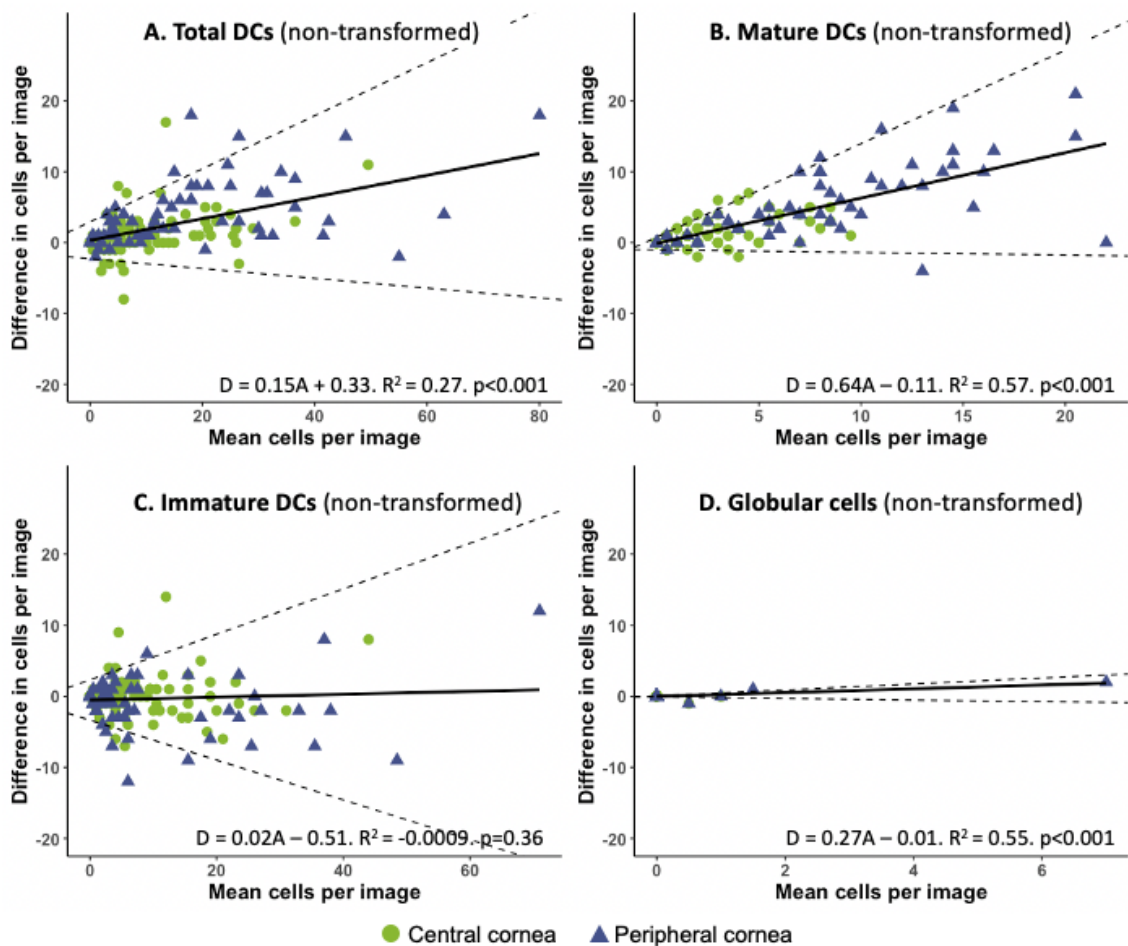


Figure D3. Bland-Altman plots of intra-observer repeatability, using the regression approach for non-transformed values, for differences in dendritic cell (DC) counts vs mean DC counts for (A) total DCs; (B) mature DCs; (C) immature DCs and (D) globular cells. Mean difference (solid line) and 95% limits of agreements (LoA; dashed line) are shown to reflect heteroscedasticity.

For counts of total DCs, mature DCs, and globular cells, significant regression lines indicate that the mean differences between observers increases with the mean number of cells per image. For immature DCs, an insignificant relationship exists such that the mean difference is constant regardless of the mean number of cells present.

For total DCs and all DC subtypes, a significant relationship was observed between the residuals and mean DCs per image. The limits of agreements in **Figure D3** were adjusted to reflect heteroscedasticity. The increasing magnitude of variability in the estimates with increasing magnitude of the measure (i.e., more cells per image) was demonstrated.

As shown in **Table D1**, more than half of the IVCM images showed fewer than 20 total DCs in the image. Moreover, over 95% of images showed fewer than 40 cells per image. To facilitate interpretation of the magnitude of the difference with increasing DC numbers within each image, **Table D3** shows the mean bias and limits of agreement for mean DC counts between 10 and 40 cells, calculated using the regression method in **Figure D3**,

Table D3. Intra-observer bias and 95% limits of agreement for images that contain a mean of between 10 to 40 dendritic cells.

	Mean number of DCs per image (for between 10-40 cells)			
	10 cells	20 cells	30 cells	40 cells
Total DCs, cells	2 (-3 to 7)	3 (-4 to 10)	5 (-4 to 14)	6 (-5 to 18)
mDCs	6 (-1 to 14)	13 (-2 to 27)	-	-
imDCs	0 (-6 to 6)	0 (-9 to 9)	0 (-12 to 12)	0 (-14 to 15)
Globular cells	-	-	-	-

Data are presented as bias (95% limits of agreement). **Abbreviations:** DC, dendritic cell; imDC, immature dendritic cell; mDC, mature dendritic cell.

Inter-observer agreement

A similar approach was used to assess inter-observer agreement. **Figure D4** shows assessment of inter-observer agreement performed on log-transformed values, using the Bland-Altman method.

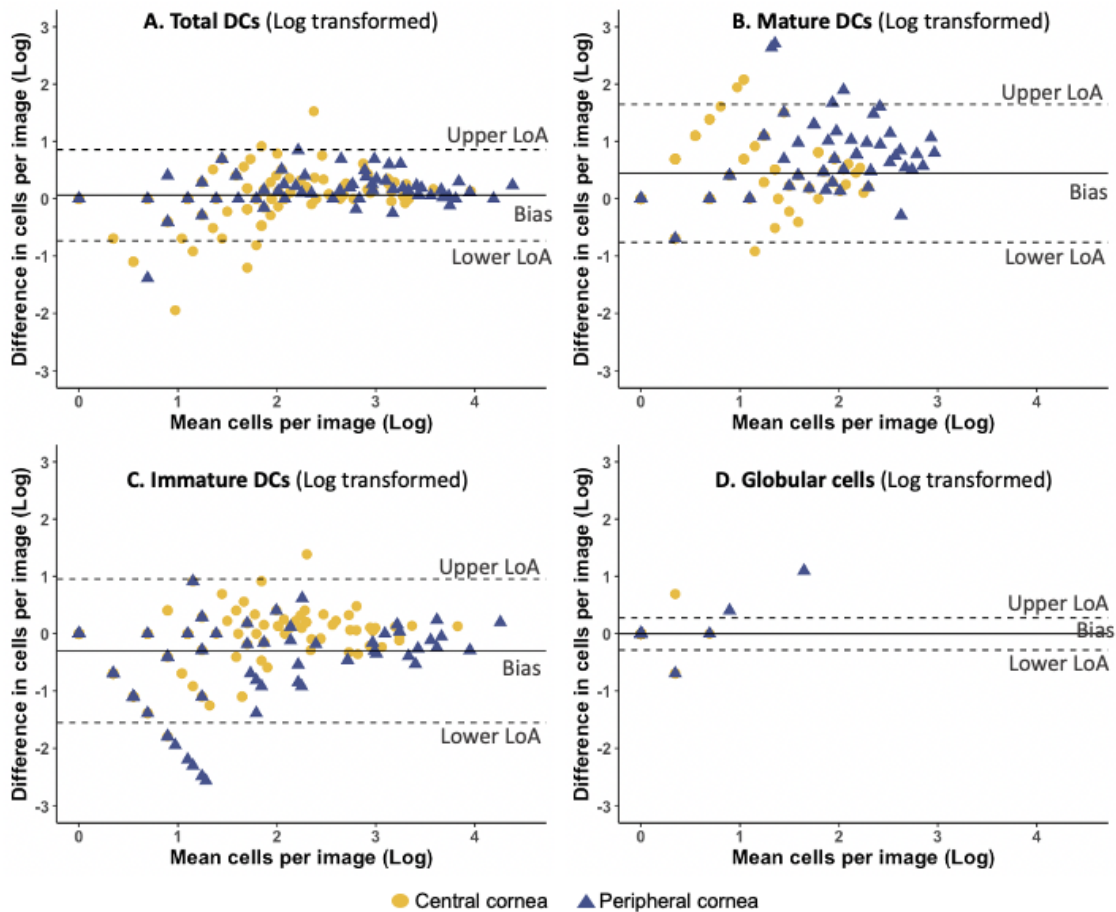


Figure D3. Bland-Altman plots showing inter-observer repeatability for log-transformed differences in dendritic cell (DC) counts vs mean DC counts for (A) total DCs; (B) mature DCs; (C) immature DCs and (D) globular cells. Geometric mean ratios represent the antilog of mean bias (solid line) and 95% limits of agreement (LoA) values (dashed line).

The geometric mean ratio in total DC count between observers was 1.06 (95% LoA: 0.48 to 2.35). For each DC morphological subtype, the geometric mean ratio for counts of mature DC counts was 1.56 (95% LoA: 0.47 to 5.20), of immature DCs was 0.74 (95% LoA: 0.21 to 2.60), and of globular cells was 1.00 (95% LoA: 0.75 to 1.32).

A regression approach for non-uniform difference was also undertaken to assess inter-observer variability, accounting for the relationship between the level of bias and mean DC counts (**Figure D5**).

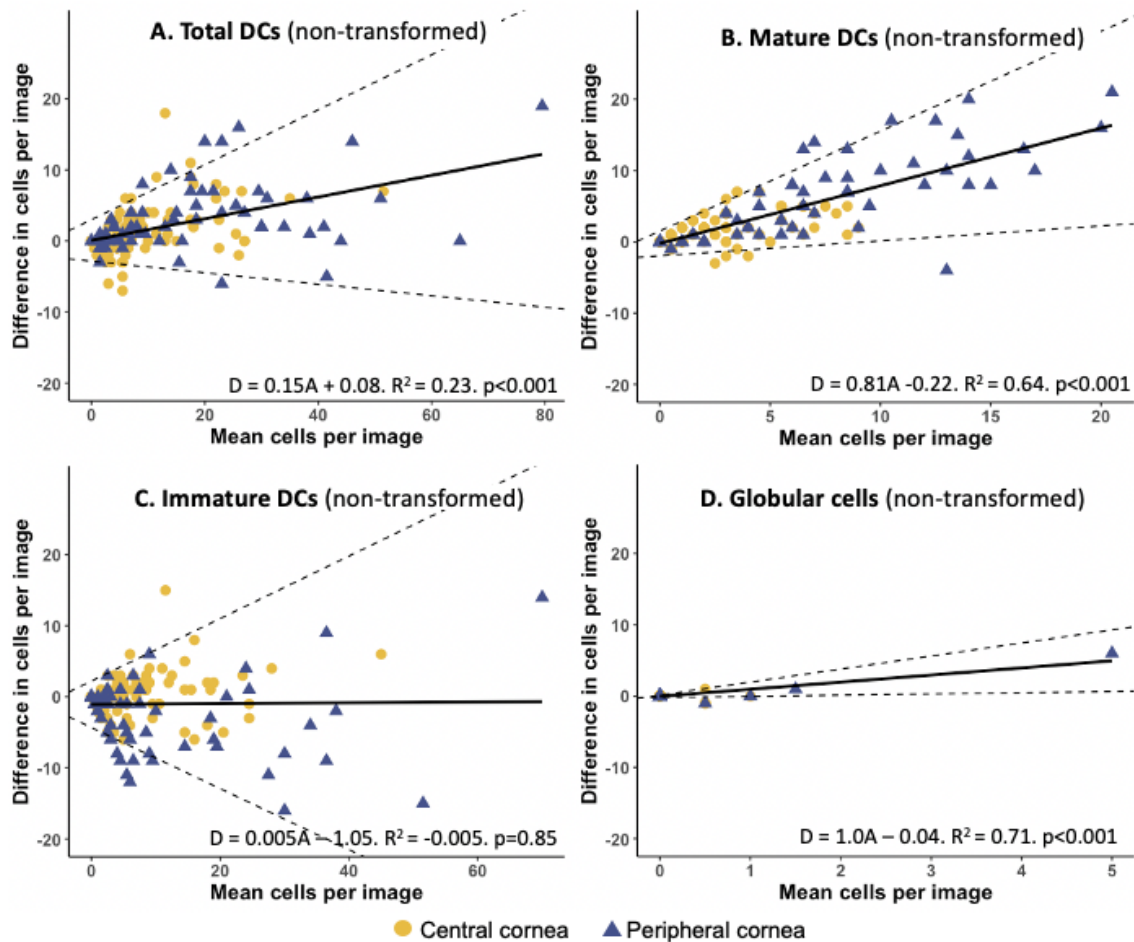


Figure D5. Bland-Altman plots of inter-observer repeatability, using the regression approach for non-transformed values, for differences in dendritic cell (DC) counts vs mean DC counts for (A) total DCs; (B) mature DCs; (C) immature DCs and (D) globular cells. Mean difference (solid line) and 95% limits of agreements (LoA; dashed line) are shown reflecting the heteroscedasticity.

Similar to intra-observer estimates, a significant regression was observed between the mean differences and the number of cells per image for total DCs, mature DCs and globular cells, but not for immature DCs.

For calculating the limits of agreement, a significant relationship was observed between the residuals and average, indicating increasing variation in the standard deviation of the estimate with the magnitude of the measure. The limits

of agreement were, therefore, modelled in recognition of the potential heteroscedasticity demonstrated by this relationship.

Using the regression method shown in **Figure D5**, the mean bias and 95% limits of agreement between observers for images having mean DC densities of between 10 to 40 cells are shown in **Table D4**.

Table D4. Inter-observer bias and 95% limits of agreement for images that contain a mean of between 10 to 40 dendritic cells.

	Mean number of DCs/image (for between 10-40 cells)			
	10 cells	20 cells	30 cells	40 cells
Total DCs, cells	2 (-4 to 7)	3 (-4 to 11)	5 (-7 to 16)	6 (-6 to 18)
mDCs	8 (0 to 16)	16 (2 to 30)	-	-
imDCs	0 (-9 to 7)	0 (-12 to 11)	0 (-12 to 12)	0 (-21 to 20)
Globular cells	-	-	-	-

Data are presented as bias (95% limits of agreement). **Abbreviations:** DC, dendritic cell; imDC, immature dendritic cell; mDC, mature dendritic cell.

D.4 Summary and interpretation

In this study, intra- and inter-observer agreement for repeatability of DC quantification was evaluated using the ICC and Bland-Altman analyses.

Total DC counts

Using the ICC, total corneal DC density showed excellent intra-observer (0.95 [95% CI: 0.89 to 0.97]) and inter-observer (0.94 [0.90 to 0.97]) agreement. However, because the ICC does not provide information on whether any differences are systematic or random, intra- and inter-observer agreements were evaluated further using Bland-Altman analysis.

Using the Bland-Altman approach on log-transformed values, the geometric mean ratios of values by intra-observer counts was 1.16 with 95% limits of agreement of 0.54 to 2.47. Therefore, the mean difference between intra-observer counts was 16%; 95% of the differences between intra-observer

measurements are between 46% lower and 147% higher. The geometric mean ratios of values by inter-observer counts of 1.06 and 95% limits of agreement 0.48 to 2.35 indicate that the mean difference between inter-observer counts was 6%, and 95% of the differences between inter-observer measurements lie between 52% lower and 135% higher. These limits are deemed to relate to the relationship between standard deviation and the magnitude of the measurement (Bland & Altman, 1999).

From the graphs of the regression model (**Figures D3 and D5**), for total DC density, both the intra- and inter-observer bias and limits of agreements increased with the mean number of DCs in the image. Thus, the repeatability in the estimation of DC counts is likely to be in better agreement in an image with fewer cells than in an image with a greater number of cells. This bias is potentially reflective of a systematic error in the manual DC counting methodology. A potential explanation is that more cellular structures leads to more DC overlap and produces ambiguity in discriminating cellular structures. This pattern is also more apparent for mature DCs, with longer cell bodies and extended dendrites, which may be more prone to exhibiting overlap (**Figure D1**).

The observed increase in mean bias was unidirectional and similar for both intra- and inter-observer counts, indicating that one observer consistently over-estimated DC density within a single trial. The estimated magnitude of the bias was 0.15 cells per mean cell in the image, for both intra- and inter-observer comparisons (as indicated by the slope of the regression lines in **Figures D3-A and D5-A**). This finding potentially indicates that a conservative approach was taken on the second count by both observers, after engaging in a consensus process.

In the present study, images were randomised from the same pool, so that any regional differences should relate only to the total number of cells in each image. Agreement in DC cell counts from the central versus peripheral cornea

were not independently assessed; however, peripheral corneal images generally have greater numbers of DCs and, therefore, more variation compared with central corneal images. Images with a mean DC count of 10 cells or less, corresponding with most images captured from the central cornea (i.e., within the 75% percentile) (**Table D1**), had intra-observer bias of 2 (95% LoA: -3 to 7) cells or less (**Table D3**), and inter-observer bias of 8 (95% LoA: 0 to 16) cells or less (**Table D4**). For images with less than 40 cells, the intra-observer bias was 6 (95% LoA: -5 to 18) cells or less and the inter-observer bias was 6 (95% LoA: -6 to 18) cells or less. Almost all central corneal images, being within this range, had variability within these limits.

DC morphological subtypes

In quantifying DC morphological subtypes, the ICC for immature cells showed excellent intra-observer (0.96 [95% CI: 0.94 to 0.97]) and inter-observer (0.92 [0.90 to 0.94]) reliability. For globular cells, intra-observer reliability was excellent (ICC: 0.94 [0.92 to 0.95]) but inter-observer reliability was poor (ICC: 0.48 [0.36 to 0.59]). For mature cells, both intra-observer (ICC: 0.63 [0.35 to 0.77]) and inter-observer (ICC: 0.51 [0.23 to 0.68]) reliabilities were moderate.

Bland-Altman analyses showed that both intra- and inter-observer limits of agreement increased with increasing cell densities for all DC subtypes. Mean intra- and inter-observer bias increased with increasing mean cell counts for mature DCs (with larger bodies and long dendrites) and globular cells (large cells with no dendrites). For immature DCs (with small bodies and minimal dendrites), mean bias was 0 regardless of the number of cells within the measurement. These findings suggest that for the quantification of DC morphological subtypes, counting of immature DCs shows less systematic bias.

This experiment evaluated the intra- and inter-observer variation of DC counts within IVCM images. However, the experiment did not evaluate how this variability may affect the estimated DC density for an individual. The individual-level variability also depends on the number of IVCM images analysed and the

degree of overlap between frames, as each image represents only approximately 0.2% of the total corneal surface area (Vagenas *et al.*, 2012). DC densities for each individual have been reported to be quantified from three to five IVCM images (Alzahrani *et al.*, 2017; Mastropasqua *et al.*, 2006; Mobeen *et al.*, 2019). Alzahrani estimated that five images from the central cornea, overlapping by less than 20%, are required to achieve a repeatable measure of DC density (Alzahrani, 2016). This value was derived from estimating the number of frames required to obtain a constant standard deviation for average DC density. However, the variability of this estimation from the 'true' mean DC density was not evaluated in this study; furthermore, potential measurement errors within each image was not considered. Findings from the current study indicate the need for further research to establish the optimum number of sampled IVCM images necessary to estimate DC density with an acceptable level of precision, within a specified proximity from the true mean, and taking into consideration the variability in DC counts within each image.

D.5 Conclusion

Both intra- and inter-observer DC counts showed excellent agreement using ICC; however, Bland-Altman analysis identified that the variability in DC counts increases systematically with mean DC numbers. For DC morphological subtypes, the quantification of immature DCs is less variable than that of mature DCs and globular cells. To improve accuracy in DC quantification, future studies should consider engaging observers in a consensus process prior to undertaking DC counting to reduce potential systematic measurement errors. This process can potentially identify observers' tendencies to over- or under-estimate and lead to improved DC repeatability.

D.6 Appendix D References

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Appendix E. Chapter 6: Supplementary materials [1 of 1]

Supplementary materials for the corresponding publication

Supplementary materials for the published article:

Zhang, A. C., De Silva, M. E. H., MacIsaac, R. J., Roberts, L., Kamel, J., Craig, J. P., Busija, L., & Downie, L. E. (2020). Omega-3 polyunsaturated fatty acid oral supplements for improving peripheral nerve health: a systematic review and meta-analysis. *Nutrition Reviews*, 78(4), 323-341.
<https://doi.org/10.1093/nutrit/nuz054>

Figure S1: Risk of bias summary

Figure S2: Forest plots of comparison for sensory NCV

Figure S3: Forest plots of comparison for distal CMAP latencies

Figure S4: Forest plots of comparison for motor NCV

Figure S5: Forest plot of comparison for adverse events

Table S1: Prespecified primary and secondary outcome measures

Table S2: Full characteristics of included studies

Table S3: Characteristics of excluded studies

Table S4: Adverse events

Appendix S1: Search strategies

Appendix S2: PRISMA checklist

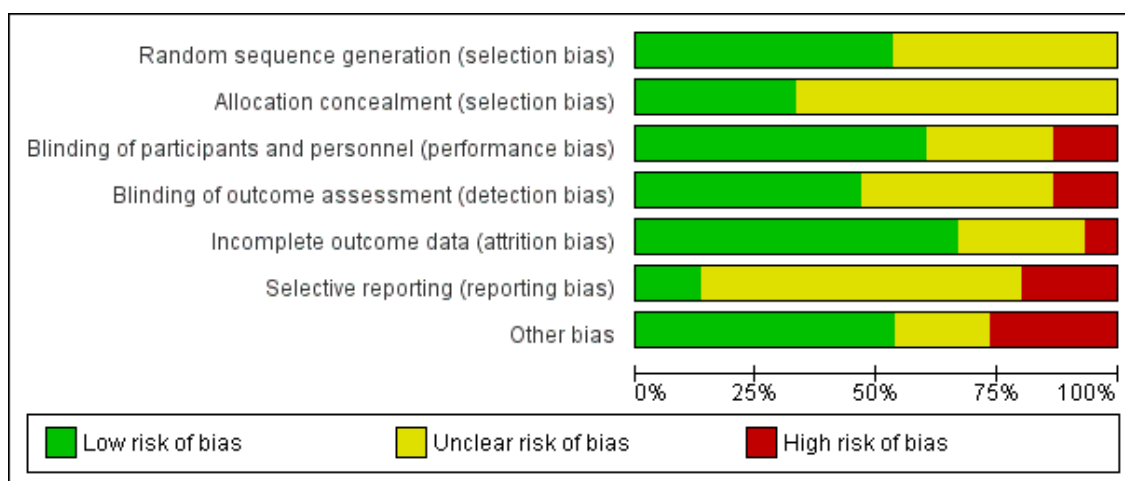


Figure S1: Risk of bias summary. Review authors' judgments about each risk of bias item presented as percentages across all included studies.

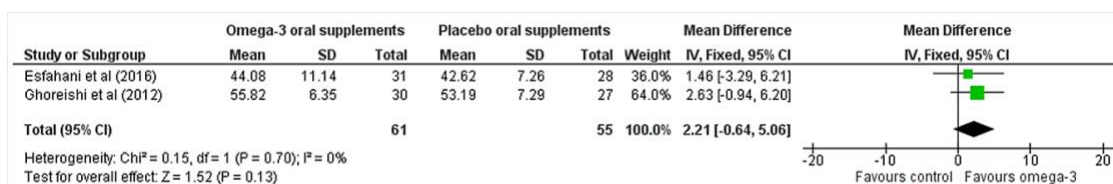


Figure S2: Forest plots of comparison for sensory NCV. Omega-3 vs placebo oral supplements for sensory nerve conduction velocities (NCV) of the ulnar nerve (ms^{-1}). Data are reported at the end of the follow-up period (ranging from 16 weeks (Ghoreishi et al.^{S8}) to 25 weeks (Esfahani et al.^{S6})).

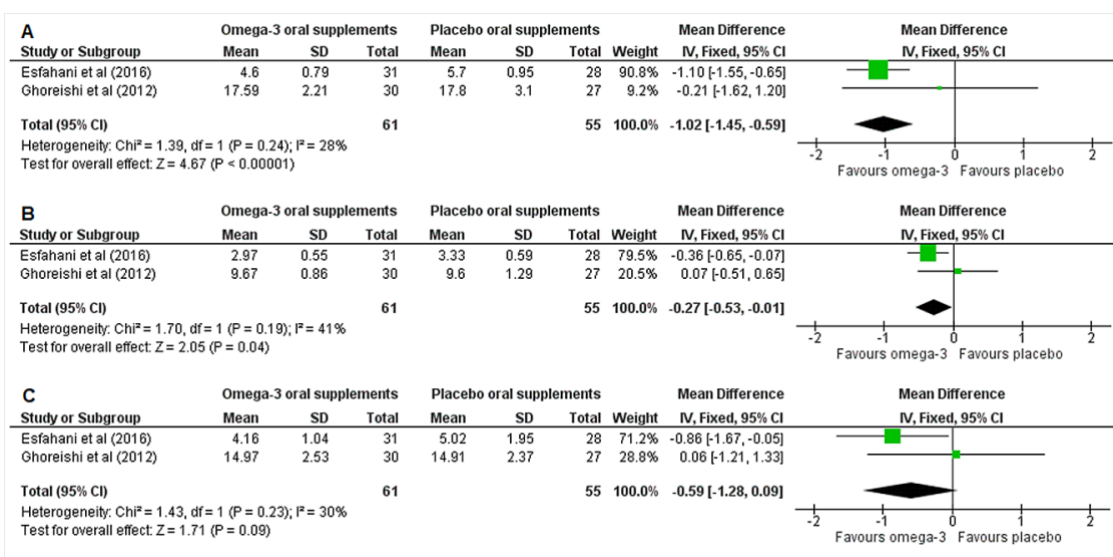


Figure S3: Forest plots of comparison for distal CMAP latencies. Omega-3 vs placebo oral supplements for distal compound motor action potential (CMAP) latencies of the (A) tibial nerve, (B) ulnar nerve and (C) peroneal nerve (ms). Data are reported at the end of the follow-up period (ranging from 16 weeks (Ghoreishi et al.^{S8}) to 25 weeks (Esfahani et al.^{S6})).

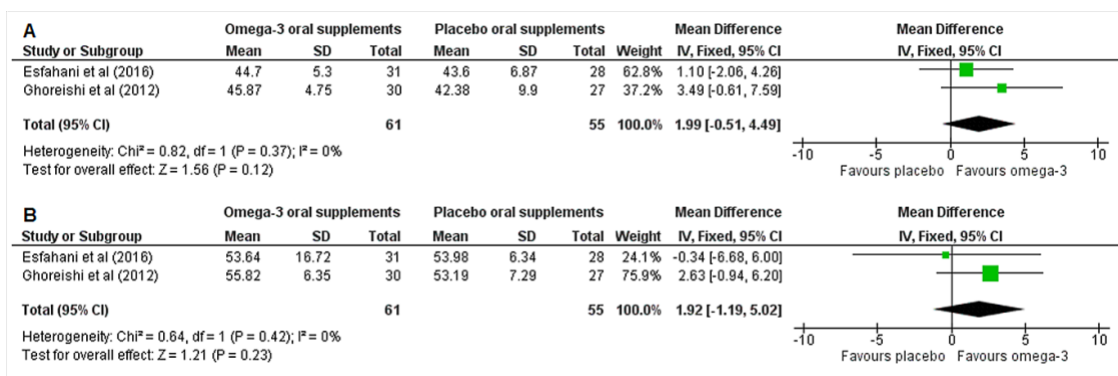


Figure S4: Forest plots of comparison for motor NCV. Omega-3 vs placebo oral supplements for motor nerve conduction velocities (NCV) of the (A) peroneal nerve, (B) ulnar nerve (ms^{-1}). Data are reported at the end of the follow-up period (ranging from 16 weeks (Ghoreishi et al.^{S8}) to 25 weeks (Esfahani et al.^{S6})).

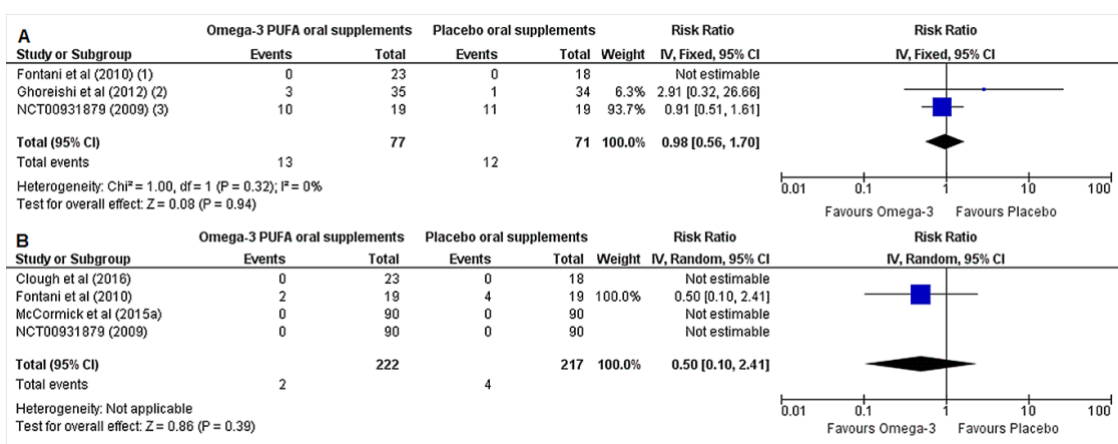


Figure S5: Forest plot of comparison for adverse events. Omega-3 vs placebo oral supplements for (A) any adverse events and (B) serious adverse events. Data are reported as the incidence of adverse events at the end of the follow-up period (ranging from 1 month (Fontani et al.^{S7}) to 18 months (McCormick et al.^{S10})).

Table S1: Prespecified primary and secondary outcome measures

Primary outcome	Change, from baseline, in PN impairments, as quantified by validated, composite (i.e., combining symptoms plus objective measures) neuropathy measures.
Secondary outcomes	Symptoms: change, from baseline, in symptoms of PN, measured by a validated, patient-assessed symptom score.
	Pain: change, from baseline, in mean scores of pain, measured by a validated, patient-assessed pain scale.
	Disability: change, from baseline, in the mean score of a patient-reported disability measure.
	Anatomical markers: Change, from baseline in CNFL, defined as the total length of nerves in a given area, measured in mm/mm ² , using a laser-scanning IVCN; Change, from baseline in IENFD.
	NCS: Change, from baseline, in nerve conduction study (NCS) parameters: SNAP amplitudes of the sural, median and ulnar nerves; SNAP latencies of the sural, median and ulnar nerves; Sensory NCV the sural, median and ulnar nerves; Distal CMAP amplitude of the peroneal, tibial, median and ulnar nerves; CMAP latency of the peroneal, tibial, median and ulnar nerves; Motor NCV of the peroneal, tibial, medium and ulnar nerves; Minimum F-wave latency of the peroneal, tibial, median and ulnar nerves.
	Sensory function in the cornea: change, from baseline, in corneal sensation, as quantified using: Contact aesthesiometry, to quantify mechanical detection thresholds using the Cochet-Bonnet aesthesiometer (measured in millimetres); Non-contact aesthesiometry, to quantify corneal sensation quantified using an air-based aesthesiometer (measured in millibars).
	Sensory function in the skin: Change, from baseline, in sensory function test scores: Mechanical detection thresholds measured using pressure aesthesiometry (e.g., von Frey hair aesthesiometer); Cold detection thresholds measured using QST methods; Warm detection thresholds measured using QST methods; Thermal pain thresholds, for cold and hot stimuli, measured using QST methods.
	Adverse events: Any adverse events Adverse events leading to discontinuation of the interventions, and Serious adverse events, being those leading to hospitalization or prolonged admission, a life-threatening event or death.

Abbreviations: CMAP, compound motor action potential; CNFL, corneal nerve fiber length; IENFD, Intraepidermal nerve fiber density; IVCN, *in-vivo* confocal microscope; NCS, nerve conduction studies; NCV, nerve conduction velocity; PN, peripheral neuropathy; QST, quantitative sensory testing; SNAP, sensory nerve action potential.

Table S2: Full characteristics of included studies*Anoushirvani et al. (2018)^{Si}*

Methods	Study design: Randomized controlled trial Study grouping: Parallel group (3 arms) How missing data were handled: Not reported Exclusions after randomisation: "Six patients were excluded from the study due to discontinuation of the drug." Losses to follow-up: "Six patients were excluded from the study due to discontinuation of the drug." Sample size calculation reported: Not reported Pre-specified primary outcome measure(s): Not reported. Pre-specified secondary outcome measure(s): Not explicitly reported. Measured: Incidence and severity of peripheral neuropathy; motor nerve conduction studies in the ulnar and peroneal nerves; sensory nerve conduction studies in the sural and ulnar nerves. Compliance measures: Not reported.
Participants	Baseline Characteristics Omega-3 PUFA oral supplements • <i>Age (mean ± SD):</i> 51.5 ± 9.2 years • <i>Gender/Sex (number of females/number of males):</i> 15/6 • <i>Underlying conditions:</i> 11 patients (52.4%) had breast cancer, followed by lung cancer (28.6%, 6 cases) and ovarian cancer (19%, 4 cases). • <i>Peripheral neuropathy diagnostic criteria:</i> Not reported • <i>Number of participants:</i> 21 Vitamin E oral supplements • <i>Age (mean ± SD):</i> 50.9 ± 10 years • <i>Gender/Sex (number of females/number of males):</i> 16/5 • <i>Underlying conditions:</i> 12 patients (57.1%) had breast cancer, followed by lung cancer (23.8%, 5 cases) and ovarian cancer (19%, 4 cases). • <i>Peripheral neuropathy diagnostic criteria:</i> Not reported • <i>Number of participants:</i> 21 Control • <i>Age (mean ± SD):</i> 52.2 ± 10.1 years • <i>Gender/Sex (number of females/number of males):</i> 15/6 • <i>Underlying conditions:</i> 13 (61.9%) had breast cancer, followed by lung cancer (28.6%, 6 cases) and ovarian cancer (9.5%, 2 cases) • <i>Peripheral neuropathy diagnostic criteria:</i> Not reported • <i>Number of participants:</i> 21 Overall • <i>Age (mean ± SD):</i> Not reported • <i>Gender/Sex (number of females/number of males):</i> 46/17 • <i>Underlying conditions:</i> 36 patients with breast cancer, 17 patients with lung cancer and 10 cases of ovarian cancer • <i>Peripheral neuropathy diagnostic criteria:</i> Not reported • <i>Number of participants:</i> 63 Inclusion criteria: "Patients with a solid tumour (lung, breast, ovaries, etc.), with a good liver and kidney status that received the first taxol treatment."

	Exclusion criteria: Previous history of chemotherapy; the presence of neuropathy due to diabetes, AIDS, alcohol abuse, thyroid dysfunction and hereditary neuropathy; the use of any dietary supplement (fish oil, vitamins and minerals) at least three months before entering the study; smoking and drugs use; low back pain and the use of other chemotherapy drugs. Pre-treatment inter-group differences: There were no significant differences between groups for gender ($p=0.861$), age ($p=0.664$), or body mass index ($p=0.780$). Location of peripheral nerve assessed: Not reported; laterality not specified. Unit of analysis: Per participant
Interventions	Intervention Characteristics Omega-3 PUFA oral supplements • <i>Type (DHA/EPA/ALA):</i> Not reported • <i>Dose DHA/EPA/ALA (milligrams/day):</i> 1920mg/day • <i>Duration of treatment (days):</i> 3 months (whilst receiving taxol) • <i>Co-interventions :</i> None • <i>Brand name:</i> Not reported Vitamin E oral supplements • <i>Type (DHA/EPA/ALA):</i> Not reported • <i>Dose DHA/EPA/ALA (milligrams/day):</i> 600 mg/day • <i>Duration of treatment (days):</i> 3 months (whilst receiving taxol) • <i>Co-interventions :</i> None • <i>Brand name:</i> Not reported Control • <i>Type:</i> Not reported • <i>Dose (milligrams/day):</i> Not reported • <i>Duration of treatment (days):</i> 3 months (whilst receiving taxol) • <i>Co-interventions :</i> None • <i>Brand name:</i> Not reported
Outcomes	Incidence and severity of peripheral neuropathy; motor nerve conduction studies in the ulnar and peroneal nerves; sensory nerve conduction studies in the sural and ulnar nerves.
Identification	Sponsorship source: Funding sources: -Present? Yes -Actual source: "This research did not receive any financial support". Conflict (or declaration) of interest statement: -Present? Yes - Nature of conflict of interest: "The authors have declared that no competing interests exist". Country: Iran. Setting: Ayatollah Khansari Hospital. Comments: Date study conducted: not reported. Trial registration number: not reported. Contacting study investigators: trial authors contacted to obtain details regarding units of error for results (22nd February 2019). Author's name: Reza Agha Bozorgi Institution: Arak University of Medical Sciences

	Email: reza.aghabozorgi@gmail.com Address: Department of Hematology and Medical Oncology, Arak University of Medical Sciences, Arak, Iran Article details: Full text Year of publication: 2018 Journal of publication: Open Access Maced J Med Sci Language: English Publication status: Published
Note	The study authors were contacted on 21 February 2019 regarding: 1) missing outcome data (SD values) of the mean and units of measurement, at the study endpoint (3 months) for both motor and sensory electrophysiology variables; 2) the scale used to measure peripheral neuropathy incidence at the end of the treatment period; and 3) the total length of time that participants were treated. We did not receive a reply from the study authors after four weeks. In the published article, inter-group differences were reported at the end of a three months evaluation period for sensory NCS amplitudes in the sural nerve (omega-3: 84.6 mV versus placebo: 98.6 mV, $p > 0.05$), and ulnar nerves (omega-3: 64.7 mV versus placebo: 13.7 mV, $p > 0.05$); sensory NCS latencies in the sural nerve (omega-3: 76.2 ms versus placebo 95.2 ms, $p > 0.05$) and the ulnar nerves (omega-3: 87.3 ms versus placebo: 68.3 ms), $p > 0.05$), and sensory NCV in the sural nerve (omega-3: 29.41 ms⁻¹ versus placebo: 41.41 ms⁻¹, $p > 0.05$), and the ulnar nerves (omega-3: 87.49 ms⁻¹ versus placebo: 31.49 ms⁻¹, $p > 0.05$). Inter-group differences at the end of three months were also reported for motor NCS amplitudes in the peroneal nerve (omega-3: 71.5 mV versus placebo: 66.5 mV, $p > 0.05$), and ulnar nerves (omega-3: 55.7 mV versus placebo: 87.7 mV, $p > 0.05$); motor NCS latencies in the peroneal nerve (omega-3: 57.6 ms versus placebo 68.6 ms) and the ulnar nerves (omega-3: 17.3 ms versus placebo 26.3 ms, $p > 0.05$); and motor NCV in the peroneal nerve (omega-3: 51.45 ms⁻¹ versus placebo: 11.45 ms⁻¹), and the ulnar nerves (omega-3: 21.5 ms⁻¹ versus placebo: 11.5 ms⁻¹, $p > 0.05$).

Risk of bias table

Bias	Authors' judgement	Support for judgement
Random sequence generation (selection bias)	Low risk	Quote: "Then, the patients were randomly divided into three equal groups according to the number table." Judgement Comment: Randomisation sequence generated using a "number table".
Allocation concealment (selection bias)	Unclear risk	Judgement Comment: Not reported how allocation administered. Trial is described as "randomised" but with no further details.
Blinding of participants and personnel (performance bias)	Low risk	Quote: "The person who registered the information was not aware of the patient's group, and the patients were not aware of their position in the relevant groups considering the medical ethics conditions."

		Judgement Comment: Clearly states that participants and personnel not aware of which treatment was received.
Blinding of outcome assessment (detection bias)	Unclear risk	Quote: "The person who registered the information was not aware of the patient's group, and the patients were not aware of their position in the relevant groups considering the medical ethics conditions." Judgement Comment: It is unclear whether all outcome assessors were masked.
Incomplete outcome data (attrition bias)	Low risk	Quote: "69 patients with different types of cancers were studied. Six patients were excluded from the study due to discontinuation of the drug. Finally, 63 patients were evaluated in three groups. In the omega-3 group, 15 women (71.4%) and 6 men (28.6%) were present. In the vitamin E group, 16 women (76.2%) and 5 men (23.8%) were admitted, while 15 (71.4%) and 6 men (28.6%) were presented in the control group." Judgement Comment: Missing data less than 20% (i.e. more than 80% follow-up) and equal follow-up in both groups and no obvious reason why loss to follow-up should be related to the outcome.
Selective reporting (reporting bias)	Unclear risk	Quote: "(Protocol number IR.ARAKMU.REC.2015.219; November 23, 2015)." Judgement Comment: A protocol is referenced, however this is no access to this protocol and the trial does not appear to be registered.
Other bias	Low risk	Judgement Comment: No other apparent sources of bias.

Carter *et al.* (2012)^{S2}

Methods	Study design: Randomized controlled trial Study grouping: Parallel group How missing data were handled: Not reported Exclusions after randomisation: Not reported Losses to follow-up: Not reported Sample size calculation reported: Not reported Pre-specified primary outcome measure(s): Not reported Pre-specified secondary outcome measure(s): Not explicitly reported. Measured: body mass index; systolic arterial pressure; diastolic arterial pressure; mean arterial pressure; heart rate; forearm blood; forearm vascular conductance; calf blood flow; calf vascular conductance; forearm vascular measurements: calf vascular measurements. Compliance measures: Subjects were asked to maintain their current diet and exercise routines. A pill diary and weekly reminders were issued to the subjects to track compliance.
Participants	Baseline Characteristics Omega-3 PUFA Normotensive - Age (mean $\pm$ SE): 24 $\pm$ 2 years - Gender/Sex (number of females/number of males): 10/9 - Underlying conditions: Normotensive - Peripheral neuropathy diagnostic criteria: Not reported - Number of participants: 19 Control Normotensive - Age (mean $\pm$ SE): 24 $\pm$ 2 years - Gender/Sex (number of females/number of males): 10/9 - Underlying conditions: Normotensive

	• <i>Peripheral neuropathy diagnostic criteria</i>: Not reported • <i>Number of participants</i>: 19 Omega-3 PUFA Pre-hypertensive • <i>Age (mean ± SE)</i>: 23 ± 1 years • <i>Gender/Sex (number of females/number of males)</i>: 0/15 • <i>Underlying conditions</i>: Pre-hypertensive • <i>Peripheral neuropathy diagnostic criteria</i>: Not applicable • <i>Number of participants</i>: 15 Control Pre-hypertensive • <i>Age (mean ± SE)</i>: 25 ± 3 • <i>Gender/Sex (number of females/number of males)</i>: 1/13 • <i>Underlying conditions</i>: Pre-hypertensive • <i>Peripheral neuropathy diagnostic criteria</i>: Not applicable • <i>Number of participants</i>: 14 Overall • <i>Age (mean ± SE)</i>: Not reported • <i>Gender/Sex (number of females/number of males)</i>: 21/46 • <i>Underlying conditions</i>: Normotensive or pre-hypertensive • <i>Peripheral neuropathy diagnostic criteria</i>: Not applicable • <i>Number of participants</i>: 67 Inclusion criteria: Classifications consistent with the Seventh report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure; normotensive: resting systolic pressure 120 mmHg and diastolic pressure 80 mmHg; prehypertensive: resting systolic pressure of 120–139 mmHg and/or a diastolic pressure of 80–89 mmHg. Participants were asked to refrain from caffeine, alcohol, and exercise for >12 h before testing. Exclusion criteria: Smoking; diabetes; hypertension; autonomic dysfunction; use of blood pressure medication; taking any omega-3 fatty acid supplements for 2 months before the start of the study. Pre-treatment inter-group differences: Not explicitly reported. Table 1 suggests no significant inter-group baseline differences for age, sex, height or body mass. Location of peripheral nerve assessed: MSNA of lower leg and forearm; laterality not specified. Unit of analysis: Per participant
Interventions	Intervention Characteristics Omega-3 PUFA Normotensive • <i>Type (DHA/EPA/ALA)</i>: DHA + EPA • <i>Dose DHA/EPA/ALA (milligrams/day)</i>: 1100 mg/day DHA + 1600mg/day EPA • <i>Duration of treatment (days)</i>: 8 weeks (56 days) • <i>Co-interventions</i>: None • <i>Brand name</i>: Not reported Control Normotensive • <i>Type (DHA/EPA/ALA)</i>: Olive oil • <i>Dose DHA/EPA/ALA (milligrams/day)</i>: 9000mg/day (olive oil) • <i>Duration of treatment (days)</i>: 8 weeks (56 days) • <i>Co-intervention</i>: None • <i>Brand name</i>: Not reported Omega-3 PUFA Pre-hypertensive • <i>Type (DHA/EPA/ALA)</i>: DHA + EPA • <i>Dose DHA/EPA/ALA (milligrams/day)</i>: 1100 mg/day DHA + 1600mg/day EPA • <i>Duration of treatment (days)</i>: 8 weeks (56 days) • <i>Co-interventions</i>: None

	• Brand name: Not reported Control Pre-hypertensive • Type: Olive oil • Dose (milligrams/day): 9000mg/day (olive oil) • Duration of treatment (days): 8 weeks (56 days) • Co-interventions: None • Brand name: Not reported
Outcomes	Body mass index; systolic arterial pressure; diastolic arterial pressure; mean arterial pressure; heart rate; forearm blood; forearm vascular conductance; calf blood flow; calf vascular conductance; forearm vascular measurements; calf vascular measurements.
Identification	Sponsorship source: Funding sources: - Present? Yes - Actual source: This project was supported by National Heart, Lung, and Blood Institute Grant HL-o88689. Conflict (or declaration) of interest statement: - Present? Yes - Nature of conflict of interest: No conflicts of interest, financial or otherwise, are declared by the author(s). Country: Not reported. Setting: Not reported. Comments: Date study conducted: Not reported. Trial registration number: Not reported. Contacting study investigators: trial authors not contacted; no additional information provided for review. Author's name: Jason R. Carter Institution: Michigan Technological University Email: jcarter@mtu.edu Address: Dept. of Kinesiology and Integrative Physiology, Michigan Technological Univ., 1400 Townsend Drive, Houghton, MI 49931 Article details: Full text Year of publication: 2012 Journal of publication: Am J Physiol Heart Circ Physiol Language: English Publication status: Published

Risk of bias table

Bias	Authors' judgement	Support for judgement
Random sequence generation (selection bias)	Unclear risk	Quote: "participants were randomly assigned" Judgement Comment: Not reported how list was generated. Trial may be described as "randomised" but with no further details.
Allocation concealment (selection bias)	Unclear risk	Quote: "participants were randomly assigned" Judgement Comment: Not reported how allocation administered. Trial described as "randomised" but with no further details.
Blinding of participants and personnel (performance bias)	Unclear risk	Quote: "Sixty-seven subjects participated in a double-blind, randomly assigned, placebo based study." Judgement Comment: Described as "double blind" with no information on who was masked.
Blinding of outcome	Unclear risk	Quote: "Sixty-seven subjects participated in a double-blind, randomly assigned, placebo based study."

assessment (detection bias)		Judgement Comment: Described as “double blind” with no information on who was masked.
Incomplete outcome data (attrition bias)	Unclear risk	Judgement Comment: Missing data >20% (i.e. follow-up <80%) but follow-up equal in both groups.
Selective reporting (reporting bias)	Unclear risk	Judgement Comment: No access to protocol or trials registry entry.
Other bias	Low risk	Quote: "No conflicts of interest, financial or otherwise, are declared by the author(s)." Judgement Comment: No other source of bias.

Carter et al. (2013)^{S3}

Methods	Study design: Randomized controlled trial Study grouping: Parallel group How missing data were handled: "if there was a missing data point for 1 min in the pretreatment mental stress, we deleted the subsequent data point in the posttreatment (and vice versa); this ensured appropriate comparisons." Exclusions after randomisation: Not reported. Losses to follow-up: Two subjects completed the complete pretesting but only were able to complete for the seated resting measurements. Additionally, one subject was unable to complete the postmental stress protocol. Accordingly, hemodynamic responses to mental stress are reported in 64 total subjects (of the 67 recruited). Complete MSNA responses to mental stress are available in 29 subjects (12 fish oil, 17 placebo), and because of shifts in neurogram, measurements of total MSNA are available in 23 subjects. Technical issues and artefact during mental stress prevented the analysis of forearm and calf blood flow in several subjects. Forearm measurements were recorded in 29 fish oil subjects and 26 placebo subjects, while calf measurements were recorded in 28 fish oil and 25 placebo subjects during mental stress. Sample size calculation reported: Not reported Pre-specified primary outcome measure(s): Not reported Pre-specified secondary outcome measure(s): Not explicitly reported. Measured: blood pressure; heart rate; muscle sympathetic nerve activity (MSNA); forearm vascular conductance (FVC); calf vascular conductance (CVC). Compliance measures: A pill diary and regular email and phone reminders were issued to the subjects to track compliance according to the study terms. Subjects were asked to maintain current diet and exercise habits during the 8-week supplementation period.
Participants	Baseline Characteristics Omega-3 PUFA oral supplements - Age (mean $\pm$ SD): Not reported - Gender/Sex (number of females/number of males): 10/24 - Underlying conditions: Normal; non-hypertensive. - Peripheral neuropathy diagnostic criteria: Not applicable - Number of participants: 34 Control - Age (mean $\pm$ SD): Not reported - Gender/Sex (number of females/number of males): 11/22 - Underlying conditions: Normal; non-hypertensive. - Peripheral neuropathy diagnostic criteria: Not applicable - Number of participants: 33 Overall

	• Age (mean $\pm$ SD): 24 $\pm$ 1 years • Gender/Sex (number of females/number of males): 21/46 • Underlying conditions: Normal; non-hypertensive. • Peripheral neuropathy diagnostic criteria: Not applicable • Number of participants: 67 Inclusion criteria: Non-hypertensive participants (resting blood pressure 140 systolic/90 diastolic). Subjects were required to refrain from caffeine, alcohol, and exercise for at least 12 hours before testing. Exclusion criteria: Smoking; diabetes; autonomic dysfunction; use of blood pressure medication. Pre-treatment inter-group differences: Table 1 suggests no significant difference between baseline values for key vascular or nerve parameters. No data relating to inter-group age comparisons were provided. Location of peripheral nerve assessed: Knee/calf (MSNA at the peroneal nerve); laterality was not specified. Unit of analysis: Per participant
Interventions	Intervention Characteristics Omega-3 PUFA oral supplements • Type (DHA/EPA/ALA): DHA + EPA • Dose DHA/EPA/ALA (milligrams/day): 1100mg/day DHA + 1600mg/day EPA • Duration of treatment (days): 8 weeks (56 days) • Co-interventions: None • Brand name: Not reported Control • Type: Olive oil • Dose (milligrams/day): 9000mg/day olive oil • Duration of treatment (days): 8 weeks (56 days) • Co-interventions: None • Brand name: Not reported
Outcomes	Blood pressure; heart rate; muscle sympathetic nerve activity (MSNA); forearm vascular conductance (FVC); calf vascular conductance (CVC).
Identification	Sponsorship source: Funding sources: - Present? Yes - Actual source: This project was supported by the National Heart, Lung, and Blood Institute Grant HL-o88689. Conflict (or declaration) of interest statement: - Present? Yes - Nature of conflict of interest: No conflicts of interest, financial or otherwise, are declared by the author(s). Country: Not reported Setting: Integrative Physiology Laboratory at Michigan Technological University Comments: Date study conducted: Not reported. Trial registration number: Not reported. Contacting study investigators: Trial authors not contacted; no additional information used for review. Author's name: J R Carter Institution: Michigan Technological University Email: jcarter@mtu.edu Address: Department of Kinesiology and Integrative Physiology, Michigan Technological University 1400 Townsend Drive, Houghton, MI, USA 49931 Article details: Full text Year of publication: 2013 Journal of publication: American Journal of Physiology-Regulatory, Integrative and Comparative Physiology

	Language: English Publication status: Published

Risk of bias table

Bias	Authors' judgement	Support for judgement
Random sequence generation (selection bias)	Unclear risk	Quote: "All subjects were randomly assigned into this double-blind, placebo-controlled investigation" Judgement Comment: Not reported how list was generated. Trial may be described as "randomised" but with no further details.
Allocation concealment (selection bias)	Unclear risk	Quote: "subjects were randomly assigned into the fish oil or placebo (olive oil) group." Judgement Comment: Not reported how allocation administered. Trial may be described as "randomised" but with no further details.
Blinding of participants and personnel (performance bias)	Unclear risk	Quote: "All subjects were randomly assigned into this double-blind, placebo-controlled investigation" Judgement Comment: Described as "double blind" with no information on who was masked.
Blinding of outcome assessment (detection bias)	Unclear risk	Quote: "All subjects were randomly assigned into this double-blind, placebo-controlled investigation" Judgement Comment: Described as "double blind" with no information on who was masked.
Incomplete outcome data (attrition bias)	Low risk	Quote: "Two subjects completed the complete pretesting but only were able to complete for the seated resting measurements. Additionally, one subject was unable to complete the postmental stress protocol. Accordingly, hemodynamic responses to mental stress are reported in 64 total subjects" Judgement Comment: Missing data less than 20% (i.e. more than 80% follow-up) and equal follow-up in both groups and no obvious reason why loss to follow-up should be related to outcome.
Selective reporting (reporting bias)	Unclear risk	Judgement Comment: No access to protocol or trials registry entry.
Other bias	Low risk	Quote: "This project was supported by the National Heart, Lung, and Blood Institute Grant HL-o88689." Quote: "No conflicts of interest, financial or otherwise, are declared by the author(s)." Judgement Comment: No other apparent sources of bias.

Chinnery et al. (2017)^{S4}

Methods	Study design: Randomized controlled trial Study grouping: Parallel group Exclusions after randomisation: None. Losses to follow-up: None. How missing data were handled: Not reported. Sample size calculation reported: Not reported. Pre-specified primary outcome measure(s): Based upon clinical trial registry information: Change from baseline at Day 90 in tear osmolarity and dry eye symptoms (OSDI, Allergan, USA).

	Pre-specified secondary outcome measure(s): Based upon clinical trial registry information: Change from baseline at Day 90 in tear stability (tear break-up time), ocular bulbar redness, meibomian gland capping, ocular surface staining, Schirmer score and tear inflammatory cytokine levels. The analysis of corneal nerve parameters using in vivo confocal microscopy was not a pre-specified outcome measure. Compliance measures: Compliance was assessed by participants returning supplement containers, with unused capsules, at each follow-up visit for counting by an independent researcher.
Participants	Baseline Characteristics Omega-3 PUFA oral supplements • Age (mean $\pm$ SD): 42 $\pm$ 20 years • Gender/Sex (number of females/number of males): 6/2 • Underlying conditions: Dry eye disease • Peripheral neuropathy diagnostic criteria: Not applicable • Number of participants: 8 Control • Age (mean $\pm$ SD): 46 $\pm$ 20 years • Gender/Sex (number of females/number of males): 3/1 • Underlying conditions: Dry eye disease • Peripheral neuropathy diagnostic criteria: Not applicable • Number of participants: 4 Overall • Age (mean $\pm$ SD): Not reported • Gender/Sex (number of females/number of males): 9/3 • Underlying conditions: Dry eye disease • Peripheral neuropathy diagnostic criteria: Not applicable • Number of participants: 12 Inclusion criteria: Adults with moderate DED, defined as an Ocular Surface Disease Index (OSDI) symptom score ≥ 18 and 65 and tear osmolality ≥ 316 mOsmol/L in at least one eye. In addition, potential participants needed to have distance best-corrected visual acuity $\geq 20/40$ (Snellen equivalent) in each eye and intra-ocular pressure ≤ 21 mmHg in both eyes. Exclusion criteria: (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 3 months prior to enrolment, (iii) Consumption of essential fatty acid oral supplement in the 3 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 3 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 6 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 3 months prior to enrolment, (xiii) History of ocular surgery/trauma, which could affect corneal sensitivity and/or tear distribution within 6 months of enrolment, or (xiv) The use of any of the following topical medications in the 3 months prior to baseline: corticosteroids, non-steroidal anti-inflammatories or cyclosporine. Pre-treatment inter-group baseline differences: Participant baseline (day 1) data are summarised in Table 1; there were no significant inter-group differences for age, sex, DED severity, corneal nerve parameters, dendritic cell density (DCD) or basal epithelial cell parameters at baseline ($p > 0.05$ for all comparisons). Location of peripheral nerve assessed: Eye with highest tear osmolarity at baseline. Unit of analysis: Per eye (one eye)
Interventions	Intervention Characteristics Omega-3 PUFA oral supplements • <i>Type (DHA/EPA/ALA):</i> DHA + EPA • <i>Dose DHA/EPA/ALA (milligrams/day):</i> ~500mg/day DHA + ~1000mg/day EPA (being either re-esterified triacylglyceride or phospholipid forms) • <i>Duration of treatment (days):</i> 90 • <i>Co-interventions:</i> None • <i>Brand name:</i> Not reported. Control • <i>Type:</i> Olive oil • <i>Dose (milligrams/day):</i> 1500mg/day (olive oil) • <i>Duration of treatment (days):</i> 90 • <i>Co-interventions:</i> None • <i>Brand name:</i> Not reported
Outcomes	Best-corrected visual acuity; OSDI score; tear osmolarity; comprehensive slit lamp examination; corneal sub-basal nerve plexus parameters (corneal nerve branch density, total corneal nerve branch density, corneal nerve branch density on the main fibre, corneal nerve fibre density, corneal nerve fibre length, nerve fibre width and area); other corneal parameters (basal epithelial cell density, dendritic cell density).
Identification	Sponsorship source: Funding sources: - Present? Yes - Actual source: This study was funded by a grant from the Rebecca L Cooper Medical Foundation (LED, 2015), ANZ Medical Research and Technology Trust grant (LED, 2015) and NHMRC project grant APP1042612 (HRC). Conflict (or declaration) of interest statement: - Present? Yes - Nature of conflict of interest: No conflicting relationship exists for any author in relation to this work. Country: Australia Setting: University of Melbourne Eye Care Clinic Comments: Date study conducted: October 2014 to November 2015. Trial registration number: ACTRN12614001019695. Contacting study investigators: trial authors not contacted; no additional information provided for review. Author's name: Laura E Downie Institution: The University of Melbourne Email: ldownie@unimelb.edu.au

	Address: Department of Optometry and Vision Sciences, The University of Melbourne, Parkville, Australia Article details: Full text Year of publication: 2017 Journal of publication: Ophthalmic and Physiological Optics Language: English Publication status: Published

Risk of bias table

Bias	Authors' judgement	Support for judgement
Random sequence generation (selection bias)	Low risk	Quote: "In brief, an independent data manager generated a participant randomisation sequence using a random number generator in Microsoft Excel" Judgement Comment: Computer generated list.
Allocation concealment (selection bias)	Low risk	Quote: "This randomisation schedule was provided to an independent compounding pharmacist (Dartnell's Pharmacy, Victoria, Australia http://www.dartnellspharmacy.com.au/) who repackaged the study supplements into identical, opaque containers using the randomisation schedule." Quote: "Eligible participants were sequentially enrolled by a masked research optometrist, who dispensed the investigational product labelled with the appropriate randomisation code." Judgement Comment: Study supplements packaged independently into identical containers.
Blinding of participants and personnel (performance bias)	Low risk	Quote: "Participants and all study personnel were masked to treatment allocations throughout the study." Judgement Comment: Clearly stated that participants and personnel not aware of which treatment received.
Blinding of outcome assessment (detection bias)	Low risk	Quote: "Participants and all study personnel were masked to treatment allocations throughout the study." Judgement Comment: The present study does not specifically state that outcome assessors are masked. However, the paper references methods taken from a larger study where participants are enrolled, that state: "All study personnel, including the principal investigator (L.E.D.), clinical outcome assessor (L.A.D.), laboratory outcome assessors (C.Y.W., D.C.J.), and co-investigators (H.R.C., A.J.V.), were masked to participant allocation."
Incomplete outcome data (attrition bias)	Low risk	Quote: "All 12 participants completed the study and exceeded the pre-specified, minimum level of compliance to the study regimen (defined, as in other nutraceutical trials 26 as $\geq 75\%$ capsule consumption), based upon capsule counts;" Judgement Comment: Missing data less than 20% (i.e. more than 80% follow-up) and equal follow-up in both groups and no obvious reason why loss to follow-up should be related to outcome.
Selective reporting (reporting bias)	Unclear risk	Clearly addressed by authors that this was a post-hoc analysis; outcomes reported are not in the original trial entry, but are reported in a referenced article.
Other bias	Low risk	Quote: "No conflicting relationship exists for any author in relation to this work." Quote: "This study was funded by a grant from the Rebecca L Cooper Medical Foundation (LED, 2015), ANZ Medical

		Research and Technology Trust grant (LED, 2015) and NHMRC project grant APP1042612 (HRC). The sponsors had no role in the design or conduct of this research." Judgement Comment: No other apparent sources of bias.

Clough et al. (2016)⁵⁵

Methods	Study design: Randomized controlled trial Study grouping: Parallel group How missing data were handled: Not reported Exclusions after randomisation: Based on the CONSORT flow diagram, 100 people were randomised, and 99 had baseline data, suggesting 1 participant was excluded after randomisation (the reason is not provided). Losses to follow-up: Based on the CONSORT flow diagram, 99 people had baseline data, and 90 had baseline and end-point data, suggesting 9 participants were lost to follow-up. Sample size calculation reported: Not reported. Pre-specified primary outcome measure(s): Not reported in the paper, but based upon the clinical trial registry entry is: Change in biomarkers for NAFLD and change in liver fat at 18 months of follow-up. Pre-specified secondary outcome measure(s): Not explicitly reported. Based upon the clinical trial registry: Change in risk factors for cardiovascular disease and type 2 diabetes at 18 months of follow-up. Based upon the paper, the following outcomes were also measured: Hot (HPT) and cold (CPT) temperature perception testing; temperature perception index (TPI); vibrotactile perception testing (VPT); microvascular function. Compliance measures: Not reported.
Participants	Baseline Characteristics Omega-3 PUFA oral supplements • <i>Age (mean ± SD):</i> 47.6 ± 10.7 years • <i>Gender/Sex (number of females/number of males):</i> 22/22 • <i>Underlying conditions:</i> Non-alcoholic fatty liver disease, without neuropathy, with and without diabetes • <i>Peripheral neuropathy diagnostic criteria:</i> Distal peripheral neuropathy in feet diagnosed based on failure to detect a 10g monofilament • <i>Number of participants:</i> 44 Control • <i>Age (mean ± SD):</i> 54.1 ± 9.6 years • <i>Gender/Sex (number of females/number of males):</i> 15/31 • <i>Underlying conditions:</i> Non-alcoholic fatty liver disease, without neuropathy, with and without diabetes • <i>Peripheral neuropathy diagnostic criteria:</i> Distal peripheral neuropathy in feet diagnosed based on failure to detect a 10g monofilament • <i>Number of participants:</i> 46 Overall • <i>Age (mean ± SD):</i> 50.9 ± 10.6 years • <i>Gender/Sex (number of females/number of males):</i> 37/53 • <i>Underlying conditions:</i> Non-alcoholic fatty liver disease, without neuropathy, with and without diabetes • <i>Peripheral neuropathy diagnostic criteria:</i> Distal peripheral neuropathy in feet diagnosed based on failure to detect a 10g monofilament • <i>Number of participants:</i> 90 Inclusion criteria: (From CTR) Men women >18 years; alcohol consumption 35 units / week for women 50 units / week for men; i) steatohepatitis

	diagnosed on normal clinical grounds (including in most cases liver biopsy/scan within 3 years of recruitment) or ii) steatohepatitis diagnosed on normal clinical grounds (including in most cases liver biopsy), assessed by Kleiner scoring system to assess severity, with no known aetiological factors for underlying liver disease (e.g. exclusion of hepatitis A, B C, primary biliary cirrhosis, autoimmune hepatitis, haemochromatosis). Exclusion criteria: Evidence of distal peripheral neuropathy in their feet (failure to detect a 10 g monofilament); evidence of diabetic eye disease at retinal screening. Pre-treatment inter-group differences: Table 1, summarising the baseline participant characteristics, suggests a baseline inter-group difference in age ($p=0.003$). Of the participants, 35 were treated with statins, 32 with antihypertensive drugs, seven with fibrates, 18 with ACE inhibitors (ACEI), 24 with metformin and 13 with antidepressants. Location of peripheral nerve assessed: Toe (left), dominant forearm Unit of analysis: Per participant
Interventions	Intervention Characteristics Omega-3 PUFA oral supplements • <i>Type (DHA/EPA/ALA):</i> DHA + EPA • <i>Dose DHA/EPA/ALA (milligrams/day):</i> 4000mg/day combined DHA + EPA • <i>Duration of treatment (days):</i> 15 - 18 months (450-540 days) • <i>Co-interventions:</i> None • <i>Brand name:</i> Omacor (Lovaza) ethyl esters Control • <i>Type:</i> Olive oil (as reported in clinical trials registry) • <i>Dose (milligrams/day):</i> 4000mg/day (olive oil) • <i>Duration of treatment (days):</i> 15 - 18 months (450-540 days) • <i>Co-interventions:</i> None • <i>Brand name:</i> Not reported
Outcomes	Temperature perception (hot perception thresholds, cold perception thresholds, threshold temperature perception index), vibrotactile perception threshold, microvascular function (Cutaneous microvascular blood flow). Notes: Individual baseline and end of study measurements for temperature perception thresholds were not quantitatively reported but shown in a figure (figure 1); multivariable regression modelling testing showed no effect of the DHA+EPA intervention on temperature perception thresholds. "There were no important adverse effects or side effects reported"
Identification	Sponsorship source: Funding sources: - Present? Yes - Actual source: National Institute for Health Research through the NIHR Southampton Biomedical Research Unit grant and by a Diabetes UK allied health research training fellowship; DHA+EPA (Omacor/Lovaza) and placebo were provided by Pronova Biopharma through Abbott Laboratories, Southampton, UK. Conflict (or declaration) of interest statement: - Present? Yes - Nature of conflict of interest: PCC serves on the Clinical Advisory Board of Pronova Biopharma and has acted as a consultant to Amarin. All other authors declare that there is no duality of interest associated with their contribution to this manuscript Country: Not reported Setting: Not reported Comments:

	Date study conducted: commenced November 2009, according to clinical trial registry, with anticipated completion date of February 2018. Trial registration number: NCT00760513. Contacting study investigators: trial authors not contacted; no additional information provided for review. Author's name: Christopher D. Byrne Institution: University of Southampton, Southampton General Hospital Email: cdtb@soton.ac.uk Address: Human Development and Health Academic Unit, Faculty of Medicine, The Institute of Developmental Sciences (IDS), MP887, University of Southampton, Southampton General Hospital, Southampton SO16 6YD, UK Article details: Full text Year of publication: 2016 Journal of publication: Diabetologia Language: English Publication status: Published

Risk of bias table

Bias	Authors' judgement	Support for judgement
Random sequence generation (selection bias)	Low risk	Quote: "Participants were randomised 1:1, using sequential numbering, by personnel independent from the trial team." Judgement Comment: Computer generated list, random table, other method of generating random list.
Allocation concealment (selection bias)	Unclear risk	Quote: "Participants were randomised 1:1, using sequential numbering, by personnel independent from the trial team." Judgement Comment: Not reported how allocation administered. Trial may be described as "randomised" but with no further details.
Blinding of participants and personnel (performance bias)	Low risk	Quote: "All participants and all members of the research team were blinded to group assignment." Judgement Comment: Clearly stated that participants and personnel not aware of which treatment received.
Blinding of outcome assessment (detection bias)	Low risk	Quote: "All participants and all members of the research team were blinded to group assignment." Judgement Comment: Clearly stated that outcome assessors were masked.
Incomplete outcome data (attrition bias)	Low risk	Quote: "Baseline and end of study thermal thresholds were obtained on 90 individuals" Judgement Comment: Missing data less than 20% (i.e. more than 80% follow-up) and equal follow-up in both groups and no obvious reason why loss to follow-up should be related to outcome.
Selective reporting (reporting bias)	Unclear risk	Quote: "In a pre-specified sub-study of the Wessex Evaluation of fatty Liver and Cardiovascular markers in NAFLD with Omacor therapy (WELCOME) trial (ClinicalTrials.gov registration no. NCT00760513) [20, 21] (a randomised double-blind placebo-controlled trial), we tested the effect of DHA+ EPA (Omacor/Lovaza) treatment on hot and cold temperature perception thresholds." Judgement Comment: The current study is a sub-study of a larger trial (referenced), however only selected outcomes are reported with some pre-specified outcomes reported in the referenced paper. VPT and MF/RF appears to only be

		reported at baseline, with results reported in the referenced paper.
Other bias	Unclear risk	Quote: "This work was supported by the National Institute for Health Research through the NIHR Southampton Biomedical Research Unit grant and by a Diabetes UK allied health research training fellowship awarded to KMcC (Diabetes UK. BDA 09/0003937)." Quote: "DHA+EPA (Omacor/Lovaza) and placebo were provided by Pronova Biopharma through Abbott Laboratories, Southampton, UK." Quote: "PCC serves on the Clinical Advisory Board of Pronova Biopharma and has acted as a consultant to Amarin." Judgement Comment: Potential conflict of interest due to industry provision of the supplements and one author acting as a consultant for a nutritional supplement company.

Esfahani et al. (2016)^{S6}

Methods	Study design: Randomized controlled trial Study grouping: Parallel group How missing data were handled: "For analyzing the results on an intention to treat (ITT) basis, multiple imputation was performed by related variables." Exclusions after randomisation: Not reported. Losses to follow-up: The rate of drop out was 5 patients in omega-3 PUFAs supplemented group and 7 patients in the placebo group (Fig. 1), so the response rate was 80.09 %. Sample size calculation reported: Yes Pre-specified primary outcome measure(s): The presence of chronic oxaliplatin-induced peripheral neuropathy (OXIPN) and grading of its severity, ascertained by reduced Total Neuropathy Score (TNSr). Pre-specified secondary outcome measure(s): a) Nerve conduction studies (NCVs): distal motor latency (DML), peak to baseline amplitude of compound muscle action potential (a-CMAP), and motor conduction velocity for tibial, Peroneal and ulnar nerves. Moreover, peak-to-peak amplitude of sensory action potentials (a-SAP) and sensory conduction velocity (antidromic technique) of sural and ulnar nerves were also measured. b) Proinflammatory cytokines: serum levels of proinflammatory cytokines: interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), and high-sensitivity C-reactive Protein (hs-CRP), measured before the onset of chemotherapy and one month after the cessation of treatment. Compliance measures: Clinical trial registry states that concentrations of omega-3 fatty acids in the serum phospholipid will be measured using gas chromatography; this outcome measure is not reported in the published article.
Participants	Baseline Characteristics • Omega-3 PUFA oral supplements • Age (mean $\pm$ SD): 54.14 $\pm$ 10.53 years • Gender/Sex (number of females/number of males): 18/18 • Underlying conditions: Metastatic colon cancer (stage III), treated with oxaliplatin, 130 mg/m² IV, administered over 2 h infusion in day 1 and oral capecitabine (1000 mg/m², b.i.d) on days 1–4, to be repeated every 3 weeks for 8 cycles. • Peripheral neuropathy diagnostic criteria: Post-chemotherapy criteria for diagnosing neuropathy: TNSr: grade 0, no OXIPN; 1 – 10, mild; 11 – 19, moderate; 20 – 28, severe OXIPN

	• <i>Number of participants:</i> 36 Control • <i>Age (mean ± SD):</i> 53.40 ± 15.70 years • <i>Gender/Sex (number of females/number of males):</i> 14/21 • <i>Underlying conditions:</i> Metastatic colon cancer (stage III), treated with oxaliplatin, 130 mg/m² IV, administered over 2 h infusion in day 1 and oral capecitabine (1000 mg/m², b.i.d) on days 1–4, to be repeated every 3 weeks for 8 cycles. • <i>Peripheral neuropathy diagnostic criteria:</i> Post-chemotherapy criteria for diagnosing neuropathy: TNSr: grade 0, no OXIPN; 1 – 10, mild; 11 – 19, moderate; 20 – 28, severe OXIPN • <i>Number of participants:</i> 35 Overall • <i>Age (mean ± SD):</i> Not reported • <i>Gender/Sex (number of females/number of males):</i> 32/39 • <i>Underlying conditions:</i> Metastatic colon cancer (stage III), treated with oxaliplatin, 130 mg/m² IV, administered over 2 h infusion in day 1 and oral capecitabine (1000 mg/m², b.i.d) on days 1–4, to be repeated every 3 weeks for 8 cycles. • <i>Peripheral neuropathy diagnostic criteria:</i> Post-chemotherapy criteria for diagnosing neuropathy: TNSr: grade 0, no OXIPN; 1 – 10, mild; 11 – 19, moderate; 20 – 28, severe OXIPN • <i>Number of participants:</i> 71 Inclusion criteria: Male or female patients with confirmed stage III colon cancer, aged between 20 and 85 years, and treated at Sheikhorrais University Clinic with oxaliplatin, 130 mg/m² IV, administered over 2 h infusion in day 1 and oral capecitabine (1000 mg/m², b.i.d) on days 1–4, to be repeated every 3 weeks for 8 cycles. In addition participants required a WHO performance status score of 0–1, and normal kidney and liver function. Exclusion criteria: Patients who had pre-existing peripheral neuropathy (PN) due to diabetes mellitus, alcoholic disease, HIV, and inherited PN-associated disorders. Patients who received other neurotoxic drugs prior to oxaliplatin therapy or those who were on any form of supplement therapy (fish oil, vitamins and minerals). Pre-treatment inter-group differences: "There was not any statistical difference between two groups in terms of gender, age, and BMI (P=.477, .816, and .806 respectively, Table 1)." Location of peripheral nerve assessed: Unilateral (right arm and leg). Unit of analysis: Per participant
Interventions	Intervention Characteristics • Omega-3 PUFA oral supplements • <i>Type (DHA/EPA/ALA):</i> DHA + EPA • <i>Dose DHA/EPA/ALA (milligrams/day):</i> 1037mg/day DHA + 192mg/day EPA • <i>Duration of treatment (days):</i> 175 (25 weeks) • <i>Co-interventions:</i> None • <i>Brand name:</i> Mor DHA Mini I.Q; manufactured by Minami Nutrition NV, Drie Eikenstraat 661, 2650 Edegem, Belgium Control • <i>Type:</i> Sunflower oil • <i>Dose (milligrams/day):</i> Not reported • <i>Duration of treatment (days):</i> 175 (25 weeks) • <i>Co-interventions:</i> None • <i>Brand name:</i> Not reported; manufactured by Dana Pharma, Tabriz, Iran.

Outcomes	Reduced Total Neuropathy Score (TNSr); Nerve conduction studies (distal motor latencies, compound muscle action potential amplitudes and motor conduction velocities in the tibial, peroneal and ulnar nerves; sensory action potential amplitudes, and nerve conduction velocities in the sural and ulnar nerves); pro-inflammatory cytokines (interleukin-6 (IL-6), tumor necrosis factor alpha (TNF- α), and high-sensitivity C-reactive Protein (hs-CRP)
Identification	Sponsorship source: Funding sources: - Present? Yes - Actual source: This study was funded by Sanofi in IRAN. Conflict (or declaration) of interest statement: - Present? Yes - Nature of conflict of interest: The authors declare that they have no competing interests. Country: Iran Setting: Sheikhorraies University Clinic Comments: Date study conducted: February 2012 to January 2015. Trial registration number: IRCT201112158397N2. Contacting study investigators: trial authors not contacted; no additional information provided for review. Author's name: Zohreh Ghoreishi Institution: Tabriz University of Medical Sciences Email: zohreh_ghoreishy@yahoo.com Address: Nutrition Research Center, Tabriz University of Medical Sciences, Tabriz, Iran Article details: Full text Year of publication: 2016 Journal of publication: Biomarker Research Language: English Publication status: Published

Risk of bias table

Bias	Authors' judgement	Support for judgement
Random sequence generation (selection bias)	Low risk	Quote: "Random allocation software (RAS) version 1.0.0. (Esfahan, Iran) was used for Permuted block randomization to assign patients to either the n-3 PUFAs supplemented group or the control group." Judgement Comment: Method for random sequence generation clearly stated.
Allocation concealment (selection bias)	Low risk	Quote: "Placebo and n-3 PUFAs pearls were similar in appearance and color and administered based on the same protocol." Judgement Comment: Identical containers used for active and placebo interventions.
Blinding of participants and personnel (performance bias)	Low risk	Quote: "Only the randomization coordinator of the study was aware of the patients' allocations." Judgement Comment: Clearly states that only the randomisation coordinator was aware of the participant allocations.
Blinding of outcome assessment (detection bias)	Low risk	Quote: "Only the randomization coordinator of the study was aware of the patients' allocations." Judgement Comment: Clearly states that only the randomisation coordinator was aware of the participant allocations.

Incomplete outcome data (attrition bias)	Low risk	Quote: "The rate of drop out was 5 patients in n-3 PUFAs supplemented group and 7 patients in the placebo group (Fig. 1), so the response rate was 80.09 %." Judgement Comment: Missing data less than 20% (i.e. more than 80% follow-up) and relatively equal follow-up in both groups and no obvious reason why loss to follow-up should be related to outcome.
Selective reporting (reporting bias)	High risk	Judgement Comment: Outcomes in protocol and/or trials registry entry selectively reported. In the clinical trial registry (IRCT201112158397N2), there are five primary outcomes listed:- Incidence and severity of oxaliplatin-induced peripheral neuropathy (measured using rTNS)- Serum concentration of interleukin-1 beta- Serum concentration of interleukin-6- Serum concentration of TNF-alpha- Serum concentration of Highly sensitive-CRPIn the paper itself, only the rTNS outcome is listed as a primary outcome measure and the pro-inflammatory cytokine are reported as secondary outcome measures, except for IL-1-beta which is excluded. Further, the nerve conduction studies are not listed as outcome measures on the clinical trial registry.
Other bias	High risk	Quote: "This study was funded by "Sanofi" in IRAN." Judgement Comment: Industry funding.

Fontani et al. (2010)⁵⁷

Methods	Study design: Randomized controlled trial Study grouping: Parallel group How missing data were handled: Not reported. Data from five participants from the sunflower (control) group were not included in the analyses as they did not complete the study. Exclusions after randomisation: None reported. Losses to follow-up: Five participants from the sunflower (control) group were not included in the analyses as they did not complete the study. Sample size calculation reported: Not reported. Pre-specified primary outcome measure(s): Not explicitly reported. Pre-specified secondary outcome measure(s): Not explicitly reported. Outcome measures included: quantitative sensory testing, questionnaires concerning anamnestic/ epidemiological data, pain characteristics and mood. In addition, arachidonic acid/eicosapentaenoic acid ratio (AA/EPA), cholesterol, triglycerides, high density lipoprotein (HDL), low density lipoprotein (LDL), glycaemia and cortisol were analysed. Compliance measures: On day 1, each subject met a dietician and received a personalized diet to avoid excesses. Diet was personalized, excluding food side, but keeping a similar caloric intake for all and a similar distribution of fat, protein, carbohydrates and n-3 fatty acids. Any other nutritional supplements or herbal remedies were excluded. The subjects were asked to fill in a diary card to record their daily events and other general information to be used by the investigators to assess possible protocol violations during the study (e.g. deviations from diet, use of drugs).
Participants	Baseline Characteristics Omega-3 PUFA oral supplements • <i>Age (mean ± SD):</i> Not reported • <i>Gender/Sex (number of females/number of males):</i> 23/0 • <i>Underlying conditions:</i> n=14 with fibromyalgia; n=9 with widespread musculoskeletal pain • <i>Peripheral neuropathy diagnostic criteria:</i> Not applicable

	• <i>Number of participants:</i> 23 Control • <i>Age (mean $\pm$ SD):</i> Not reported • <i>Gender/Sex (number of females/number of males):</i> 23/0 • <i>Underlying conditions:</i> n=13 with fibromyalgia; n=10 with widespread musculoskeletal pain • <i>Peripheral neuropathy diagnostic criteria:</i> Not applicable • <i>Number of participants:</i> 23 Overall • <i>Age (mean $\pm$ SD):</i> 44 $\pm$ 16 (unit of error not specified) • <i>Gender/Sex (number of females/number of males):</i> 46/0 • <i>Underlying conditions:</i> n=27 with fibromyalgia; n=19 with widespread musculoskeletal pain • <i>Peripheral neuropathy diagnostic criteria:</i> Not applicable • <i>Number of participants:</i> 46 Inclusion criteria: Clinical symptoms of chronic widespread musculoskeletal pain of at least moderate intensity (at least 40 on a 0-100 Visual Analogue Scale; VAS) present for at least 6 months Exclusion criteria: Obesity; hypertension; heavy smoking (more than 8 cigarettes per day); drinking more than two glasses of spirits per day; and caffeine consumption of more than two cups per day; cortisone treatment; dermatological problems; fever; invasive techniques in the three days preceding the experimental sessions. Pre-treatment inter-group differences: The two groups did not differ significantly between each other in blood parameters (Table 1), clinical symptoms and quantitative sensory testing (Table 2) and questionnaires (Table 3). Location of peripheral nerve assessed: The superficial punctuate pain threshold was assessed on the glabrous skin of the third phalanx of the second finger (non-dominant hand); cold and heat thresholds (dominant hand) Unit of analysis: Per participant
Interventions	Intervention Characteristics Omega-3 PUFA oral supplements • <i>Type (DHA/EPA/ALA):</i> DHA + EPA + Other omega-3s (alpha linolenic, stearidonic, eicosatetraenoic and ocosapentaenoic acid) • <i>Dose DHA/EPA/ALA (milligrams/day):</i> 800mg/day DHA + 1600mg/day EPA + 400mg/day other omega-3s • <i>Duration of treatment (days):</i> 35 • <i>Co-interventions:</i> None • <i>Brand name:</i> Not commercial available; manufactured by Enervit ALSO SpA (Milan, Italy) Control • <i>Type:</i> Oleic acid (sunflower oil) • <i>Dose (milligrams/day):</i> 4000mg/day oleic acid • <i>Duration of treatment (days):</i> 35 • <i>Co-interventions:</i> None • <i>Brand name:</i> Not commercial available; manufactured by Balestrini s.r.l. (Materie Prime Naturali, Milan)
Outcomes	Quantitative sensory testing (von Frey, tender points number, tender points threshold, heat pain, cold pain, cold pressure threshold. Cold pressure tolerance); questionnaires concerning anamnestic/ epidemiological data; pain characteristics and mood, haematological parameters (arachidonic acid, eicosapentaenoic acid ratio, cholesterol, triglycerides, high density lipoprotein (HDL), low density lipoprotein (LDL), glycaemia and cortisol).
Identification	Sponsorship source:

	Funding sources: - Present? Yes - Actual source: Research supported by Enervit SpA, Milan, Italy. Conflict (or declaration) of interest statement: - Present? No - Nature of conflict of interest: Not reported Country: Italy Setting: University of Siena Rheumatology Unit Comments: Date study conducted: Not reported Trial registration number: Not reported Contacting study investigators: Trial authors not contacted; no additional information used for review. Authors name: Giuliano Fontani Institution: University of Siena, Italy Email: Not provided Address: Rheumatology Unit, University of Siena, Italy Article details: Full text Year of publication: 2010 Journal of publication: Journal of Complementary and Integrative Medicine Language: English Publication status: Published

Risk of bias table

Bias	Authors' judgement	Support for judgement
Random sequence generation (selection bias)	Low risk	Quote: "patients were randomly assigned" Quote: "The recruited patients of each stratum were equally distributed in the n-3 and sunflower oil groups by drawing of lots." Judgement Comment: Method of sequence generation described by strata and drawing of lots.
Allocation concealment (selection bias)	Low risk	Quote: "Enervit ALSO SpA (Milan, Italy) prepared the omega-3 fish oil and G. Balestrini s.r.l. (Materie Prime Naturali, Milan) prepared the oleic sunflower oil capsules, which were indistinguishable in packaging, shape or taste." Judgement Comment: Study supplements were packaged in identical appearing packaging, and were similar in appearance, shape and taste.
Blinding of participants and personnel (performance bias)	Low risk	Quote: "Patients were informed that they could receive either n-3 or sunflower oil, i.e. treatment or placebo (double-blind placebo-controlled design)." Judgement Comment: Described as "double blind" and states that participants and researchers were not aware of the treatment allocation.
Blinding of outcome assessment (detection bias)	Low risk	Quote: "blindly assigned a code to the products and revealed the content to researchers after the completion of the analysis." Judgement Comment: Described as "double blind" and clearly states that the researchers were blind to the treatment allocation.
Incomplete outcome data (attrition bias)	High risk	Quote: "5 FM subjects in the SF group did not conclude the study and were not considered. Therefore only 18 subjects (8 FM and 10 MSP) were analysed. The"

		Judgement Comment: n=5 (from 23), equivalent to 11% of total participants, dropped out of one group (the sunflower oil group) only.
Selective reporting (reporting bias)	Unclear risk	Judgement Comment: No access to protocol or trials registry entry.
Other bias	High risk	Quote: "Research supported by Enervit SpA, Milan, Italy." Judgement Comment: Funded by the manufacturer of the study supplements.

Ghoreishi et al. (2012)^{S8}

Methods	Study design: Randomized controlled trial Study grouping: Parallel group How missing data were handled: Diagram (Figure 1) reporting participant flow, participants who withdrew or did not complete the study are indicated as not included in data analysis and interpretation. Exclusions after randomisation: Four patients discontinued the study due to a critical health conditions. Losses to follow-up: Eight patients were unwilling to continue after the first cycle of therapy. Sample size calculation reported: Yes. Pre-specified primary outcome measure(s): Reduced Total Neuropathy Score (rTNS) Pre-specified secondary outcome measure(s): Nerve conduction studies (NCS); serum level of EPA and DHA. Compliance measures: Compliance not tracked, however serum concentrations of EPA and DHA were measured before and after the supplementation period, and a significant difference was observed between study groups, which the authors suggest could be an indicator of participant compliance.
Participants	Baseline Characteristics Omega-3 PUFA oral supplements • Age (mean $\pm$ SD): 46.19 $\pm$ 9.76 years • Gender/Sex (number of females/number of males): 30/0 (although 35 were randomised, baseline data are only given for the 30 who completed the study) • Underlying conditions: Breast cancer, at risk of paclitaxel-induced peripheral neuropathy (PIPN). Treatment with four courses of 175 mg/m² paclitaxel for a positive node breast cancer, given over a 3-hour infusion every three weeks for 4 cycles. • Peripheral neuropathy diagnostic criteria: Not applicable • Number of participants: 30 Control • Age (mean $\pm$ SD): 45.70 $\pm$ 12.00 years • Gender/Sex (number of females/number of males): 27/0 (although 34 were randomised, baseline data are only given for the 27 who completed the study) • Underlying conditions: Breast cancer, at risk of paclitaxel-induced peripheral neuropathy (PIPN). Treatment with four courses of 175 mg/m² paclitaxel for a positive node breast cancer, given over a 3-hour infusion every three weeks for 4 cycles. • Peripheral neuropathy diagnostic criteria: Not applicable • Number of participants: 27 Overall • Age (mean $\pm$ SD): Not reported

	• <i>Gender/Sex (number of females/number of males)</i>: 57/0 • <i>Underlying conditions</i>: Breast cancer, at risk of paclitaxel-induced peripheral neuropathy (PIPN). Treatment with four courses of 175 mg/m² paclitaxel for a positive node breast cancer, given over a 3-hour infusion every three weeks for 4 cycles. • <i>Peripheral neuropathy diagnostic criteria</i>: Not applicable • <i>Number of participants</i>: 57 (although 69 were randomised) Inclusion criteria: Female; age between 30 to 70 years; treatment with 4 courses of 175 mg/m² paclitaxel for a positive node breast cancer; satisfactory kidney and liver function; WHO performance scores of 0 to 1. Exclusion criteria: All factors which predisposed patients to PN (including a medical history of prior chemotherapy treatment, pre-existing peripheral neuropathy due to diabetes mellitus, HIV, alcohol abuse, thyroid dysfunction and hereditary PN associated disorders); taking any form of nutritional supplement (fish oil, vitamins and minerals) at least three months before enrolment. Pre-treatment inter-group differences: There was no statistical significant difference between the two groups according to age ($P = 0.86$). The means of body mass index (BMI) for the omega-3 supplemented group and the control group were 45.99 ± 9.01 and 44.14 ± 8.89 respectively, without statistical differences ($P = 0.43$). Location of peripheral nerve assessed: Right leg (tibial, peroneal, sural) and hand (ulnar) nerves. Unit of analysis: Per participant
Interventions	Intervention Characteristics Omega-3 PUFA oral supplements • <i>Type (DHA/EPA/ALA)</i>: DHA + EPA • <i>Dose DHA/EPA/ALA (milligrams/day)</i>: 1037mg/day DHA + 192mg/day EPA • <i>Duration of treatment (days)</i>: 16 weeks (112 days) • <i>Co-interventions</i>: None. • <i>Brand name</i>: Mor DHA Mini I.Q. Minami Nutrition NV, Drie Eikenstraat 661,2650 Edegem, Belgium Control • <i>Type</i>: Sunflower oil (corn oil reported in clinical trial registry) • <i>Dose (milligrams/day)</i>: Not reported • <i>Duration of treatment (days)</i>: 16 weeks (112 days) • <i>Co-interventions</i>: None. • <i>Brand name</i>: Dana Pharma, Tabriz, Iran
Outcomes	Reduced total neuropathy score (rTNS); nerve conduction studies (distal motor latencies, compound muscle action potential amplitudes and motor conduction velocities in the tibial, peroneal and ulnar nerves; sensory action potential amplitudes and sensory conduction velocities in the sural and ulnar nerves); serum levels of omega-3 fatty acids.
Identification	Sponsorship source: Funding sources: - Present? No - Actual source: Not reported Conflict (or declaration) of interest statement: - Present? Yes - Nature of conflict of interest: The authors declare that they have no competing interests. Country: Iran Setting: Sheiqorrais University Clinic Comments: Date study conducted: April 2010 to October 2011.

	Trial registration number: Not reported Contacting study investigators: Trial authors not contacted; no additional information provided for review. Author's name: Seyed Ali Keshavarz Institution: Tehran University of Medical Sciences Email: sakeshavarz2012@gmail.com Address: Department of Nutrition and Biochemistry, School of Health, Tehran University of Medical Sciences, Tehran, Iran Article details: Full text Year of publication: 2012 Journal of publication: BMC Cancer Language: English Publication status: Published

Risk of bias table

Bias	Authors' judgement	Support for judgement
Random sequence generation (selection bias)	Low risk	Quote: "The randomization plan was based on a permuted block randomization." Judgement Comment: Sequence generation method documented.
Allocation concealment (selection bias)	Low risk	Quote: "Sealed closed envelopes containing random codes (A or B) were used to assign subjects to either the intervention group or the control group. The allocation of patients was only known to the randomization coordinator of the study." Quote: "omega-3 fatty acid oral supplements as soft gelatin capsules (Mor DHA Mini I.Q. Minami Nutrition NV, Drie Eikenstraat 661,2650 Edegem, Belgium) at a dose of 640 mg (54% DHA, 10% EPA) three times a day during chemotherapy with paclitaxel and one month after the end of therapy or placebo of Sun flower soft gelatin capsules, (Dana Pharma, Tabriz, Iran) that were similar in appearance to omega-3 fatty acid soft gelatin capsules and similarly administered." Judgement Comment: Sealed opaque envelopes, with identical drug containers.
Blinding of participants and personnel (performance bias)	Low risk	Quote: "The allocation of patients was only known to the randomization coordinator of the study." Judgement Comment: Described as "double blind" and states that the "allocation of patients was only known to the randomisation coordinator of the study."
Blinding of outcome assessment (detection bias)	Low risk	Quote: "The allocation of patients was only known to the randomization coordinator of the study." Judgement Comment: Described as "double blind" and states that the "allocation of patients was only known to the randomisation coordinator of the study."
Incomplete outcome data (attrition bias)	Low risk	Quote: "69 patients were randomly allocated to receive omega-3 fatty acids (n = 35) or placebo (n = 34). Four patients discontinued the study due to a critical health conditions and 8 patients were unwilling to continue after the first cycle of therapy (lost to follow up, n = 12). So 57 female patients completed the study (Figure 1)." Judgement Comment: Missing data less than 20% (i.e. more than 80% follow-up) and equal follow-up in both groups and no obvious reason why loss to follow-up should be related to outcome.

Selective reporting (reporting bias)	High risk	Judgement Comment: Outcomes in protocol and/or trials registry entry selectively reported (serum level of IL-1, IL-6, TNF-alpha and hs-CRP not reported).
Other bias	Low risk	Quote: "The authors declare that they have no competing interests." Judgement Comment: No other source of bias.

McCormick et al. (2015)^{S9}

Methods	Study design: Randomized controlled trial Study grouping: Parallel group How missing data were handled: Not reported Exclusions after randomisation: Not reported Losses to follow-up: Not reported Sample size calculation reported: Not reported. Pre-specified primary outcome measure(s): Not reported Pre-specified secondary outcome measure(s): Not explicitly reported. Measured: Vibration perception threshold (VPT); microvascular function (MF); liver fat; body fat; insulin resistance (IR) Compliance measures: Not reported.
Participants	Baseline Characteristics Omega-3 PUFA oral supplements • <i>Age (mean ± SD):</i> Not reported • <i>Gender/Sex (number of females/number of males):</i> Not reported. • <i>Underlying conditions:</i> Non-alcoholic fatty liver disease without neuropathy, with and without diabetes • <i>Peripheral neuropathy diagnostic criteria:</i> Not reported. • <i>Number of participants:</i> Not reported. Control • <i>Age (mean ± SD):</i> Not reported. • <i>Gender/Sex (number of females/number of males):</i> Not reported. • <i>Underlying conditions:</i> Non-alcoholic fatty liver disease without neuropathy, with and without diabetes • <i>Peripheral neuropathy diagnostic criteria:</i> Not reported. • <i>Number of participants:</i> Not reported. Overall • <i>Age (mean ± SD):</i> 51.4 years • <i>Gender/Sex (number of females/number of males):</i> 31/70 • <i>Underlying conditions:</i> Non-alcoholic fatty liver disease without neuropathy, with and without diabetes • <i>Peripheral neuropathy diagnostic criteria:</i> Not reported • <i>Number of participants:</i> 101 Inclusion criteria: Non-alcoholic fatty liver disease (NAFLD) Exclusion criteria: Neuropathy Pre-treatment inter-group differences: Not reported. Location of peripheral nerve assessed: Left big toe; forearm (laterality not specified) Unit of analysis: Per participant
Interventions	Intervention Characteristics • Omega-3 PUFA oral supplements • <i>Type (DHA/EPA/ALA):</i> Not reported • <i>Dose DHA/EPA/ALA (milligrams/day):</i> 4000mg/day • <i>Duration of treatment (days):</i> 450-540 days (15-18 months) • <i>Co-interventions:</i> Not reported • <i>Brand name:</i> Not reported Control

	• <i>Type</i>: Not reported • <i>Dose (milligrams/day)</i>: Not reported • <i>Duration of treatment (days)</i>: 450-540 days (15-18 months) • <i>Co-interventions</i>: Not reported • <i>Brand name</i>: Not reported
Outcomes	Vibration perception threshold (VPT); microvascular function (MF); liver fat; body fat; insulin resistance (IR). “MF and VPT were not improved with omega-3 treatment”
Identification	Sponsorship source: Funding sources: - Present? No Conflict (or declaration) of interest statement: - Present? No Country: Not reported Setting: Not reported Comments: Date study conducted: not reported. Trial registration number: not reported. Contacting study investigators: trial authors not contacted; no additional information provided for review. Authors name: KG McCormick Institution: University of Southampton Email: Not reported Address: Not reported Article details: Conference poster - Diabetes UK Professional Conference 2015 Year of publication: 2015 Journal of publication: Diabetic Medicine Language: English Publication status: Published

Risk of bias table

Bias	Authors' judgement	Support for judgement
Random sequence generation (selection bias)	Unclear risk	Judgement Comment: Not reported how list was generated. Trial described as “randomised” but with no further details.
Allocation concealment (selection bias)	Unclear risk	Judgement Comment: Not reported how allocation administered. Trial described as “randomised” but with no further details.
Blinding of participants and personnel (performance bias)	High risk	Judgement Comment: No information on blinding, where the outcome is likely to be influenced by lack of blinding. We assume that in absence of reporting on this participants and personnel were not masked.
Blinding of outcome assessment (detection bias)	High risk	Judgement Comment: No information on masking, and the outcome measurement is likely to be influenced by lack of blinding. We assume that in absence of reporting on this outcome assessors were not masked
Incomplete outcome data (attrition bias)	Unclear risk	Judgement Comment: Follow-up not reported.
Selective reporting (reporting bias)	Unclear risk	Judgement Comment: No access to protocol or trials registry entry.

Other bias	Unclear risk	Judgement Comment: Not enough information provided to make reasonable judgement.

McCormick et al. (2015a)^{S10}

Methods	Study design: Randomized controlled trial Study grouping: Parallel group How missing data were handled: Multivariable-adjusted regression models (intention-to-treat analyses) Exclusions after randomisation: Not explicitly reported. CONSORT diagram indicates that some participants (n=4-5 for omega-3 group; n=4-5 in the placebo group) did not have data for end measurements for selected outcomes (VPT and MF/RF). Losses to follow-up: Not reported. Sample size calculation reported: No a priori sample size calculation. In the Discussion the authors state: "Whilst we did not undertake prior sample size calculations for this pre-specified sub study, our results provided no hint of improvement with the intervention (Table 4). A retrospective power calculation showed that with the number of patients completing the trial and $\alpha = 0.05$, we had 99% power to detect a 20% change in MF/RF. " Pre-specified primary outcome measure(s): Vibrotactile perception (VPT); microvascular function (MR/RF) at the end of the study. Pre-specified secondary outcome measure(s): Glucose, insulin, total cholesterol, HDL cholesterol and triacylglycerol concentrations, HbA1C, HOMA-IR, blood pressure, Dual energy x-ray absorptiometry (DXA), MRI and magnetic resonance spectroscopy (MRS), liver fibrosis, Carotid intima-media thickness, QRISK risk of cardiovascular disease calculations. Compliance measures: Not reported.
Participants	Baseline Characteristics Omega-3 PUFA oral supplements • Age (mean $\pm$ SD): 48.2 $\pm$ 11.1 years • Gender/Sex (number of females/number of males): 25/24 • Underlying conditions: Non-alcoholic fatty liver disease, with and without type-2 diabetes, without overt neuropathy or microvascular disease. • Peripheral neuropathy diagnostic criteria: Failure to detect a 10g monofilament. • Number of participants: 51 Control • Age (mean $\pm$ SD): 53.8 $\pm$ 9.7 years • Gender/Sex (number of females/number of males): 17/34 • Underlying conditions: Non-alcoholic fatty liver disease, with and without type-2 diabetes, without overt neuropathy or microvascular disease. • Peripheral neuropathy diagnostic criteria: Failure to detect a 10g monofilament. • Number of participants: 49 Overall • Age (mean $\pm$ SD): Not reported. • Gender/Sex (number of females/number of males): 42/58 • Underlying conditions: Non-alcoholic fatty liver disease, with and without type-2 diabetes, without overt neuropathy or microvascular disease. • Peripheral neuropathy diagnostic criteria: Failure to detect a 10g monofilament. • Number of participants: 100

	Inclusion criteria: Diagnosis of NAFLD (based on liver biopsy or the presence of hepatic steatosis on ultrasound) Exclusion criteria: Other liver diseases; evidence of distal peripheral neuropathy in feet (diagnosed based on failure to detect a 10 g monofilament); evidence of diabetic eye disease (diagnosed at retinal screening). Pre-treatment inter-group differences: Participants at baseline were stratified by diabetes status and by randomisation status. The mean $\pm$ SD age was 51.7$\pm$10.9 years for the 69 individuals (38 men) without diabetes and 50.1$\pm$10.3 years for the 32 individuals (20 men) with type 2 diabetes. Thirteen participants were current smokers and three had suffered from ischaemic heart disease. Of the participants, 41 were taking statins, 36 were taking antihypertensive drugs (nine calcium antagonists) and 27 were taking metformin. The VPT at 125 Hz and MF/RF did not differ between individuals with and without diabetes. Of the participants without diabetes, ~50% had impaired fasting glucose or impaired glucose tolerance. Location of peripheral nerve assessed: Big toe (VPT) and forearm (MF) Unit of analysis: Per participant
Interventions	Intervention Characteristics Omega-3 PUFA oral supplements • <i>Type (DHA/EPA/ALA):</i> DHA + EPA • <i>Dose DHA/EPA/ALA (milligrams/day):</i> 1520mg/day DHA + 1840mg/day EPA • <i>Duration of treatment (days):</i> 450-540 days (15-18 months) • <i>Co-interventions:</i> None • <i>Brand name:</i> Omacor (Lovaza) ethyl esters Control • <i>Type:</i> Olive oil (~67% oleic acid, ~15% linoleic acid, ~15% palmitic acid. ~2% stearic acid and ~1% α-linolenic acid) • <i>Dose (milligrams/day):</i> 4000mg/day • <i>Duration of treatment (days):</i> 450-540 days (15-18 months) • <i>Co-interventions:</i> None • <i>Brand name:</i> Not reported.
Outcomes	Vibrotactile perception; microvascular function, biochemical and anthropometric measurements. “In multivariable-adjusted regression models (intention-to-treat analyses), DHA+EPA treatment was associated with an increase in VPT; Stratifying by diabetes status showed there was no specific benefit of DHA+EPA treatment on VPT ($p=0.36$) or MF/RF ($p=0.53$) in people with diabetes.”
Identification	Sponsorship source: Funding sources: - Present? Yes - Actual source: This National Institute for Health Research (NIHR) Southampton Biomedical Research Unit grant and by a Diabetes UK allied health research training fellowship; Omacor and placebo were provided by Pronova Biopharma through Abbott Laboratories, Southampton, UK. Conflict (or declaration) of interest statement: - Present? Yes - Nature of conflict of interest: PCC serves on the Clinical Advisory Board of Pronova Biopharma and has acted as a consultant to Amarin. All other authors declare that there is no duality of interest associated with this manuscript. Country: Not reported Setting: Not reported Comments: Date study conducted: November 2009 to February 2018.

	Trial registration number: NCT00760513. Contacting study investigators: trial authors not contacted; no additional information provided for review. Author's name: Christopher D. Byrne Institution: University Hospital Southampton Email: cdtb@soton.ac.uk Address: Human Development and Health Academic Unit, Faculty of Medicine, University Hospital Southampton, MP887, IDS Building, Tremona Road, Southampton SO166YD, UK Article details: Full text Year of publication: 2015 Journal of publication: Diabetologia Language: English Publication status: Published

Risk of bias table

Bias	Authors' judgement	Support for judgement
Random sequence generation (selection bias)	Low risk	Quote: "Participants were block randomised by an independent clinical trials pharmacist" Judgement Comment: Computer generated list, random table, other method of generating random list.
Allocation concealment (selection bias)	Low risk	Quote: "Only the clinical trials pharmacist was unblinded, and randomisation group allocation was concealed from all study members throughout the trial." Quote: "Participants were block randomised by an independent clinical trials pharmacist to treatment with identical capsules by mouth of either n-3 fatty acid ethyl esters" Judgement Comment: The pharmacist was independent to the clinical trials, and was the only one to know the "randomisation".
Blinding of participants and personnel (performance bias)	Low risk	Quote: "Only the clinical trials pharmacist was unblinded, and randomisation group allocation was concealed from all study members throughout the trial." Judgement Comment: Clearly stated that participants and personnel not aware of which treatment received
Blinding of outcome assessment (detection bias)	Low risk	Quote: "Only the clinical trials pharmacist was unblinded, and randomisation group allocation was concealed from all study members throughout the trial." Judgement Comment: Clearly stated that participants and personnel not aware of which treatment received.
Incomplete outcome data (attrition bias)	Low risk	Judgement Comment: Missing data less than 20% (i.e. more than 80% follow-up) and equal follow-up in both groups and no obvious reason why loss to follow-up should be related to outcome.
Selective reporting (reporting bias)	Low risk	Judgement Comment: The current study is a sub-study of a larger trial (referenced), all outcomes in protocol related to this sub-study are reported.
Other bias	Unclear risk	Quote: "Omacor and placebo were provided by Pronova Biopharma through Abbott Laboratories, Southampton, UK. This work was support- ed by a National Institute for Health Research (NIHR) Southampton Biomedical Research Unit grant and by a Diabetes UK allied health research training fellowship awarded to KGM"

		Quote: "PCC serves on the Clinical Advisory Board of Pronova Biopharma and has acted as a consultant to Amarin." Judgement Comment: Conflict of interest relevant but not clear that it is important to the nature of the review.

Monahan *et al.* (2004)^{SH}

Methods	Study design: Randomized controlled trial Study grouping: Parallel group Exclusions after randomisation: Not reported How missing data were handled: Not reported Losses to follow-up: Not reported Pre-specified primary outcome measure(s): Not reported. Pre-specified secondary outcome measure(s): Not explicitly reported. Measured: Ischemic Handgrip to Fatigue, Cold Pressor Test, Multifiber recordings of MSNA, Arterial Blood Pressure and Heart Rate, Blood samples. Sample size calculation reported: Not reported. Compliance measures: Efforts to increase/verify compliance to pill ingestion included review of subject pill ingestion diary, pill count, and phone-based contacts.
Participants	Baseline Characteristics Omega-3 PUFA oral supplements - Age (mean $\pm$ SE): 25 $\pm$ 1 years - Gender/Sex (number of females/number of males): 3/6 - Underlying conditions: None - Peripheral neuropathy diagnostic criteria: Not applicable - Number of participants: 9 Control - Age (mean $\pm$ SE): 24 $\pm$ 1 years - Gender/Sex (number of females/number of males): 5/4 - Underlying conditions: None - Peripheral neuropathy diagnostic criteria: Not applicable - Number of participants: 9 Overall - Age (mean $\pm$ SE): Not reported - Gender/Sex (number of females/number of males): 8/10 - Underlying conditions: None - Peripheral neuropathy diagnostic criteria: Not applicable - Number of participants: 18 Inclusion criteria: Age 18 to 35 years, normotensive, non-smoking, nonobese (body mass index 27kg/m²), non-medicated and otherwise healthy. Exclusion criteria: Not reported. Pre-treatment inter-group differences: Table of baseline subject characteristics suggests no significant baseline differences in demographics or other key clinical parameters. Location of peripheral nerve assessed: Peroneal nerve of the leg (laterality not stated) Unit of analysis: Per participant
Interventions	Intervention Characteristics Omega-3 PUFA oral supplements - Type (DHA/EPA/ALA): DHA + EPA - Dose DHA/EPA/ALA (milligrams/day): 2000mg/day DHA + 3000mg/day EPA - Duration of treatment (days): 30 days (1 month) - Co-interventions: None

	• Brand name: Pharmacist's Ultimate Health, Saint Paul, Minn Control • Type: Olive oil • Dose (milligrams/day): 10000mg/day • Duration of treatment (days): 30 days (1 month) • Co-interventions: None • Brand name: Assembled by local compounding pharmacist
Outcomes	Ischemic Handgrip to Fatigue; Cold Pressor Test; Multifiber recordings of muscle sympathetic nerve activity; arterial blood pressure and heart rate; blood samples (blood lipids, C-reactive protein (using an immunoturbidimetric assay), and prostanoids (6-Keto-PGF And thromboxane B)
Identification	Sponsorship source: Funding sources: - Present? Yes - Actual source: Grants supporting this project were received from the National Institute of Health, National Space and Biomedical Research Institute, an Established Investigator Grant from the American Heart Association, and a Pennsylvania State University Tobacco Settlement Grant. Additional support was provided by a National Institutes of Health-sponsored General Clinical Research Center with National Center for Research Resources Grants. Conflict (or declaration) of interest statement: - Present? No - Nature of conflict of interest: Not reported Country: Not reported Setting: Not reported Comments: Date study conducted: not reported. Trial registration number: not reported. Contacting study investigators: trial authors not contacted; no additional information provided for review. Author's name: Kevin Monahan Institution: Penn State College of Medicine Email: kmonahan@psu.edu Address: Penn State College of Medicine, Division of Cardiology Ho47, The Milton S. Hershey Medical Center, 500 University Dr, Hershey, PA 17033-2390. Article details: Full text Journal of publication: Hypertension Language: English Publication status: Published Year of publication: 2004

Risk of bias table

Bias	Authors' judgement	Support for judgement
Random sequence generation (selection bias)	Unclear risk	Quote: "This study was randomized, placebo-controlled, and double-blinded." Judgement Comment: Not reported how list was generated. Trial described as "randomised" but with no further details.
Allocation concealment (selection bias)	Unclear risk	Quote: "This study was randomized, placebo-controlled, and double-blinded." Judgement Comment: Not reported how allocation administered. Trial described as "randomised" but with no further details.

Blinding of participants and personnel (performance bias)	Unclear risk	Quote: "This study was randomized, placebo-controlled, and double-blinded." Judgement Comment: Described as "double-blinded" with no information on who was masked.
Blinding of outcome assessment (detection bias)	Unclear risk	Quote: "This study was randomized, placebo-controlled, and double-blinded." Judgement Comment: Described as "double-blinded" with no information on who was masked.
Incomplete outcome data (attrition bias)	Unclear risk	Judgement Comment: Follow-up not reported.
Selective reporting (reporting bias)	Unclear risk	Judgement Comment: No access to protocol or trials registry entry.
Other bias	Low risk	Quote: "Grants supporting this project were received from the National Institute of Health (HL58503, DC006459, and HL67624), National Space and Biomedical Research Institute (CA00207), an Established Investigator Grant from the American Heart Association, and a Pennsylvania State University Tobacco Settlement Grant. Additional support was provided by a National Institutes of Health-sponsored General Clinical Research Center with National Center for Research Resources Grants MO1 RR10732 and CO6 RR016499." Judgement Comment: No other source of bias.

NCT00931879 (2009)^{S12}

Methods	Study design: Randomized controlled trial Study grouping: Parallel group Exclusions after randomisation: 6 participants did not complete the study (it is unclear whether these were exclusions after randomisation or losses to follow-up): 3 in Lovaza group, 3 in placebo. How missing data were handled: Excluded from analysis based upon number of participants reported in outcome analyses. Losses to follow-up: 6 participants did not complete the study (it is unclear whether these were exclusions after randomisation or losses to follow-up): 3 in Lovaza group, 3 in placebo. Pre-specified primary outcome measure(s): The following outcome measures were measured at one-year: 1. Efficacy Measures Are Nerve Conduction Studies, Specifically Changes in Conduction Amplitude. 2. Measurements of Indices of Large and Small Fiber Nerve Function Including Heart Rate Variation Measures. 3. Measurements of Indices of Large and Small Fiber Nerve Function Using Vibration and Thermal Thresholds. 4. Percent Change in Measurements of Indices of Large and Small Fiber Nerve Function Including Vibration Thresholds. 5. Measurements of Indices of Large and Small Fiber Nerve Function Including Markers of Inflammation and Oxidative Stress. 6. Nerve Conduction Studies; Specifically, Increases in Conduction Velocity. 7. Nerve Conduction Studies, Specifically Changes in Latency. 8. Nerve Conduction Studies, Specifically Changes in F-wave Conduction. 9. Examining Increased Vascular Response to Ischemic Block and to Local Warming at the Dorsum of the Foot. Pre-specified secondary outcome measure(s): None Sample size calculation reported: Yes. Compliance measures: Not reported.

Participants	Baseline Characteristics Omega-3 PUFA oral supplements • Age (mean $\pm$ SD): 58.21 $\pm$ 1.70 years • Gender/Sex (number of females/number of males): 12/7 • Underlying conditions: Type-2 diabetes • Peripheral neuropathy diagnostic criteria: None • Number of participants: 19 Control • Age (mean $\pm$ SD): 60.58 $\pm$ 2.06 years • Gender/Sex (number of females/number of males): 11/8 • Underlying conditions: Type-2 diabetes • Peripheral neuropathy diagnostic criteria: None • Number of participants: 19 Overall • Age (mean $\pm$ SD): 59.39 $\pm$ 1.33 years • Gender/Sex (number of females/number of males): 23/15 • Underlying conditions: Type-2 diabetes • Peripheral neuropathy diagnostic criteria: None • Number of participants: 38 Inclusion criteria: Males or non-pregnant, non-lactating females age 18-80 years; type 2 diabetes mellitus according to the current ADA criteria; triglyceride levels above 149 mg/dL; minimum of 2 years after diagnosis of type 2 diabetes; prior to participation in this study, each subject must sign an informed consent document. Exclusion criteria: Presence of type 1 diabetes mellitus (defined as C-peptide 1 ng/ml or diabetes onset at 35 years of age in a non-obese patient); Presence of diabetic retinopathy that is more severe than "background" level; Presence of diabetic nephropathy, defined by urine dipstick results greater than 300 mg/100 mL for protein (proteinuria); Presence of clinically significant neuropathy that is clearly of non-diabetic origin, e.g. alcoholic or autoimmune; Bilateral amputation of lower extremities or foot ulcers involving the great toes. Presence of neuroarthropathy (Charcot deformity) is allowable; History of major macrovascular events such as myocardial infarction or stroke; Participation in another clinical trial concurrently or within 30 days prior to entry into this study; The use of ACE-inhibiting agents or angiotensin receptor blockade therapy (ARB) is allowed but must have been stable for at least 30 days prior to study entry and may not change during the course of the study. This is prudent due to their potential effects on blood flow; Patients with moderate or severe hepatic insufficiency or abnormalities of liver function defined as any liver enzymes (aspartate aminotransferase, alanine transaminase, alkaline phosphatase) greater than 3 times the upper limit of normal; Presence of pedal edema; Presence or history of heart failure New York Heart Association Class II or greater; Other serious medical conditions that in the opinion of the investigator, would compromise the subject's participation in the study; Concomitant use of medications known to exacerbate triglyceride levels, such as estrogens. Pre-treatment inter-group differences: Not reported. Location of peripheral nerve assessed: Arms, legs and foot Unit of analysis: Per participant
Interventions	Intervention Characteristics Omega-3 PUFA oral supplements • Type (DHA/EPA/ALA): Not reported • Dose DHA/EPA/ALA (milligrams/day): 4000mg/day omega-3 ethyl esters • Duration of treatment (days): 180 • Co-interventions : None

	• Brand name: Lovaza ethyl esters Control • Type (DHA/EPA/ALA): Not reported • Dose DHA/EPA/ALA (milligrams/day): Not reported. • Duration of treatment (days): 180 • Co-interventions: None • Brand name: Not reported.
Outcomes	Nerve Conduction Studies (motor conduction amplitudes, latencies, velocities and F-wave conduction in the tibial, median and peroneal nerves; sensory conduction amplitudes, latencies and velocities in the ulnar and sural nerves); heart rate variation measures; vibration and thermal thresholds; markers of inflammation and oxidative stress; adverse events.
Identification	Sponsorship source: Funding sources: - Present? Yes - Actual source: Eastern Virginia Medical School, GlaxoSmithKline. Conflict (or declaration) of interest statement: - Present? Yes - Nature of conflict of interest: "Principal Investigators are NOT employed by the organization sponsoring the study. There is NOT an agreement between Principal Investigators and the Sponsor (or its agents) that restricts the PI's rights to discuss or publish trial results after the trial is completed". Country: United States of America Setting: Strelitz Diabetes Center Comments: Date study conducted: July 2009 to May 2017. Trial registration number: NCT00931879. Contacting study investigators: trial authors contacted; additional information provided for review. The authors were contacted to confirm: 1) whether the results are reported as change from baseline or endpoint values, and 2) the total supplementation period and total time of follow-up. The authors confirmed that: 1) results are reported as endpoint values, and 2) the total supplementation period for both omega-3 and placebo were administered for six months, and the outcomes were extracted at 12 months follow-up. Author's name: Henri Parson Institution: Eastern Virginia Medical School Email: parsonhk@evms.edu Address: 825 Fairfax Ave, Norfolk, VA 23507, USA Article details: Clinical Trial Registry (ClinicalTrials.gov NCT00931879) Journal of publication: Clinical Trial Registry (ClinicalTrials.gov) Language: English Publication status: Clinical trial registry entry Year of publication: 2009

Risk of bias table

Bias	Authors' judgement	Support for judgement
Random sequence generation (selection bias)	Unclear risk	Judgement Comment: Not reported how list was generated. Trial described as "randomised" but with no further details.
Allocation concealment (selection bias)	Unclear risk	Judgement Comment: Not reported how allocation administered. Trial described as "randomised with no further details.

Blinding of participants and personnel (performance bias)	Low risk	Judgement Comment: "Masking: Triple (Participant, Care Provider, Investigator)". Clearly stated that participants and personnel not aware of which treatment received.
Blinding of outcome assessment (detection bias)	Low risk	Judgement Comment: States "Masking: Triple (Participant, Care Provider, Investigator)".
Incomplete outcome data (attrition bias)	Low risk	Judgement Comment: Missing data less than 20% (i.e. more than 80% follow-up) and equal follow-up in both groups and no obvious reason why loss to follow-up should be related to outcome.
Selective reporting (reporting bias)	Low risk	Judgement Comment: All outcomes in protocol and/or trials registry entry are reported.
Other bias	High risk	Judgement Comment: Study sponsored by pharmaceutical company.

Ochi et al. (2017)^{S13}

Methods	Study design: Randomized controlled trial Study grouping: Parallel group Exclusions after randomisation: No subject dropped out during the study period. How missing data were handled: Not reported Losses to follow-up: No subject dropped out during the study period. Pre-specified primary outcome measure(s): Not explicitly reported in paper. Clinical trial registry reports: 1. After ingestion of tested materials for 8 weeks, subjects perform eccentric contraction (ECC) with elbow flexors. During ECC, 1) electromyogram 2) isotonic contraction torque 2. After ingestion of tested materials for 8 weeks, subjects perform eccentric contraction (ECC) with elbow flexors. Before and after ECC, 1) ultrasonic imaging analysis for elbow flexors 2) reactivity of electrical micro-stimulation 3) maximal isometric contraction torque 4) range of motion 5) muscle soreness measured as a visual analog scale 6) muscle soreness measured by algometer Pre-specified secondary outcome measure(s): Not explicitly reported in the paper. Clinical trial registry reports the following (although not all of these outcomes are described in the paper): 1. After ingestion of tested materials for 8 weeks, subjects perform eccentric contraction (ECC) with elbow flexors. During ECC, analyzing ultrasonic imaging for elbow flexors. 2. After ingestion of tested materials for 8 weeks, subjects perform eccentric contraction (ECC) with elbow flexors. Before and after ECC, testing blood makers (creatine kinase, myoglobin, IL-6, TNF-alpha). Sample size calculation reported: Yes Compliance measures: Compliance to intake was assessed by daily record and a pill count at the end of the study. To help the reliability of the pill count, subjects were given an excess number of pills and asked to return any remaining pills at the end of the study.
Participants	Baseline Characteristics Omega-3 PUFA oral supplements - Age (mean $\pm$ SD): 20.7 $\pm$ 0.7 years - Gender/Sex (number of females/number of males): 0/10 - Underlying conditions: Normal participants undergoing ECC - Peripheral neuropathy diagnostic criteria: Not applicable.

	• <i>Number of participants:</i> 10 Control • <i>Age (mean ± SD):</i> 21.3 ± 0.9 years • <i>Gender/Sex (number of females/number of males):</i> 0/11 • <i>Underlying conditions:</i> Normal participants undergoing ECC • <i>Peripheral neuropathy diagnostic criteria:</i> Not applicable. • <i>Number of participants:</i> 11 Overall • <i>Age (mean ± SD):</i> Not reported • <i>Gender/Sex (number of females/number of males):</i> 0/21 • <i>Underlying conditions:</i> Normal participants undergoing ECC • <i>Peripheral neuropathy diagnostic criteria:</i> Not applicable. • <i>Number of participants:</i> 21 Inclusion criteria: Healthy male subjects, age >20 years. Exclusion criteria: regular resistance training; no exercise; serious liver, kidney, heart, respiratory, endocrine or metabolic diseases; scheduled for surgery during this study; administration of regular medication; food allergies to fish/ participation of other clinical trial during this study; restriction of exercise; any reason in the opinion of the lead principal investigator not to take part in this study. Pre-treatment inter-group differences: There was no significant difference between groups for age, weight and body mass index. Location of peripheral nerve assessed: Non-dominant arm Unit of analysis: Per participant
Interventions	Intervention Characteristics Omega-3 PUFA oral supplements • <i>Type (DHA/EPA/ALA):</i> DHA + EPA • <i>Dose DHA/EPA/ALA (milligrams/day):</i> 260mg/day DHA + 600mg/day EPA • <i>Duration of treatment (days):</i> 62 days • <i>Co-interventions:</i> None • <i>Brand name:</i> Nippon Suisan Kaisha Ltd., Tokyo, Japan Control • <i>Type:</i> Corn oil • <i>Dose (milligrams/day):</i> 2400mg/day (corn oil) • <i>Duration of treatment (days):</i> 62 days • <i>Co-interventions:</i> None • <i>Brand name:</i> Nippon Suisan Kaisha Ltd., Tokyo, Japan
Outcomes	Eccentric contractions, maximal voluntary isometric contraction torque, range of motion of the elbow joint, upper arm circumference, ultrasonography of the upper arm, muscle soreness, M-wave latency,
Identification	Sponsorship source: Funding sources: - Present? Yes - Actual source: This study was supported by Nippon Suisan Kaisha ltd. However, the sponsor was not involved in data collection or data entry and there were no restrictions on publication. Conflict (or declaration) of interest statement: - Present? Yes - Nature of conflict of interest: The authors declare that they have no competing interests. Country: Not reported Setting: Not reported Comments: Date study conducted: July 2015 to January 2016 (data analysis concluded). Trial registration number: UMIN000018285.

	Contacting study investigators: trial authors not contacted; no additional information provided for review Author's name: Eisuke Ochi Institution: Hosei University Email: ochi@hosei.ac.jp Address: Faculty of Bioscience and Applied Chemistry, Hosei University, 3-7-2, Kajino, Koganei 184-8584, Japan Article details: Full text Journal of publication: Journal of the International Society of Sports Nutrition Language: English Publication status: Published Year of publication: 2017

Risk of bias table

Bias	Authors' judgement	Support for judgement
Random sequence generation (selection bias)	Low risk	Quote: "The subjects were randomly assigned to two groups using a table of random numbers in such a manner as to minimize the inter-group differences in age, body fat, body mass index." Judgement Comment: Computer generated list, random table, other method of generating random list.
Allocation concealment (selection bias)	Unclear risk	Quote: "The sequence allocation concealment and blinding to subjects and researchers were maintained throughout this period." Judgement Comment: Not reported how allocation administered. Trial described as "randomised" but with no further details.
Blinding of participants and personnel (performance bias)	Low risk	Quote: "The sequence allocation concealment and blinding to subjects and researchers were maintained throughout this period." Judgement Comment: Clearly stated that participants and personnel not aware of which treatment received.
Blinding of outcome assessment (detection bias)	Unclear risk	Quote: "double-blind, placebo-controlled, parallel-group trial design." Judgement Comment: Trial is described as "double-blind", and also states that "researchers" were blinded, however it is unclear whether the researchers were also the outcome assessors.
Incomplete outcome data (attrition bias)	Low risk	Quote: "No subject dropped out during the study period." Judgement Comment: No losses to follow-up reported.
Selective reporting (reporting bias)	High risk	Judgement Comment: Outcomes in protocol and/or trials registry entry selectively reported (the following outcomes, listed in the clinical trial registry, were not reported in the manuscript: electromyogram, reactivity of electrical micro-stimulation, muscle soreness measured by algometer, blood makers (creatinine kinase, myoglobin, IL-6, TNF-alpha)).
Other bias	High risk	Quote: "Consent for publication Not applicable." Quote: "and Kouzaki for helpful support. Funding This study was supported by Nippon Suisan Kaisha Ltd. However, the sponsor was not involved in data collection or data entry and there were no restrictions on publication."

		Judgement Comment: Industry funded. However authors declare that sponsors are not involved in data collection, entry and no publication restrictions are involved.

Palmer *et al.* (2014)^{S14}

Methods	Study design: Randomized controlled trial Study grouping: Parallel group Exclusions after randomisation: Not reported. How missing data were handled: Not reported. Losses to follow-up: Not reported. Pre-specified primary outcome measure(s): Not reported. Pre-specified secondary outcome measure(s): Not explicitly reported. Measured: QTc duration, Erythrocyte palmitoleic acid; insulin resistance; myocardial diastolic function; vibration perception threshold. Sample size calculation reported: Not reported. Compliance measures: Not reported.
Participants	Baseline Characteristics Omega-3 PUFA oral supplements • <i>Age (mean ± SD):</i> Not reported. • <i>Gender/Sex (number of females/number of males):</i> Not reported. • <i>Underlying conditions:</i> Non-alcoholic fatty liver disease • <i>Peripheral neuropathy diagnostic criteria:</i> Not reported. • <i>Number of participants:</i> Not reported. Control • <i>Age (mean ± SD):</i> Not reported. • <i>Gender/Sex (number of females/number of males):</i> Not reported. • <i>Underlying conditions:</i> Non-alcoholic fatty liver disease • <i>Peripheral neuropathy diagnostic criteria:</i> Not reported. • <i>Number of participants:</i> Not reported. Overall • <i>Age (mean ± SD):</i> 52 ± 10.6 years • <i>Gender/Sex (number of females/number of males):</i> Not reported. • <i>Underlying conditions:</i> Non-alcoholic fatty liver disease • <i>Peripheral neuropathy diagnostic criteria:</i> Not reported. • <i>Number of participants:</i> 86 Inclusion criteria: Not reported. Exclusion criteria: Not reported. Pre-treatment inter-group differences: Not reported. Location of peripheral nerve assessed: Not reported. Unit of analysis: Per participant.
Interventions	Intervention Characteristics Omega-3 PUFA oral supplements • <i>Type (DHA/EPA/ALA):</i> Not explicitly reported • <i>Dose DHA/EPA/ALA (milligrams/day):</i> 4000mg/day purified omega-3 fatty acids • <i>Duration of treatment (days):</i> 15-18 months (450-540 days) • <i>Co-interventions:</i> Not reported. • <i>Brand name:</i> Not reported. Control • <i>Type:</i> Not reported. • <i>Dose (milligrams/day):</i> Not reported. • <i>Duration of treatment (days):</i> 15-18 months (450-540 days) • <i>Co-interventions:</i> Not reported. • <i>Brand name:</i> Not reported.

Outcomes	QTc duration; erythrocyte palmitoleic acid; insulin resistance; myocardial diastolic function; vibration perception threshold.
Identification	Sponsorship source: Funding sources: - Present? No. Conflict (or declaration) of interest statement: - Present? No. Country: Not reported. Setting: Not reported. Comments: Date study conducted: not reported. Trial registration number: not reported. Contacting study investigators: trial authors not contacted; no additional information provided for review. Author's name: CE Palmer Institution: University of Southampton Email: Not reported. Address: Human Development and Health Academic Unit, University of Southampton, Southampton, UK Article details: Conference abstract Journal of publication: Diabetic Medicine Language: English Publication status: Published Year of publication: 2014

Risk of bias table

Bias	Authors' judgement	Support for judgement
Random sequence generation (selection bias)	Unclear risk	Judgement Comment: Not reported how list was generated. Trial described as "randomised" but with no further details.
Allocation concealment (selection bias)	Unclear risk	Judgement Comment: Not reported how allocation administered. Trial described as "randomised" but with no further details.
Blinding of participants and personnel (performance bias)	Unclear risk	Judgement Comment: Described as "double blind" with no information on who was masked.
Blinding of outcome assessment (detection bias)	Unclear risk	Judgement Comment: Described as "double blind" with no information on who was masked.
Incomplete outcome data (attrition bias)	Unclear risk	Judgement Comment: Follow-up not reported.
Selective reporting (reporting bias)	Unclear risk	Judgement Comment: No access to protocol or trials registry entry.
Other bias	Low risk	Judgement Comment: No other apparent sources of bias.

Stiefel et al. (1999)^{S15}

Methods	Study design: Randomized controlled trial Study grouping: Parallel group

	How missing data were handled: Not reported. Exclusions after randomisation: Not reported. Losses to follow-up: Not reported. Sample size calculation reported: Not reported. Pre-specified primary outcome measure(s): Not reported. Pre-specified secondary outcome measure(s): Not explicitly stated. Measured: Levels of blood glucose, serum urea and creatinine; Sodium Transport Kinetics; Lipid Composition of the Erythrocyte Cell Membrane; Neural Conduction Study. Compliance measures: Participants were on a 2,500 kcal diet, containing 30% fat, 15% protein and 55% carbohydrates; during the study period, compliance of the diet (in both groups) and particularly the composition of dietary fats was routinely checked. Compliance of the supplementation (in the supplemented group) was also strictly checked.
Participants	Baseline Characteristics Omega-3 PUFA oral supplements • <i>Age (mean ± SD):</i> Not reported • <i>Gender/Sex (number of females/number of males):</i> Not reported • <i>Underlying conditions:</i> Type 1 diabetes • <i>Peripheral neuropathy diagnostic criteria:</i> Not reported • <i>Number of participants:</i> 8 Control • <i>Age (mean ± SD):</i> Not reported • <i>Gender/Sex (number of females/number of males):</i> Not reported • <i>Underlying conditions:</i> Type 1 diabetes • <i>Peripheral neuropathy diagnostic criteria:</i> Not reported • <i>Number of participants:</i> 10 Overall • <i>Age (mean ± SD):</i> 25.5 ± 6.4 years • <i>Gender/Sex (number of females/number of males):</i> 10/8 • <i>Underlying conditions:</i> Type-1 diabetes • <i>Peripheral neuropathy diagnostic criteria:</i> Not reported • <i>Number of participants:</i> 18 Inclusion criteria: Type-1 (insulin-dependent) diabetes, less than 35 years of age and with a BMI under 25 kg/m². Exclusion criteria: Chronic complications (as determined by routine clinical and eye examination) Pre-treatment inter-group differences: "There were no statistically significant differences in the studied parameter between groups at baseline (see values in tables 1–3)." Location of peripheral nerve assessed: Median nerve (first finger, wrist, elbow); sural nerve (calf, ankle); foot (extensor digitorum brevis); abductor pollicis brevis (hand); peroneal nerve (ankle, knee) Unit of analysis: Per participant
Interventions	Intervention Characteristics Omega-3 PUFA oral supplements • <i>Type (DHA/EPA/ALA):</i> DHA + EPA • <i>Dose DHA/EPA/ALA (milligrams/day):</i> 330mg/day DHA + 630mg/day EPA • <i>Duration of treatment (days):</i> 90 days • <i>Co-interventions:</i> Standard diet (2,500 kcal, containing 30% fat (the approximate composition of dietary fats was in% of polyunsaturated, monounsaturated and saturated, respectively, 10, 10 and 10), 15% protein and 55% carbohydrates. The ratio of n-6:n-3 fatty acids in the diet was 12:1) • <i>Brand name:</i> Not reported

	Control • <i>Type</i>: "Usual diet" • <i>Dose (milligrams/day)</i>: No treatment • <i>Duration of treatment (days)</i>: 90 days • <i>Co-interventions</i>: Standard diet (2,500 kcal, containing 30% fat (the approximate composition of dietary fats was in% of polyunsaturated, monounsaturated and saturated, respectively, 10, 10 and 10), 15% protein and 55% carbohydrates. The ratio of n-6:n-3 fatty acids in the diet was 12:1) • <i>Brand name</i>: Not applicable
Outcomes	Levels of blood glucose, serum urea and creatinine; Sodium Transport Kinetics; Lipid Composition of the Erythrocyte Cell Membrane; Neural Conduction Study (sensory conduction amplitudes and velocities in the sural and median nerves; motor conduction amplitude and velocities in the median and peroneal nerves).
Identification	Sponsorship source: Funding sources: - Present? No. - Actual source: Not reported. Conflict (or declaration) of interest statement: - Present? No. - Nature of conflict of interest: Not reported. Country: Not reported Setting: Not reported Comments: Date study conducted: Not reported. Trial registration number: Not reported. Contacting study investigators: Trial authors not contacted; no additional information used for review. Author's name: Valentina Ruiz-Gutierrez Institution: Instituto de las Grasas y sus Derivados (CSIC) Email: valruiz@cica.es Address: Instituto de las Grasas y sus Derivados (CSIC) Avda Padre Garcia Tejero, 4E-41012 Seville, Spain Article details: Full text Year of publication: 1999 Journal of publication: Annals of Nutrition and Metabolism Language: English Publication status: Published

Risk of bias table

Bias	Authors' judgement	Support for judgement
Random sequence generation (selection bias)	Unclear risk	Quote: "In the first visit, the patients were randomly assigned to the supplemented or non supplemented group." Judgement Comment: Not reported how list was generated. Trial is described as "randomised" but with no further details.
Allocation concealment (selection bias)	Unclear risk	Quote: "the first visit, the patients were randomly assigned to the supplemented or non supplemented group." Judgement Comment: Not reported how allocation administered. Trial may be described as "randomised" but with no further details.
Blinding of participants and personnel	High risk	Judgement Comment: Participants not masked (as either assigned to 'usual diet' or 'supplements'). No mention of masking in relation to personnel.

(performance bias)		
Blinding of outcome assessment (detection bias)	High risk	Judgement Comment: No information on masking. We assume that in absence of reporting on this outcome assessors were not masked.
Incomplete outcome data (attrition bias)	Low risk	Judgement Comment: Missing data not explicitly states but appears to be no losses to follow up based upon data presented in tables 2 and 3.
Selective reporting (reporting bias)	Unclear risk	Judgement Comment: No access to protocol or trials registry entry.
Other bias	Low risk	Judgement Comment: No other apparent sources of bias.

Table S3: Characteristics of excluded studies

Study identification	Reason for exclusion
Blommers et al. (2002) ^{S16}	Wrong outcomes (peripheral nerve structure/function not measured)
Carvalho et al (2016) ^{S17}	Wrong intervention (not omega-3 PUFAs)
ChiCTR-IOR-15006058 (2015) ^{S18}	Wrong outcomes (peripheral nerve structure/function not measured)
ChiCTR-IOR-16008351 (2016) ^{S19}	Wrong outcomes (peripheral nerve structure/function not measured)
Chous et al. (2016) ^{S20}	Wrong intervention (co-interventions not administered in the same dose and frequency)
Clark et al. (2018) ^{S21}	Wrong study design (not a randomised controlled trial)
Esfahani et al. (2014) ^{S22}	Duplicate of published study that is already included (conference abstract)
Florian et al. (2009) ^{S23}	Wrong intervention (not omega-3 PUFAs)
Florian et al. (2010) ^{S24}	Wrong intervention (not omega-3 PUFAs)
Galarraga et al. (2008) ^{S25}	Wrong outcomes (peripheral nerve structure/function not measured)
Ghoreishi et al. (2015) ^{S26}	Duplicate of published study that is already included (conference abstract)
Hershman et al. (2015) ^{S27}	Wrong outcomes (peripheral nerve structure/function not measured)
Kremer et al. (1990) ^{S28}	Wrong outcomes (peripheral nerve structure/function not measured)
Long et al. (2011) ^{S29}	Wrong study design (not a randomised controlled trial)
Maroun et al. (2015) ^{S30}	Wrong intervention (co-interventions not administered in the same dose and frequency)
Monahan et al. (2007) ^{S31}	Wrong intervention (not omega-3 PUFAs)
NCT01049295 (2011) ^{S32}	Duplicate of published study that is already included (clinical trials registry entry)
NCT01821833 (2013) ^{S33}	Wrong outcomes (peripheral nerve structure/function not measured)
NCT02034266 (2017) ^{S34}	Wrong study design (not a randomised controlled trial)
NCT02294149 (2014) ^{S35}	Wrong intervention (co-interventions not administered in the same dose and frequency)
NCT02795572 (2016) ^{S36}	Wrong study design (not a randomised controlled trial)
Nikishin et al. (2010) ^{S37}	Wrong study design (not a randomised controlled trial)
Okuda et al. (1996) ^{S38}	Wrong study design (not a randomised controlled trial)

Remans et al. (2004) ^{S39}	Wrong outcomes (peripheral nerve structure/function not measured)
Sanchez Lara et al. (2012) ^{S40}	Duplicate of published study that is already included (conference abstract)
Sanchez Lara et al. (2014) ^{S41}	Wrong outcomes (not a direct measure of nerve function)
Sergienko et al. (2013) ^{S42}	Wrong study design (not a randomised controlled trial)
Serhiyenko et al. (2015) ^{S43}	Wrong study design (not a randomised controlled trial)
Serhiyenko et al. (2015a) ^{S44}	Wrong outcomes (not a direct measure of nerve function)
Williams et al. (1986) ^{S45}	Wrong study design (not a randomised controlled trial)

Table S4: Adverse events

Study (year)	Number of participants in each group at baseline	Adverse events	Details of adverse events	Serious adverse events	Details of serious adverse events
Anoushirvani et al. (2018) ^{S1}	Omega-3 group: 21 Placebo group: 21	Not reported.	-	Not reported.	-
Carter et al. (2012) ^{S2}	Omega-3 group: 34 Placebo group: 33	Not reported.	-	Not reported.	-
Carter et al. (2013) ^{S3}	Omega-3 group: 34 Placebo group: 33	Not reported.	-	Not reported.	-
Chinner et al. (2017) ^{S4}	Omega-3 group: 8 Placebo group: 4	Not reported.	-	Not reported.	-
Clough et al. (2016) ^{S5,a}	Omega-3 group: 44 Placebo group: 46	Not reported.	-	Omega-3 group: 0 Placebo group: 0	"There were no important adverse effects or side effects reported"
Esfahani et al. (2016) ^{S6}	Omega-3 group: 36 Placebo group: 35	Not reported.	-	Not reported.	-
Fontani et al. (2010) ^{S7}	Omega-3 group: 23 Placebo group ^b : 23	Omega-3 group: 0 Placebo group: 0	"No adverse events either related or unrelated to the fish and sunflower oil supplementation were reported during the study."	Omega-3 group: 0 Placebo group: 0	"No adverse events either related or unrelated to the fish and sunflower oil supplementation were reported during the study."
Ghoreishi et al. (2012) ^{S8}	Omega-3 group: 35 Placebo group: 34	Omega-3 group: 3 Placebo group: 1	Discontinued intervention because of critical conditions:	Not reported.	-

			Omega-3 group: 3 Placebo group: 1		
McCormick et al. (2015) ^{S_{9,a}}	Not reported (total 101)	Not reported.	-	Not reported.	-
McCormick et al. (2015a) ^{10,a}	Omega-3 group: 51 Placebo group: 49	Not reported.	-	Omega-3 group: 0 Placebo group: 0	"There were no important adverse (or unexpected) effects or side effects."
Monahan et al. (2004) ^{S₁₁}	Omega-3 group: 9 Placebo group: 9	Not reported.	-	Not reported.	-
NCT00931879 (2009) ^{S₁₂}	Omega-3 group: 19 Placebo group: 19	Omega-3 group: 8 Placebo group: 7	<u>Hyperglycaemia</u> Omega-3 group: 0 Placebo group: 1 <u>Eye disorders:</u> (i) Eye discharge Omega-3 group: 1 Placebo group: 0 (ii) Dry/achy eyes Omega-3 group: 0 Placebo group: 1 <u>Gastrointestinal disorders:</u> (i) Severe-moderate diarrhoea Omega-3 group: 2 Placebo group: 0 (ii) Dehydration/severe vomiting/diarrhoea Omega-3 group: 2 Placebo group: 0 (iii) Abdominal pain Omega-3 group: 0 Placebo group: 1 (iv) Bacterial infection Omega-3 group: 0 Placebo group: 1 <u>Personal injury:</u> Omega-3 group: 0 Placebo group: 1	Omega-3 group: 2 Placebo group: 4	<u>Cardiac disorders</u> (i) Hospitalisation due to low blood pressure and fainting Omega-3 group: 0 Placebo group: 1 (ii) Overnight stay in emergency room for observation after experiencing chest pain Omega-3 group: 0 Placebo group: 1 <u>Head trauma due to accidental fall</u> Omega-3 group: 0 Placebo group: 1 <u>Atypical chest pain</u> Omega-3 group: 1 Placebo group: 0 <u>Surgical and medical procedures</u> (i) Planned surgery to treat achalasia/oesophageal motility disorder Omega-3 group: 0 Placebo group: 1 (ii) Emergency surgery to relieve bile duct obstruction Omega-3 group: 1 Placebo group: 0

			<u>Musculoskeletal and connective tissue disorders</u> (i) Upper limb weakness Omega-3 group: 0 Placebo group: 1 (ii) Back pain Omega-3 group: 0 Placebo group: 1 <u>Nervous system disorders</u> (i) Fainting/dizziness: Omega-3 group: 4 Placebo group: 0 (ii) Bell's palsy: Omega-3 group: 0 Placebo group: 1 <u>Respiratory, thoracic and mediastinal disorders</u> (i) Upper respiratory infection Omega-3 group: 1 Placebo group: 0 (ii) Bronchitis Omega-3 group: 0 Placebo group: 1 <u>Cranial/facial lesions</u> Omega-3 group: 1 Placebo group: 0 <u>Bladder suspension with sling</u> Omega-3 group: 0 Placebo group: 1		
Ochi et al. (2017) ^{Si3}	Omega-3 group: 10 Placebo group: 11	Not reported.	-	Not reported.	-
Palmer et al. (2014) ^{Si4, a}	Not reported (total number of participants: 86)	Not reported.	-	Not reported.	-

Stiefel et al. (1999) ^{S15}	Omega-3 group: 8 Placebo group: 10	Not reported.	-	Not reported.	-

^aAs these studies are derived from the same trial (the WELCOME study), data may derive from the same cohort of participants.

^bFive participants were lost to follow-up in this group.

Appendix S1: Search strategies

MEDLINE (OviD) search strategy

Ovid MEDLINE(R) Epub Ahead of Print, In-Process & Other Non-Indexed Citations, Ovid MEDLINE(R) Daily and Ovid MEDLINE(R) 1946 to February 2019

1. randomized controlled trial.pt.
2. controlled clinical trial.pt.
3. (randomised OR randomized).ab,ti.
4. placebo.ab.
5. drug therapy.fs.
6. randomly.ab.
7. trial.ab.
8. groups.ab.
9. 1 OR 2 OR 3 OR 4 OR 5 OR 6 OR 7 OR 8
10. exp animals/ not humans.sh.
11. 9 not 10
12. exp Diabetic Neuropathies/
13. exp Diabetic Foot/
14. exp Peripheral Nervous System Diseases/
15. neuropath*.tw.
16. exp Neuralgia/
17. neuralgia.tw.
18. exp Charcot-Marie-Tooth Disease/
19. exp Facial Nerve Diseases/
20. exp Nerve Regeneration/
21. exp Nerve Degeneration/
22. nerve*.tw.
23. exp Hyperalgesia/
24. allodynia.tw.
25. exp Sensation/
26. sensation.tw.
27. exp Sensation Disorders/
28. an?esthesiomet*.tw.
29. esthesiomet*.tw.
30. aesthesiomet*.tw.
31. (guillain and barre).tw.
32. (polyradiculoneuritis OR polyradiculoneuropath* OR polyradiculopath*).tw.
33. (inflammatory adj3 (polyneuropath* OR mononeuropath* OR polyradiculoneuropath*)).tw.
34. (amyloid adj3 neuropath*).tw.
35. (motor and sensory and neuropath* and hereditary).mp.
36. (hereditary and sensory and autonomic and neuropath*).mp.
37. (heredit* adj6 neuropath*).mp.
38. or/12-37
39. (omega-3 OR omega 3).tw.
40. (PUFA* OR LCPUFA* OR polyunsaturated OR poly-unsaturated).tw.
41. eicosapentaenoic.tw.
42. EPA.tw.
43. docosahexaenoic.tw.
44. DHA.tw.
45. exp Alpha-Linolenic Acid/
46. alpha-linolenic.tw.
47. Fatty acids/
48. Fatty Acids, Unsaturated/
49. Fatty Acids, Essential/
50. exp Fatty Acids, Omega-3/

51. (Fatty adj3 acid*).tw.
52. or/39-51
53. 11 and 38 and 52

EMBASE (OviD) search strategy

Embase Classic + Embase 1947 to February 2019

1. exp Randomized Controlled Trial/
2. Exp controlled clinical trial
3. (randomised OR randomized).ab,ti.
4. placebo.ab.
5. drug therapy.fs.
6. randomly.ab.
7. trial.ab.
8. groups.ab.
9. 1 OR 2 OR 3 OR 4 OR 5 OR 6 OR 7 OR 8
10. exp animals/ not humans.sh.
11. 9 not 10
12. exp neuropathy/
13. exp neuralgia/
14. exp nerve regeneration/
15. exp peripheral nervous system/
16. neuropath*.tw.
17. neuralgia.tw.
18. nerve*
19. an?esthesiomet*.tw.
20. esthesiomet*.tw.
21. aesthesiomet*.tw.
22. sensation*.tw.
23. exp sensation/
24. exp hyperalgesia/
25. exp allodynia/
26. hyperalgesia.tw.
27. allodynia.tw.
28. (guillain and barre).tw.
29. (polyradiculoneuritis OR polyradiculoneuropath* OR polyradiculopath*).tw.
30. (inflammatory adj3 (polyneuropath* OR mononeuropath* OR polyradiculoneuropath*)).tw.
31. (amyloid adj3 neuropath*).tw.
32. (motor and sensory and neuropath* and hereditary).mp.
33. (hereditary and sensory and autonomic and neuropath*).mp.
34. (heredit* adj6 neuropath*).mp.
35. or/12-34
36. exp unsaturated fatty acid/
37. exp fish oil/
38. (PUFA* OR LCPUFA* OR polyunsaturated OR poly-unsaturated).tw.
39. (omega-3 OR omega 3).tw.
40. eicosapentaenoic.tw.
41. docosahexaenoic.tw.
42. alpha-linolenic.tw.
43. (Fatty adj3 acid*).tw.
44. EPA.tw.
45. DHA.tw.
46. or/36-45
47. 11 and 35 and 46

Cochrane Central Register of controlled Trials (CENTRAL) search strategy

1. MeSH descriptor: [Diabetic Neuropathies] explode all trees
2. MeSH descriptor: [Diabetic Foot] explode all trees
3. MeSH descriptor: [Peripheral Nervous System Diseases] explode all trees
4. MeSH descriptor: [Neuralgia] explode all trees
5. MeSH descriptor: [Charcot-Marie-Tooth Disease] explode all trees
6. MeSH descriptor: [Facial Nerve Diseases] explode all trees
7. MeSH descriptor: [Nerve Regeneration] explode all trees
8. MeSH descriptor: [Nerve Degeneration] explode all trees
9. (neuropath* OR Neuralgia* OR nerve*):ti,ab,kw
10. MeSH descriptor: [Hyperalgesia] explode all trees
11. MeSH descriptor: [Sensation] explode all trees
12. MeSH descriptor: [Sensation Disorders] explode all trees
13. (sensation* OR hyperalgesia OR allodynia):ti,ab,kw
14. (an?esthesiomet* OR esthesiomet* OR aesthesiomet*):ti,ab,kw
15. #1 OR #2 OR #3 OR #4 OR #5 OR #6 OR #7 OR #8 OR #9 OR #10 OR #11 OR #12 OR #13 OR #14
16. (omega-3 OR omega 3 OR ω-3 OR ω3 OR "ω 3"):ti,ab,kw
17. (PUFA* OR LCPUFA* OR polyunsaturated OR poly-unsaturated):ti,ab,kw
18. MeSH descriptor: [Eicosapentaenoic Acid] explode all trees
19. (Eicosapentaenoic OR EPA):ti,ab,kw
20. MeSH descriptor: [Docosahexaenoic Acids] explode all trees
21. (Docosahexaenoic OR DHA):ti,ab,kw
22. MeSH descriptor: [Alpha-Linolenic Acid] explode all trees
23. (Alpha-Linolenic):ti,ab,kw
24. MeSH descriptor: [Fatty acids] this term only
25. MeSH descriptor: [Fatty Acids, Unsaturated] this term only
26. MeSH descriptor: [Fatty Acids, Essential] this term only
27. MeSH descriptor: [Fatty Acids, Omega-3] explode all trees
28. (Fatty near/3 acid*):ti,ab,kw
29. #16 OR #17 OR #18 OR #19 OR #20 OR #21 OR #22 OR #23 OR #24 OR #25 OR #26 OR #27 OR #28
30. #15 and #29

US National Institutes of Health Clinical Trials Registry (ClinicalTrials.gov)

search strategy

Condition = neuropathy OR nerve OR sensation

Intervention = omega-3 OR "omega 3" OR polyunsaturated OR PUFA OR "docosahexaenoic" OR DHA OR "eicosapentaenoic" OR EPA OR "fish oil" OR "fish-oil"

World Health Organisation International Clinical Trials Registry Platform (WHO ICTRP) search strategy (www.who.int/ictrp/)

Condition = nerve disorders OR nerve disease OR neuropathy OR nerve OR sensation

Intervention = omega-3 OR omega 3 OR polyunsaturated OR PUFA OR docosahexaenoic OR eicosapentaenoic OR fish oil

Appendix S2: PRISMA checklist^{S46}

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	3-5
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	5
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	5
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	5
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	6
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	Appendix S1
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	6
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	6
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	6; Table 1
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	6
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	7-8

Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	8
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	7-8
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	7-8
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	Figure 1
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	Table S2
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	10, Figure 2, Figure S2
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	11-16; Figures 3-5; Figures S3-S5
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	Figures 3-5; Figures S3-S5
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	Figure 2; Figure S2
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	N/A (see page 9)
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	17-22
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	22-25
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	26-27
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	27

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Appendix F. Chapter 7: Supplementary materials [1 of 3]

nPROOFS₁ Clinical Trial Protocol

Protocol for the randomised controlled clinical trial:

“Investigating the neuroPRotective effect of Oral Omega-3 Fatty acid
Supplementation in type 1 diabetes (nPROOFS₁): a randomised, placebo-
controlled trial”

PROTOCOL

“A randomised controlled trial to evaluate the effects of omega-3 fatty acid supplementation on peripheral nerve health in type-1 diabetes

Protocol Number: nPROOFS-1-v1.2

Phase: IIb

Sponsor: The University of Melbourne

Ethics approval: St Vincent's Hospital, Melbourne

Principal investigator: Dr Laura Downie
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Study personnel:

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Dr Leslie Roberts, Consultant Neurologist and Director of the Neurophysiology
Department of St Vincent's Hospital, and Honorary Senior Fellow - Faculty Medicine, Dentistry and Health Sciences - University of Melbourne

Dr Jordan Kamel, Consultant Neurologist at St Vincent's Hospital, Melbourne.

- Associate investigators

A/Prof Jennifer Craig, Department of Ophthalmology, University of Auckland, New Zealand (co-supervisor of Ms Alexis Ceecee Zhang)

1. Study overview

1.1. Administrative information

1.1.1. Trial registration

This study has been prospectively registered on the Australia New Zealand Clinical Trials Registry (ANZCTR) <http://www.anzctr.org.au/> (ACTRN12618000705280).

1.1.2. Sponsor

This is an investigator-initiated study, sponsored by The University of Melbourne.

1.1.3. Funding

This study will be funded by the Downie research laboratory funds from the Department of Optometry and Vision Science, The University of Melbourne. Support for the project includes a Melbourne Neuroscience Interdisciplinary grant and a Rebecca L Cooper Medical Foundation research grant.

1.1.4. Expected duration of study

This study is expected to run for a total of 2.5 years from recruitment to the completion of the last participant's last visit. The length of treatment for an individual participant, including all follow-up visits, is six months.

1.1.5. Contribution to study

Name	Summary of contribution
Dr Laura Downie Principal Investigator Dept of Optometry & Vision Sciences University of Melbourne, Parkville VIC	Conceived idea for clinical trial; led protocol design and drafting, including development of methodologies for ocular testing; providing funding for the study.
Ms Alexis Ceecee Zhang PhD Student Investigator Dept of Optometry & Vision Sciences University of Melbourne, Parkville VIC	Co-investigator, provided input into methodologies for ocular testing, and design and drafting of trial protocol.
Prof Richard MacIsaac Director, Department of Endocrinology & Diabetes St Vincent's Hospital, Melbourne and	Co-investigator, contributed to protocol, in particular assessments relating to diabetes status.
Dr Leslie Roberts Director, Department of Neurophysiology St Vincent's Hospital, Melbourne	Co-investigator, contributed to protocol, in particular assessments relating to neurophysiology testing.
Dr Jordan Kamel Neurologist St Vincent's Hospital, Melbourne	Co-investigator, contributed to protocol, in particular assessments relating to neurophysiology testing.

1.2. Background and rationale

Diabetic neuropathy is the most common complication of diabetes mellitus, affecting >50% of individuals with the condition (Dyck & Dyck, 1999). The Diabetes Control and Complications Trial demonstrated that intensive blood glucose control could reduce the incidence of neuropathy and potentially slow progressive nerve damage (Albers *et al.*, 2010; Nathan *et al.*, 1993); however, there are currently no clinical therapies for attenuating the onset or progression of neuropathy.

Omega-3 polyunsaturated fatty acids (PUFAs) are essential fatty acids that cannot be synthesised by the human body. Increased consumption of omega-3 PUFAs

endogenously generates anti-inflammatory and pre-resolving mediators that have been shown to be beneficial in a range of inflammatory conditions (Simopoulos, 2002). Once ingested, omega-3 PUFAs are incorporated into cell membranes, and play a role in cellular signalling and gene expression (Calder & Grimble, 2002), and maintaining nerve integrity. In animal models of diabetes, omega-3 supplementation can attenuate adverse changes in nerve structure and function (Gerbi *et al.*, 1999; Yee *et al.*, 2010). Derivatives of omega-3 fatty acids have also been shown to restore corneal nerve integrity and restore damage after corneal injury in rabbits (Cortina *et al.*, 2010). However, there has been limited investigation in humans, particularly as related to diabetic neuropathy.

There is some clinical evidence to suggest that omega-3 oral supplements may be useful in treating peripheral neuropathy in diseases other than diabetes. For example, omega-3 supplementation was shown to reduce the severity of symptoms and improve total neuropathy score in paclitaxel-induced peripheral neuropathy (Ghoreishi *et al.*, 2012). Our laboratory has recently shown that omega-3 fatty acids promote corneal nerve regeneration in dry eye disease, which is characterised by a mild degree of corneal neuropathy (Chinnery *et al.*, 2017). Furthermore, a recent open-label, proof of concept study involving a single-arm of participants with type-1 diabetes, treated for 12 months with omega-3 PUFAs, reported a 20% increase in small nerve fibres using corneal nerves as a primary outcome measure (Lewis *et al.*, 2017). Together, these promising findings provide the rationale for the present proposal, being a randomised, double-masked, placebo-controlled clinical trial to assess the effects of a six-month period of supplementation with omega-3 PUFAs on small nerve fibre structure and function, measured both in the eye and the extremities of the body, in individuals with type-1 diabetes.

JUSTIFICATION OF ASSESSMENT APPROACHES: Ocular manifestations of diabetes in the anterior eye include changes in tear film integrity, impaired wound healing and diabetic cataract, and in the posterior eye, diabetic retinopathy (Negi & Vernon, 2003). Studies suggest that there is an association between peripheral neuropathy and ocular surface complications in diabetes, especially in relation to reduced corneal nerve density and sensitivity, where both reductions in corneal nerve structure and function have been shown to correlate with the severity of diabetic peripheral neuropathy (Malik *et al.*, 2003; Pritchard *et al.*, 2010). Damage to small nerve fibres, including unmyelinated C-fibres and thinly myelinated A δ -fibres, is recognised to precede the involvement of larger nerve fibres (Breiner *et al.*, 2014; Greene *et al.*, 1999).

Corneal *in vivo* confocal microscopy (IVCM) is the only technique that allows non-invasive imaging of the peripheral nerves in the body. It has been proposed to detect the earliest stages of change that precede the development of large fibre neuropathy in diabetes (Pritchard *et al.*, 2015), and to serve as a marker to monitor changes in neuropathy (Tavakoli *et al.*, 2013). In this study, the structure of the corneal nerves will be visualised using IVCM; we will quantify changes in corneal nerve parameters, as a primary efficacy outcome measure, between baseline and the primary endpoint (six months).

Other ocular parameters to be examined include: tear film integrity, examined using standard clinical techniques (e.g., tear osmolarity, ocular surface staining, inflammatory tear markers) and corneal nerve function (i.e., using corneal aesthesiometry). Routine dilated fundus examination will be performed, being a standard clinical procedure in

individuals with diabetes, to assess for any changes of retinal health throughout the study.

In addition to the eye health investigations, we will assess whether there are changes to small nerve fibre function in the peripheral body (ie., feet) using electrophysiology testing methods (i.e., cutaneous silent periods, which assesses thinly myelinated A δ -fibres) and autonomic testing (i.e., quantitative sudomotor axonal reflex testing, which assesses unmyelinated post-ganglionic sudomotor C-fibres) and quantitative sensory testing, to assess thermal (cold: A δ -fibres and warm: C-fibres) and vibration (medium myelinated fibres: A β) thresholds. Routine nerve conduction studies will also be performed to assess any large nerve fibre damage, and nerve excitability testing, using similar techniques, will detect any changes in these nerve fibres that may precede large nerve fibre damage.

JUSTIFICATION OF COMPARATOR: Olive oil is chosen as the comparator for omega-3 interventions, as an inert control. The main constituents of olive oil are omega-9 oleic acid (C18:1), which has shown to have no effect in the inflammatory mediators in the body. Olive oils also matches in caloric intake of the active oral supplementation. Participants of double-masked clinical trials have demonstrated a poor ability to differentiate between active fish oil and inactive olive oil as their given intervention, even if they notice a fishy aftertaste (Damico *et al.*, 2002).

2. Study design and participants

2.1. Study objectives

2.1.1. Primary objective

The aim of this randomised, double-masked, placebo-controlled clinical trial is to evaluate the effects of a six month supplementation period with omega-3 polyunsaturated fatty acid (PUFA) supplements on peripheral nerves in individuals with type-1 diabetes.

2.2. Study design

This study is a single-centre, double-masked, randomised, two-arm, parallel-group interventional study comparing the effects of omega-3 EFA oral supplements with olive oil (placebo) supplements over six months. Participants will be randomised according to a 1:1 allocation ratio to omega-3 PUFA supplements (defined below) or an olive oil (placebo) supplement.

The primary outcome measure is the change in central corneal nerve fibre length (CNFL) from baseline (Day 1); the primary analysis endpoint is Day 180 (six months).

2.3. Study setting

This study will be conducted at the Department of Optometry and Vision Sciences at The University of Melbourne, with recruitment and data collection undertaken both at this site and the Endocrinology and Neurology Departments at St Vincent's Hospital, Melbourne.

2.4. Study population and entry criteria

2.4.1. Number of participants

A total of 50 patients will be randomised over the study duration.

Given a two-arm parallel design, with the pre-specified primary outcome of CNFL (taken as a continuous measure), for 80% power at a confidence level of 95%, using an estimated true difference between intervention arms of 2.9 units, mm/mm² (Chinnery *et al.*, 2017), based on an expected standard deviation of 3.2 units, the required sample size is 21 participants per group. To allow for 15% participant attrition, the sample size within each group will be increased by this amount, giving a need to recruit a total of 25 participants in each interventions arm (n=50 for the trial).

2.4.2. Study population characteristics

Participants will be individuals with type-1 diabetes mellitus, for any length of duration, with or without diagnosed neuropathy.

2.4.3. Inclusion criteria

The following are requirements for entry into the study at Visit 1 (Baseline):

1. Male or female, ≥ 18 years of age
2. MNSI > 2
3. Written informed consent and documentation, in accordance with privacy requirements, obtained prior to performing any study procedures;
4. Distance best-corrected visual acuity of at least 6/12 Snellen equivalent in **each** eye using a standard visual acuity chart;
5. Intraocular pressure (IOP) ≤ 21 mm Hg in both eyes;
[Note: Participants with primary open-angle glaucoma or ocular hypertension (OHT) may be eligible to participate, provided they are on **stable** monotherapy **bilaterally**, with both eyes IOPs controlled and under 21 mmHg at the baseline visit. Any topical IOP-lowering medications must have a start date of ≥ 3 months prior to Visit 1, with the dosage not expected to change during the study.]
6. Ability to understand and follow study instructions, with the intention of completing all required study visits.

2.4.4. Exclusion criteria

The following are criteria for exclusion from participating in the study at Visit 1 (Baseline):

Systemic

1. Any uncontrolled systemic disease, other than sub-optimally controlled diabetes mellitus;
2. Confirmed neuropathy secondary to causes other than diabetes (e.g., alcohol polyneuropathy. Vitamin B-12 deficiency, folate deficiency, chronic renal failure, hypothyroidism, neurotoxic drug use including chemotherapy);
3. Any of the following general medical conditions: bipolar disorder, atrial fibrillation, implanted defibrillator, familial adenomatous polyposis, systemic

immunocompromise;

4. Scheduled or planned systemic surgery over the course of the study;
5. Any known bleeding disorders;
6. Current consumption of a systemic anti-coagulant medication other than aspirin;
7. Females who are currently pregnant or breastfeeding;
8. females of childbearing potential who are planning a pregnancy over the course of the study;

*[Definition: For the purposes of this study, females will be considered of childbearing potential unless they are: naturally postmenopausal or permanently sterilised (i.e., a **full** [not partial] hysterectomy). Natural menopause is defined as the permanent cessation of menstrual periods, determined retrospectively after a woman has experienced 12 months of amenorrhea without any other obvious pathological or physiological cause. If a female participant does become pregnant over the course of the study, the participant will be asked to attend for a 'Study Exit' final visit, and will then be exited from the study with appropriate follow-up **under the direction of the principal investigator**. The **principal investigator** will notify the patient's GP that the patient was being treated with an oral supplement; if necessary, the mask can be broken.]*

9. Inability to sit/lie supine comfortably during the examination procedures for any reason.

Ophthalmic

10. Known allergy to or previous reaction to any ocular agents used in the study (i.e., ocular anaesthetics, sodium fluorescein, lissamine green, ocular mydriatics);
11. Scheduled or planned ocular surgery or procedure over the course of the study;
12. Any history of rigid contact lens wear;
13. Grading of diabetic retinopathy worse than moderate retinopathy according to the simplified ETDRS (Wisconsin) classification (ETDRS Research Group, 1991);
14. Presence of any of the following ocular conditions: active ocular infection or inflammation that in the judgment of the investigator may interfere with the interpretation of the study results;
15. Corneal abnormalities or damage that could disrupt normal corneal nerve morphology, including keratoconus, bullous keratopathy, advanced corneal dystrophies, history of neurotrophic keratopathy including herpes keratitis, severe Sjogren's associated dry eye disease;
16. History of refractive surgery or trauma within the past 12 months;
17. Use of autologous serum eye drops within the past three months, or their anticipated use during the course of the study;
18. Participant has a medical or ocular condition, or is in a situation, which in the principal investigator's opinion, may put the participant at significant risk, may confound the study results, or may interfere significantly with participation in the study.

Interventional

19. Current or previous regular consumption of any omega-3 oral supplements (>3

- times/week) in the three months preceding Visit 1;
20. Current participation in another interventional drug or device study or anticipated entry into such a within 1 month of enrolment;
 21. Known allergy or hypersensitivity to any components of the study supplements;
 22. Cultural or religious beliefs that exclude the consumption of certain or all animal products.

2.4.5. Recruitment methods

Initial contact with potential participants will be made in the following ways:

- i) After the treating clinician at St Vincent's Hospital or the University of Melbourne eye clinic has identified that a patient who may be eligible to participate in the study, the patient will be asked if they give verbal consent to be contacted by the investigators (either by phone/email) to receive further information. The potential participant will then also be provided with a hard copy of the PDCF, and the investigator will contact the potential participant directly to discuss the study.
- ii) The co-investigator (Alexis Ceecee Zhang) will have information available about the study for patients who are waiting for clinical appointments at the type-1 diabetes outpatient clinic at St Vincent's Hospital. She will discuss the study with patients whilst they are waiting, and if they are interested in participating, provide them with a hard copy of the PDCF and obtain permission to follow-up with them via phone and/or email.
- iii) Potential participants who have provided written consent to be contacted for research studies at the University of Melbourne eye care clinic will be identified by chart review (by one of the investigators), with a subsequent email (first preference) or mail-out (if no email details were provided) invitation. Those who respond to the invitation will be provided with the PDCF, and then followed up via phone and/or email with regard to arranging a time for a baseline study visit.
- iv) Potential participants who respond to study advertisements will be contacted by one of the listed investigators, either via email or phone (depending upon the patient preference), and provided with further information (including the PDCF).

3. Study Interventions and Formulations

3.1. Interventions

3.1.1. Omega-3 oral supplements

Treatment arm: triglyceride form omega-3 oral supplement (Caruso's Natural Health Triple Strength fish oil). Each 1500mg capsule contains active ingredients of 540mg Eicosapentaenoic Acid (EPA) and 360mg Docosahexaenoic Acid (DHA).

Participants will be asked to consume two capsules per day; the total active dose of omega-3 triglycerides consumed will be 1080mg EPA +720mg DHA per day for 180 days.

3.1.2. Placebo oral supplement

Control arm: Olive oil (1500mg) oral supplements, two capsules per day, manufactured by BJP Laboratories Pty Ltd, for 180 days.

3.2. Treatment regimen and dosing

Each participant will be instructed to consume two capsules each day, at any time during the day, with or without food, at least two hours before or after medication. Participants will be instructed that they may or may not notice a fishy aftertaste with either treatment options, to alter their expectations, to strengthen the double-masked.

Participants will be provided with a supplement log to document their consumption of capsules each day. Participants will be advised not to 'make up' for missed doses with additional capsules on a given day; a reason for a 'missed dose' should be documented on the supplement log, which will be returned along with any remaining capsules at Visits 3, 4 and 6.

Dose alteration is not permitted. Should participants wish to discontinue dosage for any reason, they must inform the Principal investigator immediately to withdraw from the study.

3.3. Compliance to treatment

Compliance to treatment will be monitored by the analysis of systemic fatty acid profiles at the end of the study (180 days) from baseline.

Assessment of the return of unused capsules at follow-up visits, for counting by an independent research assistant, will also be used to monitor compliance to treatment.

3.4. Concomitant medications and care

Concomitant medications and care, including insulin pumps, with a start date ≥ 3 months prior to Visit 1 are permitted during the study.

Aspirin use is permitted during the study. Other blood thinning medications (e.g., warfarin, enoxaparin, dipyridamole, clopidogrel) are not permitted; participants taking these medications will not be eligible to participate in the study.

Participants will be asked to maintain their current dietary habits throughout the study and notify the study investigators of any major dietary changes.

3.5. Storage of study product

The study product will be stored in the Downie Laboratory, within the Department of Optometry and Vision Science at the University of Melbourne, in a secure (locked) area, at room temperature (20° to 25° Celsius, in a controlled chamber). Study products will be administered only to participants who have enrolled in the study, at no cost to the participant, in accordance with the conditions specified in this protocol.

3.6. Study product inventory

A detailed inventory will be kept for the study products (e.g., date of dispense, randomisation number, by whom, date of return and accountability of capsules. Study product will only be dispensed by an appropriately-qualified study personnel member. The study product is to be used in accordance with the protocol by participants.

3.7. Assignment of Interventions**3.7.1. Method of masking**

Implementation of study allocation protocols will be conducted by an independent data manager. All trial participants and study investigators, including outcome assessors and data analysts, will be masked from the participant allocation.

Oral supplements will be packaged into identical opaque containers by a compounding pharmacist (Dartnell's Pharmacy, Surrey Hills, Victoria), to maintain double-masking. Participants' adherence to treatment will be monitored at follow-up visits by assessment of returned unused capsules by an independent researcher.

The investigators involved in the collection and analysis of data will not be involved in the reconciliation of returned study products, to maintain masking.

3.7.2. Unmasking the study product

During the trial, only when necessary for the safety and proper treatment of a participant, is it permissible for the principal investigator to request the independent data manager to unmask the participant's treatment assignment, to determine which treatment has been assigned, and institute appropriate follow-up care.

Only the principal investigator, following consultation with the independent data manager, is authorised to perform an unmasking procedure. The reason for breaking the code must be recorded in the participant's source documents.

3.7.3. Treatment allocation ratio and method of participant randomisation

A randomised allocation sequence will be generated, in advance, by the independent data manager, using Microsoft Excel, to assign equal numbers of participants in the treatment and placebo groups.

The randomisation schedule will be kept by the independent data manager and will be provided to an independent entity (i.e., compounding pharmacist) for labelling of the investigational product, in sequential order, to a corresponding participant number. Eligible participants will be enrolled and assigned, by the sequential participant number, to either the treatment group or the placebo group, the treatment allocation for which will not be known to the study personnel who was involved in enrolment (i.e., the study investigator) or the participant.

4. Participant's timeline and schedule of study visits

Test	Repeats	Visit 1 (Day -35)	Visit 2 Day:1	Visit 3 Day:30±14	Visit 4 Day:90±14	Visit 5 Day: 180+ /24	Visit 6 Day: 180+ /24	Notes	Time Expected (mins)	Location
Location		UoM	SVH	UoM	UoM	SVH	UoM			
Eligibility Determination										University of Melbourne
PICF	-								20	
Demographics	-								10	
Medical History	-							Medications, concurrent procedures		
Ocular History	-									
Adverse Events	-									
Diet Inquiry	-									
Pregnancy Test	-							Females of childbearing potential only	10	
Habitual VA	-								5	
Anterior Eye Health										
MNSI questionnaire										
OSDI Questionnaire	-								5	
EQ-5D-5L questionnaire										
iMTA questionnaire										
Tear Osmolarity										
Non-contact aesthesiometry	Central							Double-staircase threshold	20	
	Inferior									
Cooled threshold	Central									
	Inferior									
Basal tear collection									15	
Phenol red thread test										
Slit lamp										
Slit lamp exam									5	
Anterior chamber VH grading	-							Assessment of anterior chamber angle		

Instill NaFl									
NaFl TBUT									
NaFl staining	Corn ea							Mag 16X, Max Intensity, Blue Light, Wratten filter Oxford Grading Scale	
Instill topical anaesthetic									
IOP								Goldmann tonometer	5
Instill mydriatic agent (Visits 1 and 6 only)									
Heidelberg Rostock Tomographer – Corneal module									
Corneal confocal microscopy	Cent ral								10
	Infer ior								
Systemic fatty acid level analysis									
Blood pin-prick test									5
Retinopathy Grading									
Retinal imaging	Right								10
	Left								
Recheck eligibility									
Randomisation									
Randomisation	-								5
Dispense study supplements	-								
Collection of unused supplements	-								
Peripheral Nerve Function									
MDNS									10
CSP									15
QSART									15
Nerve Excitability									15
Nerve Conduction									15
Systemic Health									
Fasting blood test									10

University of Melbourne

St Vincent's Hospital

University of Melbourne

St Vincent's Hospital

5. Response measures and summary of data collection methods

5.1. Efficacy measures:

5.1.1. Primary efficacy measures:

Primary Outcome:	Central Corneal Nerve Fibre Length (CNFL) (measured in mm/mm ² using a Heidelberg Rostock Tomographer III with corneal module, quantified using a fully-automated method (ACCMetrics))
Timepoint:	Change in CNFL from Day 1 at Days 90 +/- 14, 180 +/- 24

5.1.2. Secondary efficacy measures:

Secondary Outcome:	Central Corneal Nerve Fibre Density (CNFD) (measured in mm/mm ² using a Heidelberg Rostock Tomographer III with corneal module, quantified using a fully-automated method (ACCMetrics))
Timepoint:	Change in CNFD from Day 1 at 90 +/- 14, 180 +/- 24
Secondary Outcome:	Central Corneal Nerve Branch Density (CNBD) (measured in mm/mm ² using a Heidelberg Rostock Tomographer III with corneal module, quantified using a fully-automated method (ACCMetrics))
Timepoint:	Change in CNBD from Day 1 at Days 90 +/- 14, 180 +/- 24
Secondary Outcome:	Central corneal thresholds to a room-temperature air stimulus (measured in mBar using a non-contact corneal aesthesiometer)
Timepoint:	Change from Day 1 at Days 90 +/- 14, 180 +/- 24
Secondary Outcome:	Central corneal thresholds to a cooled stimulus (measured in mBar using a non-contact corneal aesthesiometer)
Timepoint:	Change from Day 1 at Days 90 +/- 14, 180 +/- 24
Secondary Outcome:	Change in health state (index score and visual analogue score as measured using the EQ-5D-5L questionnaire).
Timepoint:	Change from Day 1 at Day 90 +/- 14, 180 +/- 24

Secondary Outcome:	Michigan Neuropathy Screening Score (MNSI) composite score
Timepoint:	Change from Day 1 at Day 180 +/- 24
Secondary Outcome:	Michigan Diabetic Neuropathy Score (MDNS) (measured through clinical examination)
Timepoint:	Change from Day 1 at Day 180 +/- 24
Secondary Outcome:	Routine nerve conduction studies (NCS) –sural sensory amplitude (measured using Dantec Keypoint G4 Workstation)
Timepoint:	Change from Day 1 at Day 180 +/- 24
Secondary Outcome:	Routine nerve conduction studies (NCS) – peroneal motor velocity (measured using Dantec Keypoint G4 Workstation)
Timepoint:	Change from Day 1 at Day 180 +/- 24
Secondary Outcome:	Routine nerve conduction studies (NCS) – tibial motor minimum F-wave latency (measured using Dantec Keypoint G4 Workstation)

Timepoint:	Change from Day 1 at Day 180 +/- 24
Secondary Outcome:	Nerve excitability profile: Depolarising threshold electrotonus (TEd) (peak), TEd (90- 100 ms), hyperpolarising threshold electrotonus (Teh) (90-100 ms), TEd (10- 20 ms), superexcitability, subexcitability, resting current-voltage (I/V) slope, strength duration time constant (measured with QTRAC software using TROND protocol)
Timepoint:	Change from Day 1 at Day 180 +/- 24
Secondary Outcome:	Quantitative sudomotor axonal reflex testing (QSART), measured at the foot in sweat volume in nL using the QSWEAT system.
Timepoint:	Change from Day 1 at Day 180 +/- 24
Secondary Outcome:	Cutaneous silent period testing: upper and lower limb (measuring silent period onset latency and duration)
Timepoint:	Change from Day 1 at Day 180 +/- 24

5.2. Safety measures:

Safety Outcome:	Adverse events (AEs) reported
Timepoint:	Change from Day 1 at Days 30 +/- 14, 90 +/- 14, 180 +/- 24
Safety Outcome:	Change in habitual visual acuity measured using a standard eye chart
Timepoint:	Change from Day 1 at Days 30 +/- 14, 90 +/- 14, 180 +/- 24
Safety Outcome:	Change in intraocular pressure (IOP) measured using a Goldmann tonometer
Timepoint:	Change from Day 1 at Day 180 +/- 24
Safety Outcome:	Diabetic retinopathy graded using the Wisconsin Scale grading system
Timepoint:	Change from Day 1 at Day 180 +/- 24
Secondary Outcome:	Blood samples (measuring fasting glucose, fasting lipid profile [LDL, HDL, total cholesterol, triglycerides], FBC, ESR, CRP, Vitamin B-12, Folate, TSH, UEC, HbA1c, liver function, electrolytes)
Timepoint:	Change from Day 1 at Day 180 +/- 24

5.3. Compliance measures:

Compliance Outcome:	Total omega-3 status obtained from blood finger spot test (Waite Lipid Analytical Services)
Timepoint:	Change from Day 1 at Day 180 +/- 24
Compliance Outcome:	Total omega-9 status obtained from blood finger spot test (Waite Lipid Analytical Services)
Timepoint:	Change from Day 1 at Day 180 +/- 24
Compliance Outcome:	Total omega-6 status obtained from blood finger spot test (Waite Lipid Analytical Services)
Timepoint:	Change from Day 1 at Day 180 +/- 24
Compliance Outcome:	Omega-6:Omega-3 ratio obtained from blood finger spot test (Waite Lipid Analytical Services)
Timepoint:	Change from Day 1 at Day 180 +/- 24

Exploratory outcome measures:

1. Compliance outcome: supplement count at Day 180±24
2. Efficacy of masking: Guessing of treatment at Day 180±24
 - Guessing by participant
 - Guessing by examiner
3. Change in central corneal dendritic cell count (CDC) (measured in cells/mm² using a Heidelberg Rostock Tomographer III with corneal module) from Day 1 at Days 90±14, 180±24
4. Change in peripheral corneal nerve fibre length (CNFL) (measured in mm/mm² using a Heidelberg Rostock Tomographer III with corneal module) from Day 1 at Days 90±14, 180±24
5. Change in peripheral corneal dendritic cell count (CDC) (measured in cells/mm² using a Heidelberg Rostock Tomographer III with corneal module) from Day 1 at Days 90±14, 180±24
6. Change in peripheral corneal thresholds to a room air stimulus (measured in mBar using a non-contact corneal aesthesiometer) from Day 1 at Days 90±14, 180±24
7. Change in peripheral corneal thresholds to a cooled stimulus (measured in mBar using a non-contact corneal aesthesiometer) from Day 1 at Days 90±14, 180±24
8. Routine nerve conduction studies (NCS) – ulnar sensory amplitude, velocity and latency (measured using Dantec Keypoint G4 Workstation) from Day 1 at Day 180±24
9. Routine nerve conduction studies (NCS) – median sensory velocity, amplitude and latency (measured using Dantec Keypoint G4 Workstation) from Day 1 at Day 180±24
10. Routine nerve conduction studies (NCS) – sural sensory velocity and latency (measured using Dantec Keypoint G4 Workstation) from Day 1 at Day 180±24
11. Routine nerve conduction studies (NCS) – median motor velocity, amplitude and latency (measured using Dantec Keypoint G4 Workstation) from Day 1 at Day 180±24
12. Routine nerve conduction studies (NCS) – tibial motor amplitude and latency (measured using Dantec Keypoint G4 Workstation) from Day 1 at Day 180±24
13. Routine nerve conduction studies (NCS) – peroneal motor amplitude and latency (measured using Dantec Keypoint G4 Workstation) from Day 1 at Day 180±24
14. Change in sweat volume in the forearm measured using quantitative sudomotor axonal reflex testing (QSART) from Day 1 at Day 180±14

15. Change in sweat volume in the proximal leg measured using quantitative sudomotor axonal reflex testing (QSART) from Day 1 at Day 180±14
16. Change in sweat volume in the distal leg measured using quantitative sudomotor axonal reflex testing (QSART) from Day 1 at Day 180±14
17. Change in nerve excitability profile (additional parameters) from Day 1 at Day 180±14
 - Stimulus (mA) for 50% max response
 - Rheobase (mA)
 - Stimulus-response\slope
 - Peak response\ (mV)
 - Minimum I/V slope
 - Temperature (°C)
 - RRP (ms)
 - Polarizing current\(% threshold)
 - Polarizing current\ (mA)
 - Latency (ms)
 - TEd(40-60ms)
 - TEh(10-20ms)
 - TEd(undershoot)
 - TEh(overshoot)
 - S2 accommodation
 - Accommodation half-time (ms)
 - Hyperpol. I/V slope
 - Refractoriness at 2.5ms (%)
 - TEh(20-40ms)
 - TEh(slope 101-140ms)
 - Refractoriness at 2 ms (%)
 - Superexcitability at 7 ms (%)
 - Superexcitability at 5 ms (%)
 - TEd20 (peak)
18. Change in dry eye symptoms (measured using the validated Ocular Surface Disease Index (OSDI) questionnaire) from Day 1 at Days 90±14, 180±24
19. Change in tear stability measured using corneal sodium fluorescein from Day 1 at Days 90±14, 180±24

20. Change in tear osmolarity: electrolyte concentration of the tears (measured in mOsm/L using the TearLab system (TearLab Corporation Pty Ltd) from Day 1 at Day 180±24
21. Change in tear production measured using the phenol red thread test (R+L) from Day 1 at Days 90±14, 180±24
22. Additional slit lamp microscopy grading
 - Blepharitis grading from 0 - 4
 - Meibomian gland dysfunction grading (MGD) from 0 - 4
 - Conjunctival redness grading from 0 - 4
 - Limbal redness grading from 0 - 4
23. Change in corneal sodium fluorescein (NaFl) staining score, as the average of two eyes, from Day 1 at Day 90±14, Day 180±24
 - Corneal staining graded from 0 - 4
24. Change in tear composition markers from Day 1 at Day 180±24
25. Change in indirect healthcare costs measured using the iMTA-iPCQ health economics questionnaire at Day 1 and Day 180
26. Change in ONH structure retinal neuro-retinal fibre layer (RNFL) thickness in the right eye measured using Optic Coherence Tomography (OCT) from Day 1 at Day 180±24
27. Change in macular thickness in the right eye measured using Optical Coherence Tomography (OCT) from Day 1 at Day 180±24
28. Change in foveal avascular zone area in the right eye, measured using Optical Coherence Tomography Angiography (OCT-A) from Day 1 at Day 180±24
29. Change in retinal vascular calibre in the right eye, measured using Optical Coherence Tomography Angiography (OCT-A) from Day 1 at Day 180±24

6. Methods of data collection and study reports

6.1. Summary

All study data will be uploaded or manually entered (as appropriate) onto REDCap, a secure web-based application for research data capture, management and analysis (hosted on the University of Melbourne data centre infrastructure, provided via the National eResearch Collaboration tools and Resources project).

The co-investigator, Alexis Ceecee Zhang, will be responsible for ensuring that data are properly recorded and that all hard copy documents are securely stored.

Any protocol deviations will be documented and must also be reported to the Principal Investigator. Any actions arising from the protocol deviation will also be noted. Deviations identifying issues for protocol review will be amended as per Section 9.4.

6.2. Data Collection

Source documents in this study will include participants' ophthalmic records, relevant medical records, completed questionnaires, the patient study files (case report forms, CRFs), as well as the results of any diagnostic tests, such as blood tests and systemic fatty acid profiles.

Identifiable information will be collected and stored in a separate password-protected file that is only accessible to study investigators. The following data and information will be recorded:

- Identifiable participant information at baseline: name; contact details; study enrolment date
- A statement that written informed consent (including the date) was obtained prior to any study procedures being performed
- Participant ID number
- Dates of all study visits
- Demographic information at baseline (age, sex, date of birth, ethnicity)
- Height and weight at baseline
- Medical and ophthalmic histories at baseline
- Pre-study and concomitant medications, including insulin type, administration method and dosage
- Study procedures performed at the University of Melbourne (UoM)
 - Habitual distance refractive correction in each eye at baseline.
 - Habitual distance visual acuity in each eye at baseline, day 30 $\pm$ 14, day 90 $\pm$ 14 and day 180 $\pm$ 24.
 - EQ-5D-5L questionnaire score at baseline, day 30 $\pm$ 14, day 90 $\pm$ 14 and day 180 $\pm$ 24.
 - Ocular Surface Disease Index (OSDI) questionnaire score at baseline, day 90 $\pm$ 14 and day 180 $\pm$ 24.
 - Tear osmolarity in each eye at baseline and day 180 $\pm$ 24.
 - Corneal sensitivity thresholds in the right eye at baseline, day 90 $\pm$ 14 and day 180 $\pm$ 24.

- Phenol red thread test score in each eye at baseline, day 90 $\pm$ 14 and day 180 $\pm$ 24.
- Tear collection time and volume collected at baseline and day 180 $\pm$ 24.
- Slit lamp examination gradings in each eye at baseline, day 90 $\pm$ 14 and day 180 $\pm$ 24.
- Intraocular pressure (IOP) in each eye at baseline and day 180 $\pm$ 24.
- Diabetic retinopathy (DR) and diabetic macular oedema grading (Wisconsin classification) in each eye at baseline and day 180 $\pm$ 24.
- Optical coherence tomography angiography (OCT-A) imaging at baseline and day 180 $\pm$ 24.
- Corneal sub-basal nerve plexus and dendritic cell parameters, measured using *in vivo* confocal microscopy (IVCM) in the right eye at baseline, day 90 $\pm$ 14 and day 180 $\pm$ 24.
- Systemic fatty acid concentrations at baseline and day 180 $\pm$ 24.
- Study procedures performed at St Vincent's Hospital Melbourne (SVHM)
 - Michigan Neuropathy Screening Instrument (MNSI) and Michigan Diabetic Neuropathy Score (MDNS) examination score at baseline and day 180 $\pm$ 24.
 - Nerve conduction study (NCS) parameters for the median motor, peroneal motor, tibial motor, median sensory, sural sensory, ulnar sensory nerves at baseline and day 180 $\pm$ 24.
 - Cutaneous silent periods (CSP) measurements of the upper and lower limbs at baseline and day 180 $\pm$ 24.
 - Quantitative Sudomotor Axonal Reflex Test (QSART) measurements of the forearm, proximal leg, distal leg and foot at baseline and day 180 $\pm$ 24.
- Blood test results
 - Blood pathology test results at baseline and day 180 $\pm$ 24.
 - Systemic fatty acid profiles at baseline and day 180 $\pm$ 24.
- Occurrence, grading and status of any adverse events, including their potential association with the investigational product or a study procedure
- Number and proportion of supplements returned for each participant
- Participant's and examiner's forced-choice guesses of the treatment allocation (omega-3 PUFAs or placebo) on day 180 $\pm$ 24.
- The date the participant exited the study, and a notation as to whether participant completed the study,

The co-investigator (Alexis Ceecee Zhang) and Principal Investigator will be jointly responsible for ensuring that data are properly transferred from CRFs and correctly recorded.

6.3. Data Management

6.3.1. Data coding

All data collected directly by the investigators for this study will be de-identified using a participant ID code, assigned to the participant when they sign the informed consent form. This ID code will be used as an identifier on all study documentation, and be used for the labelling of body tissue samples (i.e., blood, tears), which are processed off-site. The only individuals who will have the capacity to identify the participant's study results (by having access to the code linking the participant ID code to the participant's

identity) will be the investigators listed on this application, and the participant themselves. The data will not be identifiable to any other persons.

6.3.2. Security and storage of data

Study data will be collected in both paper (hard copy) files and electronic formats.

For paper source documents, de-identified data, recorded during study examinations, will be stored in a binder (marked with the participant's ID code), and subsequently entered into the REDCap database (using each participant's ID code). The paper study source documents will be kept in a locked research room – The Downie Laboratory within the Department of Optometry and Vision Sciences, accessible only by research staff and students of the Downie Laboratory.

Data captured during study examinations in an electronic format will be labelled with the participant's ID code, and subsequently uploaded into the REDCap database. Only researchers name on the project application will have direct access to the raw study data.

6.4. Individual Data Reporting

Should a participant ask for details about the results of any of their study tests, immediate verbal feedback can be given by one of the study investigators. A report, summarising key findings relating to a participant's eye and/or peripheral nerve health, can also be made available, further to the participant's request, to potentially assist in guiding the participant's ongoing clinical care.

6.5. Study summary

At the conclusion of the study, a study summary report will be made available to participants who indicate wishing to receive this report during the written informed consent process.

6.6. Retention of study documentation

All study related data, participant records, consent forms, patient privacy documentation, records of the distribution and use of all study products, and copies of case report forms will be maintained on file. Electronic data and paper files will be kept for a minimum of 15 years, but possibly longer if the data is judged of continuing value at that time.

7. Safety measures

7.1. Summary

Safety measures will include:

- Adverse events query (Visits 1, 3, 4 and 6)
- Habitual distance visual acuity, BCVA (Visits 1, 3, 4 and 6)
- Intraocular pressure, IOP (Visits 1 and 6)
- Retinal imaging and ocular fundus examination (Visits 1 and 6)
- Basic blood tests (Visits 2 and 5)

7.2. Data Monitoring

This study is a single-site, investigator-initiated clinical trial (with procedures being undertaken at two Melbourne locations – St Vincent’s Hospital and the University of Melbourne). Professor Richard MacIsaac, Director - Director, Department of Endocrinology and Diabetes at St Vincent’s Hospital, will act as the medical safety monitor for the trial. He possesses the requisite expertise to assist and advise the HREC about reports of serious adverse events. All adverse events will be discussed by the Principal Investigator (Dr Laura Downie) and the medical monitor before reporting to the relevant HREC or other regulatory agencies, as required.

7.3. Adverse events (AEs)

7.3.1. Assessments and documentations of adverse events

All AEs and SAEs will be immediately (within 24 hours) reported to the Principal Investigator by the research team member.

At Visit 1, after informed consent and before the initial study products are administered, all pre-treatment adverse events will be documented (e.g., allergic response to topical anaesthetic). Conditions that are present at screening and do not deteriorate will not be considered adverse events.

After the study product supply, all treatment-emergent post-treatment adverse events will be documented. Abnormal laboratory values will not be considered adverse events unless deemed clinically significant by the medical monitor, and documented as such.

If adverse events occur, the first concern will always be the safety of the study participants. All adverse events, including severity, action taken and likely relationship to the study product will be documented throughout the study.

At each visit, the co-investigator or principal investigator will begin the examination by querying for adverse events by asking each participant a general, non-directed question such as “How have you been feeling since the last visit?” Directed questioning and examination will then be carried out as appropriate.

The description of each AE recorded will include:

- A description of the AE;
- The onset date, duration, date of resolution;
- Severity (assessed as per section 7.4;

- Seriousness (i.e., is it an SAE?);
- Actions taken;
- The outcome;
- The likelihood of the relationship of the AE to the study treatment (Unrelated, Possible, Probable, Definite).

For each adverse event reported, the number and percent of participants affected will be tabulated. Separate adverse event tables will be generated for pre-treatment AEs and post-treatment AEs. All adverse events will be presented in the listings. Other safety variables, including biomicroscopy findings, BCVA, IOP, retinal fundus photograph and blood test results will be analysed.

DEFINITIONS:

- An adverse event is **any** untoward medical occurrence in a participant or clinical investigation of participant administered a pharmaceutical product. It does not necessarily have a causal relationship with treatment.

An adverse event can therefore be **any** unfavourable or unintended sign, symptom, or disease temporally associated with the use of a study (investigational) product, whether or not it is thought to be related to the study (investigational) product. In addition, during the screening examination (Visit 1), pre-treatment adverse events will be assessed regardless of the administration of a pharmaceutical product.

All adverse events judged as having a reasonable possibility of a causal relationship to an investigational medicinal product will qualify as adverse reactions. The expression 'reasonable causal relationship' means to convey, in general, that there is evidence or argument to suggest a causal relationship.

Note: Details relating to adverse events must be collected once informed consent has been obtained, regardless of whether or not the patient has been administered study product. Progression of treatment indication including new or worsening of anticipated clinical signs or symptoms, which are collected as clinical efficacy variables and assessed as unequivocally associated with the disease progression and /or lack of efficacy, should NOT be reported as adverse events unless the disease progression is greater than anticipated in the natural course of the disease.

7.3.2. Serious adverse events (SAEs)

- A serious adverse event is any adverse event that results in any of the following outcomes:
 - death
 - a life-threatening adverse event
 - inpatient hospitalisation or prolongation of existing hospitalisation (for any reason)
 - a persistent or significant disability/incapacity
 - a congenital anomaly/birth defect
 - a diagnosis of cancer
 - any abortion (spontaneous or non-spontaneous)

- A **Suspected Unexpected Serious Adverse Reaction (SUSAR)** is any SAE that is both suspected to be related to the study treatment and is unexpected (i.e., not consistent with applicable product information)

Important medical events that may not result in death, be life-threatening, or require hospitalisation may be considered a serious adverse event when, based upon appropriate medical judgment, they may jeopardise the participant or may require medical or surgical intervention to prevent one of the outcomes listed in this definition. (See below for procedures for reporting a serious adverse event.)

Pre-planned surgeries or procedures for pre-existing, known medical conditions, for which a participant requires hospitalisation are not reportable as a serious adverse event. Any pre-planned surgery or procedure should be clearly documented in the site source documents by the medically qualified investigator at the time of the participant's entry into the study. If it has not been documented at the time of the patient's entry into the study, then it should be documented as a serious adverse event and reported as such.

7.4. Severity of AEs

A clinical determination will be made of the intensity of **every** adverse event. The severity assessment for a clinical adverse event must be completed using the following definitions as guidelines:

- Mild - awareness of sign or symptom, but easily tolerated.
- Moderate - discomfort enough to cause interference with usual activity.
- Severe - incapacitating with inability to work or do usual activity.
- Not applicable - in some cases, an adverse event may be an 'all or nothing' finding which cannot be graded.

7.5. Relationship to study product or study procedure

A determination will be made in relation to the likely relationship (if any) between an adverse event and the study product or study procedure, as applicable. A causal relationship is present if a determination is made that there is a reasonable possibility that the adverse event may have been caused by the study product or study procedure.

Adverse events will subsequently be classified according to the safety reporting flowchart in the NHMRC safety monitoring and reporting guidelines. Any serious adverse event occurring during the study period (beginning with informed consent) and for at least 28 days after the last dose of study product must be immediately reported according to HREC guidelines, no later than 24 hours after learning of a serious adverse event. All SAEs will be immediately (within 24 hours) reported to the medical monitor and the principal investigator. The medical monitor will make a judgment in relation to whether the SAE is likely related to the study product, and whether this outcome affects the potential safety of other participants in the study.

7.6. Procedures for reporting adverse events

All adverse events will be recorded on the appropriate sections in REDCap and the principal investigator will be informed within 24 hours. Any adverse event that is marked as 'ongoing' at the exit visit will be followed-up, as appropriate.

All SUSARs will be reported to the Institution (University of Melbourne Human Research Ethics Committee) and the Therapeutic Goods Administration (TGA) as soon as possible, and no later than seven calendar days for fatal or life threatening SUSARs, and 15 calendar days for all other SUSARs.

An annual safety report will be generated by the Principal Investigator and provided to the governing institutional human research ethics committee (HREC), as required by the HREC, local regulations, and the governing health authorities to provide a clear summary of the evolving safety profile of the trial. The annual safety report will include

- a brief description and analysis of new and relevant findings (as permissible);
- a brief discussion of the implications of the safety data to the trial's risk-benefit ratio;
- a description of any measures taken or proposed to minimise risk

The Principal Investigator will complete the annual review and provide the HREC and investigators with updates/addenda to the Investigator's Brochure, should new or relevant information become available.

The Principal Investigator will notify the Institution, the TGA and investigators (co-investigators and associate investigators) of all significant safety issues, amendments, temporary halt or early termination of a trial. Significant safety issues that meet the definition of an urgent safety measure should be notified within 72 hours, and all other significant safety issues should be notified within 15 calendar days

Definitions:

Significant safety issues are issues that adversely affect the safety of participants or materially impact on the continued ethical acceptability or conduct of the trial. These may require an urgent safety measure, an amendment, a temporary halt or an early termination of a trial.

Urgent Safety Measures (USMs) are significant safety issues where sponsors or trial investigators act immediately to protect participants from an immediate hazard to their health and safety.

7.7. Administrative items

This study protocol is to be conducted in accordance with the applicable Good Clinical Practice (GCP) regulations and guidelines (e.g., the International Conference on Harmonisation (ICH) Guideline on GCP).

All safety monitoring and reporting will be conducted according to the NHMRC 'Safety monitoring and reporting in clinical trials involving therapeutic goods' handbook.

8. Statistical Analysis

8.1. Statistical methods

A separate statistical analysis plan will be developed and finalised prior to study unmasking.

Data relating to participant demographics and baseline clinical characteristics will be analysed with a repeated measures model or ordered logistic regression model, as appropriate. For the primary outcome, analyses will be performed on data from right eyes. For slit lamp examination, analyses will be performed on the average of data from both eyes. For neurophysiological data, analyses will be performed on data from one limb only.

8.1.1. Populations to be analysed

The statistical analysis will follow an intention-to-treat approach (i.e., will include data from all participants randomised into the study, regardless of whether they received the study intervention). This approach preserves the prognostic balance in the study arms achieved by randomisation.

8.1.2. Handling of missing data

The proposed statistical approach provides valid inference in the presence of missing data if the missing data mechanism is ignorable (missing completely at random or missing at random) and includes participants who have a baseline and at least one post-baseline measurement of the primary outcome measure (CNFL). Missing values will be checked and reported across treatment arms and follow-up time points.

8.1.3. Methods of analysis

The primary outcome measure is the change in central corneal nerve fibre length (CNFL) from baseline (Day 1); the primary analysis endpoint is Day 180 (six months).

A range of secondary outcome measures, including safety outcomes, will be investigated (as detailed in Section 5.1).

For continuous outcome measures, a repeated measures model will be used to assess for differences in the post-treatment means between groups (i.e., the main effect of group) at each time point, with the baseline (Day 1) values used as a covariate. For binary and categorical outcome measures, an ordered logistical regression model will be used. For all analyses, an alpha level of 0.05 will be adopted for statistical significance. Unless otherwise specified, data will be expressed as mean plus/minus standard deviation.

8.2. Interim analysis

We do not intend to undertake interim data analyses, unless there is a concern in relation to safety. In this case, an independent statistician will perform the analyses, and consult with the medical monitor, who will make a judgment with regard to the termination of the trial.

9. Ethics and dissemination

9.1. Compliance with informed consent regulations

Written informed consent must be obtained from each potential participant prior to any study-related activities or procedures in the study. Consent will be obtained by a named study investigator on this project.

9.2. Compliance with human research ethics committee (HREC) regulations

This study is to be conducted in accordance with HREC regulations or applicable IEC regulations, in full conformance with principles of the “Declaration of Helsinki”. The investigator must obtain approval from a properly constituted HREC prior to initiating the study and re-approval or review at least annually.

9.3. Compliance with Good Clinical Practice (GCP)

This protocol is to be conducted in accordance with the applicable GCP regulations and guidelines.

9.4. Protocol amendments

This study will be conducted in compliance with the current version of the protocol. Any modifications made to the protocol that may affect the scientific intent, study design, participant safety or participant’s willingness to continue participation will be written as an amendment to the current protocol, and will be submitted to the HREC for approval prior to becoming effective.

9.5. Participant confidentiality

Participant confidentiality will be strictly held in trust by the study investigators and all associated research staff. This confidentiality extends to all biological samples and all clinical information collected relating to participants. The study protocol, documentation and all data generated as part of this study will be held in strict confidence. No information concerning the study or the data collected will be released to any unauthorised third party.

All laboratory specimens, evaluation forms or any other reports that leave the study sites will be identified only by the participant identification number to maintain participant confidentiality. Clinical information will not be released without the permission of the participant, except as necessary for monitoring by HREC or other regulatory agencies.

Any publications of the study results will not include identifiable information about any participants. Appropriate precautions will be taken to maintain confidentiality of medical records and personal information.

9.6. Declaration of Interests

No declarations of interests are reported by any of the participating investigators or study personnel.

9.7. Dissemination policy

A study summary to participants will be made available at the conclusion of the study. Participants will also be advised that they are welcome to phone or email the researchers if they are interested in receiving more information about any aspect of the research program.

Participants can request a copy of their individual study findings from the investigators; this information can either be provided verbally on the day, or in the form of a written report.

The findings from this study will be published as part of Alexis Ceecee Zhang's PhD thesis and are also expected to be presented at relevant scientific meetings and/or submitted for publication to refereed journals. For the purpose of dissemination of the research results, all participants will be non-identifiable, as data are collated and averaged with the data of other participants in each group.

The full protocol, participant level dataset and statistical codes formed as part of this study will not be available for public access.

10. Study visit schedule and procedures

10.1. Overview of entry procedures

Prospective participants, as defined by the inclusion and exclusion criteria, will be considered for enrollment into this study.

10.2. Informed consent and privacy

After the study is discussed with the potential participant, a person wishing to participate must provide written informed consent prior to any study-related procedure being undertaken. Each potential participant who provides informed consent will be assigned a participant ID code that will be used on all documentation and labelling related to this study. Eligible participants will also be assigned a randomisation number that will be used to assign treatment throughout the study.

After informed consent is obtained at Visit 1 and before randomisation into the study occurs, all pre-treatment adverse events will be documented.

10.3. Treatment period

Participants entering the study product treatment period must continue to meet all inclusion and exclusion criteria.

10.4. Visits and associated procedures

Clinical evaluations are intended to be performed by the same clinician throughout the study, whenever possible. If it is not possible to use the same evaluator to follow the patient, then evaluations should ideally overlap (i.e., examine the patient together and discuss findings) for at least one visit.

Visit 1 (including Screening), The University of Melbourne, 2 hours

The following procedures will be performed at Visit 1

- Written informed consent
- Demographic information
- Medical and ophthalmic histories
- Inclusion/exclusion criteria assessment
- Pre-treatment adverse event assessment
- Diet questioning
- Urine pregnancy test (for females of childbearing potential only)
- Habitual/ best corrected visual acuity
- MNSI questionnaire
- OSDI questionnaire
- EQ-5D-5L questionnaire
- iMTA questionnaire
- Tear osmolarity
- Basal (non-stimulated) tear collection
- Non-contact aesthesiometry
- Slit lamp assessment

- Anterior chamber angle assessment
- TBUT
- Ocular surface staining (Oxford scale)
- IOP (with anaesthesia and fluorescein using a Goldmann tonometer)
- Corneal confocal microscopy (with anaesthesia)
- Dilated fundus examination (with mydriatic eye drops) and non-invasive retinal imaging
- Pin-prick blood sample for omega-3 fatty acid analysis
- Re-check all eligibility requirements
- Dispense masked study products (if the participant meets the required qualifications)

Randomised participants will be provided with a supplement intake log (to mark off when they consume their supplements each day), given instructions with regard to how to take their supplements and be advised about the dose regimen to be followed. The supplement label (which specifies details with regard to: optimal storage conditions, clinical trial use only, keep out of reach of children, etc.) should also be read through and explained with the participant.

Participants will be asked to commence taking oral supplements (Day 1) on the day that their baseline nerve functions tests are conducted at Visit 2.

Visit 2 (Day 1; St Vincent's Hospital, 2 hours)

This visit will assess peripheral nerve fibre function, as per routine nerve conduction examinations, for diabetic neuropathy. The following procedures will be performed at this visit:

- Brief medical history (including related neuropathy symptoms)
- Michigan Diabetic Neuropathy Score
- Cutaneous silent period testing
- Quantitative sudomotor axonal reflex testing
- Nerve conduction studies
- Nerve excitability tests
- Blood samples

Visit 3 (23 to 37 days, The University of Melbourne, 1 hour)

The following procedures will be performed at Visit 3:

- Adverse event assessment
- Diet questioning
- Medical and ophthalmic histories
- Habitual/best corrected visual acuity
- Slit lamp examination

Visit 4 (76 to 104 days, The University of Melbourne, 1 hour)

The following procedures will be performed at Visit 4:

- Adverse event assessment
- Diet questioning
- Medical and ophthalmic histories
- OSDI questionnaire
- Habitual/best corrected visual acuity
- Non-contact aesthesiometry
- Slit-lamp examination
- TBUT
- Ocular surface staining (Oxford scale)
- Corneal confocal microscopy (with anaesthesia)
- Dispense of masked study product

Visit 5 (156 to 204 days, St Vincent's Hospital Melbourne, 2 hours)

The following procedures will be performed at Visit 5:

- Brief medical history (including related neuropathy symptoms)
- Michigan Diabetic Neuropathy Score
- Cutaneous silent period testing
- Quantitative sudomotor axonal reflex testing
- Nerve conduction studies
- Nerve excitability tests
- Blood samples

Visit 6 (156 to 204 days, The University of Melbourne, 2 hours) or Early exit

- Adverse event assessment
- Medical and ophthalmic histories
- Diet questioning
- Urine pregnancy test (for females of childbearing potential only)
- Habitual/ best corrected visual acuity
- MNSI questionnaire
- OSDI questionnaire
- EQ-5D-5L questionnaire
- iMTA questionnaire
- Tear osmolarity
- Basal (non-stimulated) tear collection
- Non-contact aesthesiometry
- Slit lamp examination
- Anterior chamber angle assessment
- TBUT
- Ocular surface staining (Oxford scale)
- IOP (with anaesthesia and fluorescein using a Goldmann tonometer)
- Corneal confocal microscopy (with anaesthesia)
- Dilated fundus examination (with mydriatic eye drops) and non-invasive retinal imaging
- Pin-prick blood sample
- Collection of unused study supplements

Unscheduled visits

Unscheduled visits may be arranged, as necessary, to ensure the safety and well-being of participants during the study. Source documentation should be completed for all parameters measured for each unscheduled visit. If the unscheduled visit is an exit visit, then all exit visit procedure data should be captured and the Visit 6 CRF should be completed.

At a minimum, the following procedures should be performed at an unscheduled visit:

- Adverse event assessment
- Concomitant medication assessment
- Concurrent procedures assessment
- BCVA
- Slit lamp biomicroscopy

All other procedures should be performed on indication, as indicated by the participant's presenting symptoms and signs.

10.5. Instructions to eligible participants

Eligible participants will be asked to attend a total of 6 study visits; four visits will be at the University of Melbourne, and two will be performed at St Vincent's Hospital (at the start and end of the supplementation period), within four weeks of the initial and final visits.

At Visit 1, eligible participants will be dispensed study products consisting of their randomised treatment allocation. Study investigators will counsel the participant to consume 2 capsules each day, with or without food, at least two hours before or after medications. Participants will be provided with a supplement log to document their consumption of capsules each day. Participants will be advised not to 'make up' for missed doses with additional capsules on a given day; a reason for a 'missed dose' should be documented on the supplement log, which will be returned along with any remaining capsules at Visits 3, 4 and 6.

An appointment will be arranged for participants within four weeks of Visit 1, to attend Visit 2 at St Vincent's Hospital, Melbourne. Participants will be asked to start their first dose on the day of Visit 2 (Day 1), following their assessments at St Vincent's Hospital. Participants will be advised that they should maintain their regular dietary habits and medications (including ocular and systemic) and notify us of any change in medications of dosage throughout the study.

Appointments for Visits 2 and 5 at St Vincent's Hospital will be arranged for the participants by the study investigators, at Visits 1 and 4 respectively.

Participants will be encouraged to contact the Principal Investigator at any time should they have any questions about the study or the instructions they have been given.

10.6. Compliance with the protocol

If a participant is inadvertently enrolled with significant deviation(s) from the specified inclusion and exclusion criteria, he/she may be discontinued from the study at the discretion of the Principal Investigator.

Participants will be asked at Visits 3, 4 and 6 whether they have had any changes to their medications (including dosage) since their last study visit. To ensure compliance with usage of study product after randomisation, participants will be queried about their study product usage. The examiner will ensure that, based upon how the participant answers such questions, that the participant has been compliant with the protocol study product usage instructions.

Participants will be scheduled for follow-up visits and these will be timed to occur as close as possible to the day specified in the visit schedule. If a patient is unable to come to their next scheduled follow-up visit within the visit tolerance window for that visit, every effort will be made to reschedule the visit as soon as possible. If a patient is absolutely unable to come in for that visit, then that visit will be considered a missed visit.

10.7. Early discontinuation of participants

Participants may voluntarily withdraw from the study at any time. If a participant decides to voluntarily withdraw from the study and he/she alerts a member of the study personnel by phone, the participant will be asked to fill in a form of withdrawal and return to the site for an early exit (equivalent to Visit 6) assessment.

Upon early discontinuation, participants will be asked to return all unused study product. If it is determined during a study visit that a patient should be discontinued from the study early for safety or other reasons, the study investigators must immediately consult the principal investigator to discuss this matter. Whenever possible, all early exit procedures and assessments will be conducted.

10.8. Withdrawal criteria

A participant can be withdrawn from the study if he/she is not able or willing to use the study product in compliance with the protocol; a female of child-bearing potential becomes pregnant; or, if he/she is not willing to comply with the required study procedures at each visit.

Withdrawal of a participant **must** be discussed with the principal investigator, who will directly advise the participant of their need to withdraw. A 'Form of Withdrawal' must be completed by the participant indicating their intent to withdraw from the study.

11. Examination procedures, tests, equipment and techniques

11.1. Ophthalmic Examinations and fatty acid analysis (University of Melbourne)

11.1.1. Examination room

All study visits will be undertaken in a quiet, ophthalmic examination room that is maintained at an average temperature of $20 \pm 5^{\circ}\text{C}$, and humidity of $55 \pm 5\%$. Full room lighting is to be maintained for all procedures except for the examination of ocular surface staining, tear-break-up-time (TBUT) with sodium fluorescein and retinal fundus photographs.

11.1.2. Medical and ophthalmic histories and adverse events

At Visit 1, the study investigator will take a thorough medical and ophthalmic history. All current medications (including prescription and over-the-counter products) will be recorded on the appropriate CRF. Ophthalmic and general surgical histories will be documented. General medical conditions and current medications will be carefully checked against the study exclusion criteria (see Section 2.4.4). All known allergies will be documented, with an indication regarding the severity of the allergic reaction (if known).

At Visits 3, 4 and 6, the study investigator will ask the participant with regard to whether their general health or medications have changed, in any way, since the previous visit (this includes dosages/form of medications). Any procedures that the participant has undergone (e.g., an X-ray) will be documented on the appropriate CRF. Adverse events and serious adverse events will be documented as detailed in Section 7.3.1).

11.1.3. Diet questioning

At Visits 1, 3, 4 and 6, the study investigator will ask participants about their prior month intake of foods that are known to be naturally high in omega-3 essential fatty acids, EFAs (i.e., specific fish, oils and nuts). This information will be used to approximately assay the natural dietary intake of omega-3 EFAs.

11.1.4. Pregnancy test

At Visits 1 and 6, females of childbearing potential (see Section 2.4.4 for definition of 'childbearing potential' as applicable to this study) will undergo a urine pregnancy test, which will be performed by the examiner.

This procedure involves the (potential) participant providing a urine sample (approximately 20mL volume) into a collection cup, in the privacy of the clinic toilet. The participant will be advised to leave their urine sample on the bathroom bench and to return to the clinical examination room. The examiner will then immediately test the urine sample by following the directions provided in a commercially available urine pregnancy test. Medical examination gloves should be worn by the examiner when handling the sample and performing the pregnancy test with the participant's urine sample. Upon completion of the pregnancy test, the urine sample should be immediately disposed of via toilet flushing. The collection cup should also be disposed of.

A potential participant must have a negative pregnancy test result in order to be eligible to participate in this study. In the event that a pregnancy test result is returned as 'positive', the researcher will recommend that the potential participant consult their GP

(or another medical doctor if they do not have a GP). A letter will be provided to the participant to take to their doctor. The investigator will advise the patient that the doctor will be able to perform additional testing to confirm or refute whether they are pregnant and to facilitate appropriate medical care. The study optometrist will offer the patient the option of helping them facilitate this appointment with their chosen medical practitioner if required.

11.1.5. Habitual/best corrected visual acuity

At each visit, the habitual visual acuity will be measured using a Snellen visual acuity chart for testing at 3 metres. If the habitual visual acuity in each eye at Visit 1 is equal to or better than 6/12, this correction will be used for ALL study visits. If the habitual visual acuity in either eye is worse than 6/12, subjective refraction will be performed at Visit 1 to obtain the best spectacle corrected visual acuity (BSCVA) in each eye. If this is equal to or better than 6/12, provided that the participant meets all other requirements, participant will be enrolled into the study. If the BSCVA in either eye is worse than 6/12, the participant will not be able to participate in the study. The BSCVA will be recorded on the participant's CRF.

To provide standardised assessments of visual acuity during the study, all assessments should be consistently carried out using the same lighting conditions during the entire study (i.e., under normal consulting room lighting conditions such that the chart is evenly lit without glare).

The right eye will be tested first, with the left eye occluded by the examiner. The patient will be asked to start reading from the 6/24 line and to read the letters from left to right on each line; the patient should be informed that the chart contains letters only and no numbers. The patient should be asked to read slowly to achieve best identification of each letter and not to proceed to the next letter until he/she has given a definite answer.

If the patient changes a response (e.g., "that was a C not an O") before he/she has read aloud the next letter, then the change must be accepted. If the patient changes a response after having read the next letter, then the change will not be accepted. The examiner must not point to specific letters during the test.

When the letters become difficult to read, or if the patient identifies a letter as one of 2 letters, he/she should be asked to choose one letter and, if necessary, to guess. Patients should be encouraged to continue trying even when letters become difficult to read. Visual acuity testing should stop when 3 or more errors are made on the same line.

The left eye will then be tested using the same procedures outlined above.

11.1.6. EQ-5D-5L questionnaire

At Visits 1, 3, 4, and 6, participants will complete the EQ-5D-5L questionnaire (see below). The two-part questionnaire is a standardised measure of health status that provides a measure of health for clinical and economic appraisal (Herdman *et al.*, 2011).

The first part is a descriptive system comprises 5 domains: mobility, self-care, usual activities, pain/discomfort, and anxiety/depression. Each domain will be rated by the participant as one of five levels, from 1 (no problem) to 5 (extreme problems), for quantifying their general health state. Only one response will be permitted per domain. A five-digit code, generated from the ratings (e.g., 11111), will be converted into an index

value using the 'EQ-5D-5L Crosswalk Index Value Calculator' using the value set developed for the United Kingdom (available from <https://euroqol.org/eq-5d-instruments/eq-5d-5l-about/valuation-standard-value-sets/crosswalk-index-value-calculator/>).

The second part of the questionnaire is a visual analogue scale ranging from 0 (the worst

Figure 1: EQ-5D-5L (UK English sample version)

Under each heading, please tick the **ONE** box that best describes your health **TODAY**.

MOBILITY

I have no problems in walking about ☐

I have slight problems in walking about ☐

I have moderate problems in walking about ☐

I have severe problems in walking about ☐

I am unable to walk about ☐

SELF-CARE

I have no problems washing or dressing myself ☐

I have slight problems washing or dressing myself ☐

I have moderate problems washing or dressing myself ☐

I have severe problems washing or dressing myself ☐

I am unable to wash or dress myself ☐

USUAL ACTIVITIES (e.g. work, study, housework, family or leisure activities)

I have no problems doing my usual activities ☐

I have slight problems doing my usual activities ☐

I have moderate problems doing my usual activities ☐

I have severe problems doing my usual activities ☐

I am unable to do my usual activities ☐

PAIN / DISCOMFORT

I have no pain or discomfort ☐

I have slight pain or discomfort ☐

I have moderate pain or discomfort ☐

I have severe pain or discomfort ☐

I have extreme pain or discomfort ☐

ANXIETY / DEPRESSION

I am not anxious or depressed ☐

I am slightly anxious or depressed ☐

I am moderately anxious or depressed ☐

I am severely anxious or depressed ☐

I am extremely anxious or depressed ☐

We would like to know how good or bad your health is **TODAY**.

This scale is numbered from 0 to 100.

100 means the best health you can imagine.

0 means the worst health you can imagine.

Mark an X on the scale to indicate how your health is **TODAY**.

Now, please write the number you marked on the scale in the box below.

YOUR HEALTH TODAY =

The best health you can imagine

100

95

90

85

80

75

70

65

60

55

50

45

40

35

30

25

20

15

10

5

0

The worst health you can imagine

health you can imagine) to 100 (the best health you can imagine). Participants are asked to rate their health at the present moment (i.e., on the day of testing) by marking an 'X' on the scale and writing the number they have marked in the corresponding box.

11.1.7. Productivity costs questionnaire

At Visits 1 and 6, participants will complete the productivity costs questionnaire developed by the Institute for Medical Technology Assessment (iMTA) (Erasmus Universiteit Rotterdam, Rotterdam, 2013). This questionnaire estimates the indirect healthcare costs outside of the healthcare system that is associated with productivity losses (Bouwman *et al.*, 2015). The questionnaire will be administered in writing by the participant. The questionnaire includes general questions about the participant's education attainment and current work status and questions about productivity loss.

Productivity losses will be valued according to the iProductivity Cost Questionnaire (iPCQ) manual (<https://www.imta.nl/questionnaires/>). Scoring will be performed for the following modules: i) absenteeism; ii) presenteeism; iii) productivity losses due to unpaid work. Absenteeism will be scored based on the number of days a participant has missed work in the last four weeks as a result of being sick. If long-term absences were noted, defined as absences from work of longer than four weeks, absenteeism will be scored using the friction-cost method as described in the questionnaire scoring manual. Presenteeism will be calculated as (Number of workdays impaired) times by $(1 - (\text{efficiency score}/10))$ times by the number of hours per workday for that participant. Lost productivity due to unpaid work will be calculated as (the number of days missed) multiplied by (the number of house help was needed per day to make up the work).

11.1.8. Ocular surface disease index (OSDI) questionnaire

At Visits 1, 4 and 6, participants will complete an OSDI questionnaire (see below). The purpose of this assessment is to capture a range of ocular surface symptoms, including symptoms related to dry eye, their severity, and their impact on the patient's ability to function, scaled into a 0 (no disease) to 100 (maximum severity of disease) score. The participant's OSDI score will be calculated as (the sum of scores for all questions answered $\times 100$) divided by (total number of questions answered $\times 4$).

Have you experienced any of the following during the last week?	All of the time	Most of the time	Half of the time	Some of the time	None of the time	
1. Eyes that are sensitive to light? ..	4	3	2	1	0	
2. Eyes that feel gritty?	4	3	2	1	0	
3. Painful or sore eyes?	4	3	2	1	0	
4. Blurred vision?	4	3	2	1	0	
5. Poor vision?	4	3	2	1	0	
Have problems with your eyes limited you in performing any of the following during the last week?	All of the time	Most of the time	Half of the time	Some of the time	None of the time	N/A
6. Reading?	4	3	2	1	0	N/A
7. Driving at night?	4	3	2	1	0	N/A
8. Working with a computer or bank machine (ATM)?	4	3	2	1	0	N/A
9. Watching TV?	4	3	2	1	0	N/A
Have your eyes felt uncomfortable in any of the following situations during the last week?	All of the time	Most of the time	Half of the time	Some of the time	None of the time	N/A
10. Windy conditions?	4	3	2	1	0	N/A
11. Places or areas with low humidity (very dry)?	4	3	2	1	0	N/A
12. Areas that are air conditioned?...	4	3	2	1	0	N/A

OSDI questionnaire

11.1.9. Tear osmolarity

At Visits 1 and 6 tear osmolarity will be assessed. Following appropriate monthly (hyperosmolar control solutions) and daily (electronic test cards) calibration (as recommended by the manufacturer) of the measurement pen labelled '2', tear osmolarity will be measured at each visit in each eye. A 50 nanolitre tear sample will be taken from the inferior temporal tear meniscus using the TearLab™ Osmolarity System (TearLab™ Corp., San Diego, CA). Measurements will be taken once in each eye, beginning with the right eye and followed by the left eye. To avoid contaminating factors, participants will be instructed to not have instilled any eye drops at least two hours to their appointment.

If the testing card touches any eye lashes, the testing card will be discarded, and the measurement will be repeated in that eye. Tear osmolarity for both eyes will be recorded. For analysis, the tear osmolarity value used will be the higher value of the two eyes, taken from one eye per participant.

11.1.10. Non-contact corneal aesthesiometry

At Visits 1, 4 and 6, corneal sensitivity will be measured using a non-contact corneal aesthesiometer (NCCA). Both room temperature and cooled air will be used to stimulate nerves with different sensory receptors in different locations of the cornea. The instrument will be positioned to fire a small stream of air, with a predetermined pressure (measured in mBar) as set by the examiner, from 1 cm away from the cornea. Both

central cornea (i.e., half way between the temporal and nasal limbus) and inferior peripheral cornea (i.e., at 1mm above the most inferior limbus point) will be measured.

The participant will be seated comfortably on the slit lamp biomicroscope, where the instrument will be mounted, and asked to wear over-the-ear headphones, which will transmit a series of auditory cues to run each trial. The participant will also be given a small number pad with tactile pads to register their response to detecting a stimulus (i.e., yes and no).

The auditory cue consists of a short beep (0.5s) followed by a brief break (2s) and a long beep (4 seconds). For each individual trial, participants will be instructed that they should blink as frequently as they wish when they hear the first beep, but to stop blinking as soon as they hear the start of the second (i.e., long) beep as they may or may not feel a small stream of air during that time. The audio cue will be terminated by a double beep to indicate the end of the trial; after hearing this cue, the participant should register their response (i.e., yes or no) on the keypad.

The sensory threshold will be determined using a double-staircase threshold technique designed using a customised MATLAB software. Trials will be repeated at discrete increments of 0.1 mbar for three reversals, until a final threshold can be calculated.

If participants respond 'yes' to two stimuli at 0.1 mbar, false positives will be checked by the examiner by initiating the testing sequence, but not releasing a stimuli. If participants respond 'yes' to two false positive checks, testing will stop, and the participants will be reinstructed. After instructions are given to the participant, testing will re-start from the beginning of the double staircase algorithm for that measurement.

Corneal sensitivity thresholds will be measured for the central and peripheral cornea to both room and cooled stimuli (4 measurements). For the cooled air setting, the instrument cooling will be turned on for three minutes prior to re-initiation of testing, as per manufacturer's instructions.

11.1.11. Basal tear sample collection

At Visits 1 and 6, tear samples will be collected using disposable 20µL glass microcapillary tubes (Drummond Scientific Co, USA). The participant will lie in a lateral decubitus position on a medical examination table. A 10µL basal (non-stimulated) tear sample will be collected via capillary flow from each eye, from the lateral tear meniscus, with the lateral canthus gently opposed. Tear samples will be collected from the right eye first, followed by the left eye. To minimise any potential reflex tearing, utmost care will be taken not to directly contact the conjunctiva, cornea or eyelid margins with the micro-capillary tube during tear collection. Tear collection flow rate will be monitored for each sample collected. Any samples with a flow rate greater than 10µL/min will be discarded to exclude dilution effects caused by reflex tearing. The collected tear samples will be immediately transferred to appropriately-labelled 50µL Eppendorf vials (Eppendorf, Westbury, NY) and stored at 4°C. Within four hours of collection, the vials will be securely stored, in locked facilities, at -80°C until required for multiplex-CBA analysis.

Note: All Eppendorf vials will be double-labelled (with permanent marker on the cap of the vial and with a paper/sticky-tape label on the vial, according to the pre-defined labelling schema).

11.1.12. Phenol red thread test

At Visits 1 and 6, phenol red thread test will be performed as a measure of resting tear volume. The phenol red thread (Entol Research Cell, London, UK) will be gently folded and removed by the examiner from the sterile packaging without touching the thread. The folded end will be hooked in the participant's inferior lid by gently pulling down the inferior lid margin, approximately a third of the way from the temporal canthus. After exactly 15 seconds, as timed by a stopwatch, the thread will be removed, and the wetted length will be measured immediately using the ruler supplied on the packaging and recorded. Examination will be performed in both eyes; the right eye will be assessed first.

11.1.13. Assessment of anterior chamber angle

At Visits 1 and 6, the anterior chamber angle will be assessed using the Van Herick technique to estimate the depth of the angle, before instillation of the mydriatic (0.5% tropicamide) eye drops. This assessment will be performed at the slit lamp using a single optic section projected onto the peripheral cornea at 16X magnification, using the brightest illumination.

The peripheral anterior chamber depth will be estimated against the peripheral corneal depth to obtain a subjective estimation of the depth of the peripheral anterior chamber angle.

11.1.14. Slit lamp biomicroscopy examination

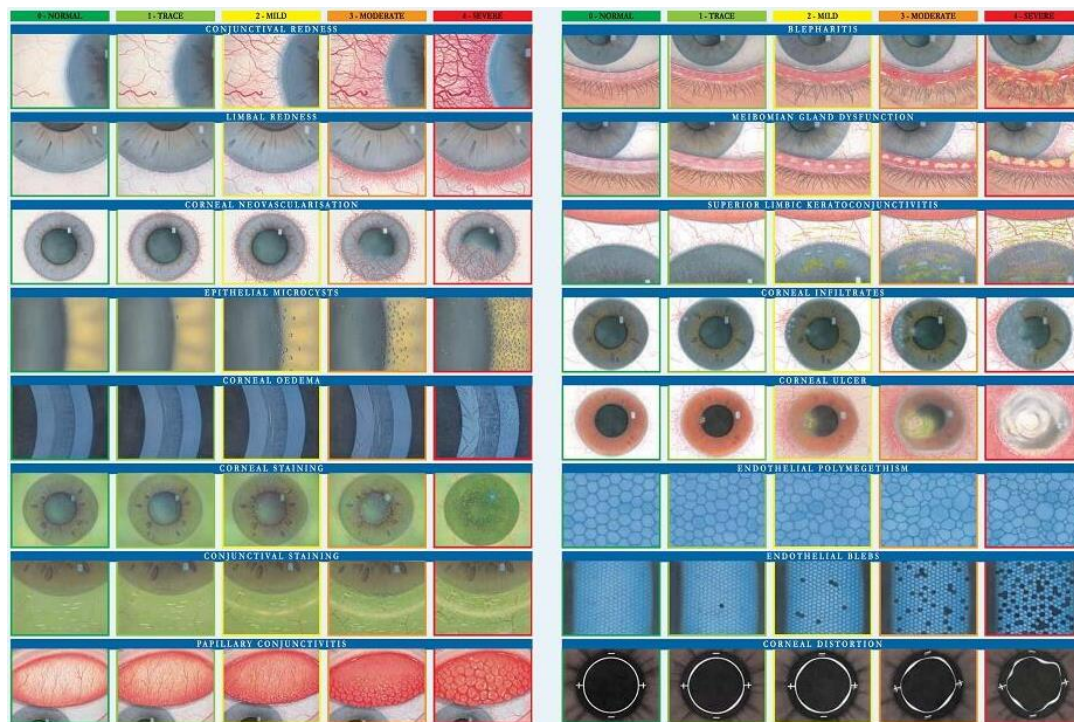
At each visit, slit lamp biomicroscopy (without pupil dilation) will be performed on both eyes, with the room lights off. Observations for the slit lamp biomicroscopy examination (using white light and 10-16x magnification) will be graded using the standardised Efron grading scale for:

- Conjunctiva: nasal and temporal redness
- Limbal redness
- MG dysfunction: extent of capping
- Blepharitis (anterior)

Grades range from 0 to 4, and will be recorded in 0.1 step increments. Observations with regard to clinically notable findings will be recorded for the conjunctiva, cornea, and tear film.

The presence of anterior chamber inflammation will be evaluated using a maximum intensity white light, under 25X magnification and a 1x1mm slit beam. The presence of cells and flare will be graded using the Standardization of Uveitis Nomenclature (SUN) grading scales (as per the grading scheme below).

SUN Grading Scheme for Anterior Chamber Cells		SUN Grading Scheme for Anterior Chamber Flare	
Grade	Cells in 1mm x 1 mm slit lamp beam	Grade	Description
0	<1	0	None
0.5+	1-5	1+	Faint
1+	6-15	2+	Moderate (Iris and lens details clear)
2+	16-25	3+	Marked (Iris and lens details hazy)
3+	26-50	4+	Intense (Fibrin or plastic aqueous)
4+	>50		

SUN Grading Schemes for Anterior Chamber Cells and Flare (Jabs *et al.*, 2005)

Efron anterior eye grading scale

11.1.15. Tear break up time (TBUT) with fluorescein

At Visits 1, 4 and 6, TBUT with fluorescein will be measured in both eyes. The sodium fluorescein strips (Amcon dry eye tests, DET Nomax Inc., St Louis MO) will be moistened with non-preserved saline and applied onto the superior bulbar conjunctiva with the upper eyelid retracted and the patient in downgaze to deliver an approximate fluid volume of 1µL to each eye. The NaFL strip will not be shaken after wetting with saline. A single instillation of sodium fluorescein should be used for both TBUT and corneal staining.

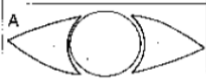
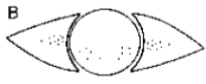
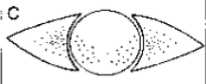
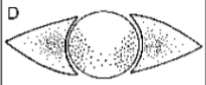
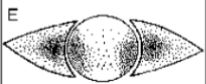
TBUT will be performed with a slit lamp at 10X magnification using a cobalt blue illumination and a yellow barrier filter (Wratten filter) to enhance contrast, set at the highest illumination intensity. Participants will be asked to first blink normally 2 to 3 times to evenly spread the fluorescein then to not blink for as long as they can. The

timing of the stopwatch will begin as soon as they start holding the blink. The stopwatch is stopped on the first occurrence of true tear break-up, not just local thinning or tear film irregularity. Three consecutive TBUTs will be performed per eye and the time in seconds for each of the three measurements will be recorded (to two decimal places).

11.1.16. Sodium fluorescein (NaFl) staining – Oxford scale

At each visit, NaFl staining of the cornea will be evaluated in each eye. Sodium fluorescein staining will be evaluated using a slit lamp with full slit width and 16X magnification with cobalt blue illumination and a yellow barrier filter (Wratten filter) to enhance contrast. The yellow barrier filter must be placed directly in front of the objective lens of the slit lamp. The slit lamp's light source must also be set to high intensity (increased voltage) when the cobalt blue and enhancement filters are in place.

NaFl staining should be graded two minutes after instillation of the sodium fluorescein, during which time TBUT is measured. Corneal staining will be assessed using the 5-point Oxford scale (see below), ranging from 0 to 5, in 0.1 grading steps.

PANEL	GRADE	VERBAL DESCRIPTOR
	0	Absent
	I	Minimal
	II	Mild
	III	Moderate
	IV	Marked
>E	V	Severe

Oxford grading scale

11.1.17. Intra-ocular pressure (IOP)

At Visits 1 and 6, intraocular pressure (with anaesthesia and fluorescein) will be measured for each eye. One drop of 0.5% proxymetacaine hydrochloride (Alcon Laboratories, French Forest, NSW, Australia) will be instilled into the lower conjunctival sac. After exactly four minutes, the participant should gently close their eyes and any excess fluid should be gently dabbed away from the eyelids using a tissue. Direct, active blotting of the *cul de sac* is not permitted.

Measurements should be taken using a calibrated Goldmann applanation tonometer. Additional sodium fluorescein can be applied if needed. One measurement should be recorded for each eye (in mmHg); the time of measurement should also be noted.

11.1.18. Corneal confocal microscopy

At Visits 1, 4 and 6, corneal confocal microscopy will be performed to capture *in-vivo* images of the corneal sub-basal nerve plexus, using a Heidelberg Retinal Tomograph III with the Rostock Corneal Module (Heidelberg Engineering GmbH, Heidelberg, Germany). Topical anaesthesia will be instilled using the methods described above. A

high-viscous eye gel (GenTealEyes. Novartis, North Ryde. NSW, Australia) will couple a single-use, sterile polymethylmethacrylate 'Tomocap' to the microscope objective lens.

Participants will be asked to fixate on a central target with the non-dominant eye while the right eye (i.e., same eye tested as NCCA) is examined. A sufficient number of images will be captured using "sequence" mode setting on the microscope of the central corneal region as a representation of that area. Participants will then be asked to fixate on a superior target while the inferior cornea (1mm above the inferior most limbus) will be imaged using the same methods as above.

Raw images will be saved on a secure server that is only accessible to the investigators. All captured images that are blurred, where the imaging depth was inconsistent, or where excessive vignette obstructs nerve visibility will be removed from the analysis sample.

For each participant, 12 images from the central cornea and eight images from the peripheral cornea will be randomly selected for analysis. Images will be checked by the investigator to ensure that any overlap is kept minimal (i.e., less than 20%).

Central corneal sub-basal nerve parameters will be quantified using an automatic image analysis software (ACCMetrics software v2, The University of Manchester) (Dabbah *et al.*, 2011). Sub-basal nerve parameters for each participant will be taken as the average value of the twelve images analysed for the following corneal nerve parameters: corneal nerve fibre length (CNFL; mm/mm²), corneal nerve fibre density (CNFD; fibres/mm²), corneal nerve branch density (CNBD; branches on main fibre/mm²) and corneal nerve total branch density (CTBD; total branches/mm²).

For the peripheral cornea, images will be analysed using ImageJ software (ImageJ; U. S. National Institutes of Health, Bethesda, Maryland, USA; <https://imagej.nih.gov/ij/>). Each image will be manually traced using NeuronJ software (Meijering *et al.*, 2004). For each image, CNFL (mm/mm²) will be derived as (The Sum of total nerve length) divided by 0.16. For each participant, the CNFL will be taken as the average value of the eight images analysed.

Central and peripheral dendritic cell count will be performed for each participant using the same set of randomly selected images as for corneal sub-basal nerve parameters. Dendritic cell count will be performed using the cell count plugin on ImageJ. For the central cornea, cell count will be stratified into three morphological phenotypes according to Lagali *et al.*, (2018), as the following: i) type-1, putative mature dendritic cells (mDCs), as bright, reflective cells with extensions of long-dendrites; ii) type-2; putative immature dendritic cells (imDCs), as small, reflective bodies either without dendrites or with short dendrites, and iii) type-3; putative globular cells, as round or oval cells bodies, that are round and larger than DCs and without any visible dendrites (Lagali *et al.*, 2018). For the peripheral cornea, the total dendritic cell count per image will be quantified (without any putative subtype classification). The number of cells per meters squared (cells/mm²) will be calculated as (the number of cells per image) divided by 0.16.

11.1.19. Systemic fatty acid pin-prick test

At Visits 1 and 6, a finger pin-prick test will be taken to obtain a small amount of blood samples for fatty acid analysis. Gloves will be worn by the examiner while performing the procedure. The side of the participant's finger will be lightly rubbed to stimulate blood flow and thoroughly rubbed with isopropyl alcohol. A trigger of the pin-prick device will be released to release a small, shallow lancet against the testing area to create a pin-prick puncture. 2-3 drops of blood will be released after gentle squeezing and mounted directly onto PUFAcoat paper supplied in the testing kit.

All test kits will be labelled with the participant's corresponding code, sealed and couriered to Waite Lipid Analytic Service (The University of Adelaide) for analysis within four weeks from collection date, as per manufacturer's instructions.

11.1.20. Diabetic retinopathy grading

At Visits 1 and 6, diabetic retinopathy will be assessed through a dilated pupil, and retinopathy stages will be graded using the simplified ETDRS (Wisconsin) classification below.

Retinopathy stage	Definition
Minimal NPDR (level 20)	Ma only
Mild NPDR (level 35)	Ma and one or more of: retinal haem, HEx, CWS, but not meeting Moderate NPDR definition
Moderate NPDR (levels 43, 47)	H/Ma $\geq$ std photo 2A in at least one quadrant and one or more of: CWS, VB, IRMA, but not meeting Severe NPDR definition
Severe NPDR <i>preproliferative</i> (level 50+)	Any of: H/Ma $>$ std photo 2A in all four quadrants, IRMA $>$ std photo 8A in one or more quadrants, VB in two or more quadrants
PDR (level 60+)	Any of: NVE or NVD $<$ std photo 10A, vitreous/ preretinal haem and NVE $<$ ½ disc area (DA) without NVD
High-risk PDR (level 70+)	Any of: NVD $>$ ¼ to ½ disc area, or with vitreous/ preretinal haem, or NVE $>$ ½ DA with vitreous/ preretinal haem
Advanced PDR	High-risk PDR with tractional detachment involving macula or vitreous haem obscuring ability to grade NVD and NVE
Macular Oedema	Retinal thickening within 2 disc diameters of macular centre
Clinically Significant Macular Oedema (CSME)	Retinal thickening within 500µm of macular centre or hard exudates within 500µm of macular centre with adjacent thickening

Classification of diabetic retinopathy into retinopathy stages derived from NHMRC "Clinical Practice Guidelines for the Management of Diabetic Retinopathy"

In addition, non-invasive images will be taken of the retina, including retinal fundus photographs and optical coherence tomography (OCT) images, as a permanent record of

posterior segment health. For retinal fundus photography, two 30-degree retinal fundus photographs will be taken from each eye at macula and disc fixations. The following OCT images will be captured: 1) Two OCT-angiography scans of the central macula (3.0x3.0 mm) in the right eye only; 2) Two OCT-angiography scans of the optic nerve head (6.0x6.0 mm) in the right eye only; 3) One standard OCT scan of the optic nerve head in the right eye only; 4) One standard OCT scan of the central macula (horizontal) in both eyes.

11.2. Nerve fibre function testing (St Vincent's Hospital, Melbourne)

At Visits 2 and 5, detailed neurophysiological assessment of small and large peripheral nerve fibres will be performed. These testing procedures will involve:

11.2.1. Michigan Neuropathy Screening Index

The Michigan Neuropathy Screening Index (MNSI) will be administered by the investigator (see below). Participants must score >3 in the two-part screening instrument, across both part A and part B, to be eligible for inclusion in the study. The physical assessment component (Part B) will be performed by a trained examiner.

Patient Version

MICHIGAN NEUROPATHY SCREENING INSTRUMENT

A. History (To be completed by the person with diabetes)

Please take a few minutes to answer the following questions about the feeling in your legs and feet. Check yes or no based on how you usually feel. Thank you.

1. Are you legs and/or feet numb?	☐ Yes	☐ No
2. Do you ever have any burning pain in your legs and/or feet?	☐ Yes	☐ No
3. Are your feet too sensitive to touch?	☐ Yes	☐ No
4. Do you get muscle cramps in your legs and/or feet?	☐ Yes	☐ No
5. Do you ever have any prickling feelings in your legs or feet?	☐ Yes	☐ No
6. Does it hurt when the bed covers touch your skin?	☐ Yes	☐ No
7. When you get into the tub or shower, are you able to tell the hot water from the cold water?	☐ Yes	☐ No
8. Have you ever had an open sore on your foot?	☐ Yes	☐ No
9. Has your doctor ever told you that you have diabetic neuropathy?	☐ Yes	☐ No
10. Do you feel weak all over most of the time?	☐ Yes	☐ No
11. Are your symptoms worse at night?	☐ Yes	☐ No
12. Do your legs hurt when you walk?	☐ Yes	☐ No
13. Are you able to sense your feet when you walk?	☐ Yes	☐ No
14. Is the skin on your feet so dry that it cracks open?	☐ Yes	☐ No
15. Have you ever had an amputation?	☐ Yes	☐ No

Total: _____

MICHIGAN NEUROPATHY SCREENING INSTRUMENT

B. Physical Assessment (To be completed by health professional)

1. Appearance of Feet

	Right		Left
a. Normal	☐ 0 Yes ☐ 1 No		Normal ☐ 0 Yes ☐ 1 No
b. If no, check all that apply:			If no, check all that apply:
Deformities	☐		Deformities ☐
Dry skin, callus	☐		Dry skin, callus ☐
Infection	☐		Infection ☐
Fissure	☐		Fissure ☐
Other	☐		Other ☐
specify: _____			specify: _____
	Right		Left
	Absent Present		Absent Present
	☐ 0 ☐ 1		☐ 0 ☐ 1
2. Ulceration			
	Present/Reinforcement		Present/Reinforcement
	Absent		Absent
	☐ 0 ☐ 0.5 ☐ 1		☐ 0 ☐ 0.5 ☐ 1
3. Ankle Reflexes			
	Present		Present
	☐ 0		☐ 0
	Decreased		Decreased
	☐ 0.5		☐ 0.5
	Absent		Absent
	☐ 1		☐ 1
4. Vibration perception at great toe			
	Normal		Normal
	☐ 0		☐ 0
	Reduced		Reduced
	☐ 0.5		☐ 0.5
	Absent		Absent
	☐ 1		☐ 1
5. Monofilament			
	Normal		Normal
	☐ 0		☐ 0
	Reduced		Reduced
	☐ 0.5		☐ 0.5
	Absent		Absent
	☐ 1		☐ 1

Signature: _____

Total Score _____ /10 Points

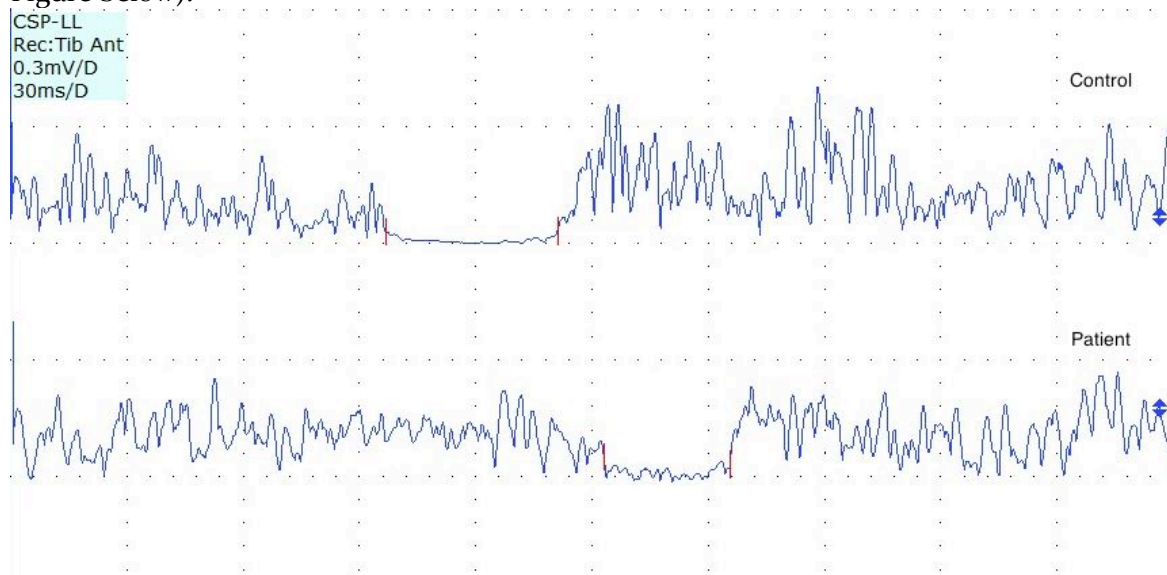
11.2.1 Michigan Diabetic Neuropathy Score (MDNS) and routine nerve conduction studies (NCS)

The first part of the MDNS is a clinical examination, which is then followed by routine nerve conduction studies. Vibratory threshold perception is assessed with a 128 Hz tuning-fork. Light touch is assessed with a 10g monofilament applied to the dorsum of the great toe. Deep tendon reflexes and muscle strength testing formed the remainder of the clinical examination score. Nerve conduction studies are then performed using an electromyographic (EMG) device (Dantec Keypoint G4 Workstation), examining the right upper and lower extremities, and included median, peroneal and tibial motor studies, and sural, median and ulnar sensory studies. Abnormal parameters are defined as amplitudes, conduction velocities or latencies that exceeded two standard deviations from the normal values of our laboratory. Testing is performed with a target temperature of 32 degrees Celsius in the upper extremity and 30 degrees Celsius in the lower extremity. Participants are given a composite score (from class 0 to 3) based on their clinical examination and nerve conduction study results.

11.2.2 Cutaneous Silent Periods (CSPs)

The CSP is recorded from both upper and both lower extremities using the same EMG device above. Filters are set at 20 Hz to 10 kHz, sweep speed is 300 ms, and sensitivity is 300 μ V. In the upper extremities, electrical stimulations are delivered to the second digit using ring electrodes (median nerve). The sensory threshold is first determined by gradually increasing electrical current stimulations of 0.5 ms duration, and the participant indicated the lowest current intensity that is felt. Standard recording electrodes are placed with the recording electrode over the contracting abductor pollicis brevis (APB) muscle, and the reference electrode at the base of the thumb. The patient

abducts the thumb at steady sub-maximal contraction. The technician provides downwards resistance, using EMG sound to provide auditory feedback and semi-quantitatively ensure that contraction is approximately 50% of maximal. While the participant is maintaining this contraction, 10 consecutive electrical stimuli of 15 times the sensory threshold are delivered to the index finger. If the CSPs obtained are poorly formed or absent, and the patient does not perceive the stimuli as painful, then stimulus intensity is increased in 10 mA increments up to 100 mA. Traces are then averaged and rectified. The CSP onset latency is then quantitatively defined by the time after stimulation when the EMG signal drops below 50% of baseline, and the CSP duration is defined by the time period when the EMG signal was less than 50% of the baseline (see Figure below).



Top – Control: normal onset latency and duration

Bottom – Patient: abnormal prolonged onset latency and shortened duration

The process is repeated in the lower extremities in a similar manner, using a stimulating probe behind the lateral malleolus (sural nerve), with the recording electrode over the belly of tibialis anterior whilst the patient maintains steady ankle dorsiflexion.

Reference values for CSP are taken from St Vincent's Hospital Melbourne reference values for small fibre testing, as below

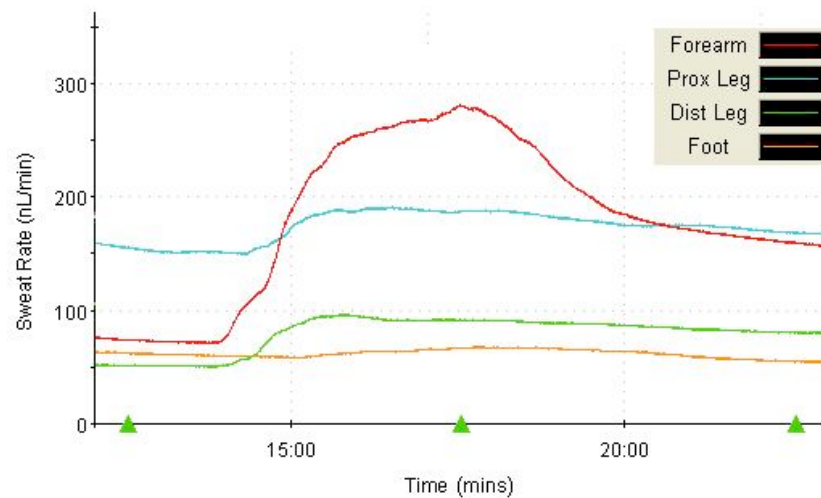
	Onset (ms)	SD (ms)	ULN (ms)	Duration (ms)	SD (ms)	LLN (ms)
Upper extremity	72.9	7.9	86.4	51.1	9.6	33.8
Lower extremity	100.7	9.2	118.7	54.4	10.7	38.3

n=50 (26 M, 24F). Reference limits are in **bold**.

11.2.3 Quantitative Sudomotor Axonal Reflex Testing (QSART)

QSART is performed using the Q-Sweat unit (WR Medical Electronics Co., Stillwater, Minnesota), which uses a desiccant pack as its dry air source. Four multi-compartment sweat capsules are placed over standard recording sites on the right side at the forearm, proximal leg, distal leg and foot. A 10% acetylcholine gel is iontophoresed into the skin at a constant current of 2 mA for five minutes to stimulate postganglionic sudomotor

unmyelinated nerves, and the sweat responses are assessed during this time plus an additional five minutes (see Figure below).



Example of abnormal reduced sweat volume from foot site.

Reference values for QSART are taken from St Vincent's Hospital Melbourne reference values for small fibre testing, as below

	Total vol (uL)	LLN (uL)	ULN (uL)	Latency	LLN (s)	ULN (s)
Forearm						
Men	2.0	0.5	3.7	102	28	191
Women	1.1	0.3	2.9	82	35	148
Prox Leg						
Men	1.6	0.4	3.9	107	47	197
Women	1.4	0.4	3.9	84	37	153
Distal Leg						
Men	1.9	0.3	3.3	95	24	190
Women	1.2	0.4	3.2	79	38	143
Foot						
Men	1.1	0.2	2.6	128	45	190
Women	0.8	0.2	1.5	116	42	353

n=40 (20 M, 20F). Reference limits are in **bold**.

11.2.4 Nerve Excitability Test

Sensory and motor axonal excitability studies are undertaken on the median nerve. Axonal excitability studies are performed using an automated computerized system (Qtrac® Institute of Neurology, Queens Square, UK). Stimulation is computer controlled and converted to current using an isolated linear bipolar constant current simulator (maximal output ± 50 mA; DS5, Digitimer, Welwyn Garden City, UK). The median nerve is stimulated at the wrist with an anode electrode placed 10 cm proximal over bone. Compound motor action potentials (CMAPs) are recorded from the abductor pollicis brevis muscle with the reference electrode placed 4 cm distal. Compound sensory action potentials (CSAPs) are recorded from the second digit, utilizing ring electrodes placed at the proximal and distal interphalangeal joints for recording and reference, respectively. Electronic noise is removed (via Hum Bug 50/60 Hz Noise Eliminator, Quest Scientific Instruments, North Vancouver, Canada). Temperature is monitored to ensure this is maintained above 32 degrees Celsius.

Multiple excitability parameters are recorded via the TROND protocol including: (i) threshold electrotonus (TE), a measure of inter-nodal conductances and membrane potential; (ii) recovery cycle (RC) of excitability, an assessment of the recovery of excitability following an action potential marking the function of nodal Na⁺ channels and (iii) current–threshold relationship (I/V), a measure of the rectifying properties of the axon. Target amplitude for threshold tracking is automatically set to approximately 40% of supramaximal response amplitude, utilizing the area of steepest slope of the stimulus response curve. Changes in the threshold current required achieving the target amplitude are tracked on-line, with the tracking steps proportional to the error between target amplitude and actual response.

11.3 Basic blood samples

Blood tests to assess diabetes control will be performed, including fasting blood sugar level and HbA_{1c}. Patients that have any disorder other than diabetes that could cause symptoms or signs of peripheral neuropathy will be excluded from participating. Thus, participants will be tested for B₁₂ levels, folate levels, lipid profile, inflammatory markers (CRP, ESR), liver function and electrolytes.

12 APPENDICES

12.2 Informed consent materials

Participant information sheet/consent form

12.3 Biological Specimens

The following biological specimen will be collected for this study

- Tear samples (as per section 11.1.10)
- Blood capillary samples (as per section 11.1.18)
- Basic blood samples (as per section 11.3)
- Urine samples (as per section 11.1.4)

At the time of collection, tubes containing the tear samples will be labelled with a participant ID number (assigned by the researchers) and the laterality (R or L eye). Similarly, PUFA-coated test cards with the blood spot assays will be labelled with a participant ID number. This method allows the privacy of the donor to be protected. This system also keeps the samples identity unknown to the people involved in the analyses of the samples

Females of childbearing potential will undergo a urine pregnancy test, to confirm their eligibility to participate, which will be performed by the study investigator. Upon completion of the pregnancy test, the urine sample and collection will be immediately disposed of.

All other samples (including tear samples and blood spot assay samples) will be destroyed once the research is completed. The samples will be disposed of according to standard laboratory practices in a manner that does not risk the confidentiality of the participants.

Blood samples will be collected for this study and can form part of the participant's routine care by St Vincent's Pathology. Testing will be performed by experienced

personnel at St Vincent's Pathology, upon request with a pathology slip as provided by Prof Richard MacIsaac. Results collected as a part of this study will then be entered into the participants CRF in coded forms as required.

12.4 Abbreviation

UoM	The University of Melbourne
SVHM	St Vincent's Hospital (Melbourne)
PUFA	Polyunsaturated Fatty Acids
EPA	Eicosapentaenoic Acid
DHA	Docosahexaenoic Acid
CNFL	Corneal Nerve Fibre Length
CNFD	Corneal Nerve Fibre Density
CNBD	Corneal Nerve Branch Density
CDC	Corneal Dendritic Cell
IVCM	In-Vivo Confocal Microscopy
NCCA	Non-Contact Corneal Aesthesiometry
BCVA	Best Corrected Visual Acuity
NaFl	Sodium Fluorescein
TBUT	Tear Break-Up Time
IOP	Intraocular Pressure
OSDI	Ocular Surface Disease Index
OPAS	Ocular Pain Assessment Score
DFE	Dilated Fundus Examination
AACG	Acute Angle Closure Glaucoma
HbA _{1c}	Glycated Hemoglobin
FBC	Full Blood Count
CRP	C-Reactive Protein
ESR	Erythrocyte Sedimentation Rate
LDL	Low-density lipoprotein
HDL	High-density lipoprotein
MDNS	Michigan Diabetic Neuropathy Score
QSART	Quantitative Sudomotor Axonal Reflex Test
CSP	cutaneous silent period
NCS	Nerve Conduction Studies
QST	Quantitative Sensory Test
CPI	Coordinating Principal Investigator
AEs	Adverse Events
SAEs	Serious Adverse Events
SUSAR	Suspected Unexpected Serious Adverse Reaction
TGA	Therapeutics Goods Administration
WLAS	Waite Lipid Analytical Services
REDCap	Research Data Capture
CRF	Case Report Form

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Appendix G. Chapter 7: Supplementary materials [2 of 3]

nPROOFS₁ Statistical Analysis Plan

Statistical analysis plan for the randomised controlled clinical trial:

“Investigating the neuroPRotective effect of Oral Omega-3 Fatty acid
Supplementation in type 1 diabetes (nPROOFS₁): a randomised, placebo-
controlled trial”

Statistical Analysis Plan

“A randomised controlled trial to evaluate the effects of omega-3 fatty acid supplementation on peripheral nerve health in type-1 diabetes”

Version: 1.4

Authors:

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Date: <30-06-2020>

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1. Introduction

1.1 Primary objective

The aim of this randomised, double-masked, placebo-controlled superiority clinical trial is to evaluate the effects of a six-month supplementation period with long-chain omega-3 polyunsaturated fatty acid (PUFA) supplements on peripheral nerve parameters in individuals with type-1 diabetes.

1.2 Study design

This study is a single-centre, double-masked, randomised, two-arm, parallel-group interventional trial comparing the effects of oral omega-3 PUFA supplements relative to olive oil (placebo) supplements over six months. Participants are randomised according to a 1:1 allocation ratio to either long-chain omega-3 PUFA supplements (defined below) or an olive oil (placebo) supplement.

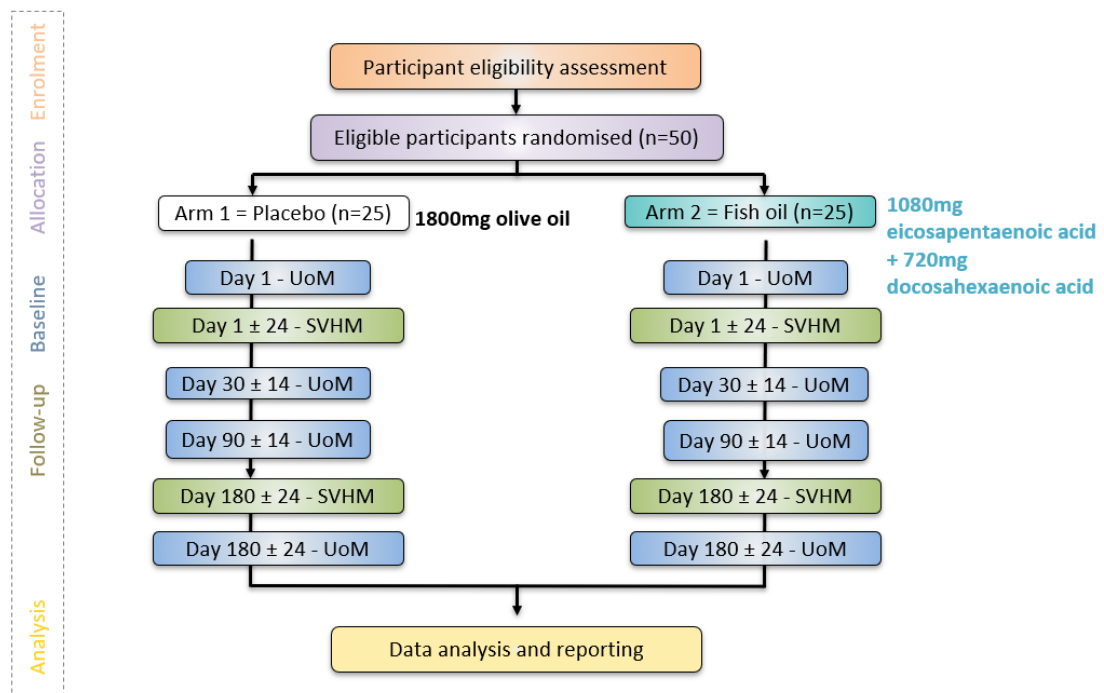
1.3 Study setting

This study is being conducted at the Department of Optometry and Vision Sciences at The University of Melbourne, with recruitment and data collection undertaken both at this site and the Departments of Endocrinology and Neurology at St Vincent's Hospital, Melbourne.

1.4 Participants

A total of 50 participants with type-1 diabetes mellitus were planned to be recruited. The first participant was enrolled on July 6, 2018; the last participant's last visit took place on March 10, 2020.

Given a two-arm parallel design, with a pre-specified primary outcome of corneal nerve fibre length (CNFL), taken as a continuous measure, for 80% power at a confidence level of 95%, using an estimated true difference between intervention arms of 2.9 units, mm/mm², (Chinnery et al., 2017), based on an expected standard deviation of 3.2 units, the required sample size was determined to be 21 participants per group. To allow for 15% participant attrition, the sample size within each group was increased by this amount, giving a need to recruit a total of 25 participants in each intervention arm (n=50 for the trial; Figure 1). During the recruitment phase, the target sample size was reduced to 43 in total, in view of the timeline feasible for completing the study and having had fewer dropouts than anticipated.

Figure 1. Planned study flow diagram

Abbreviations: SVHM, St Vincent's Hospital Melbourne; UoM, University of Melbourne.

1.5 Data source

Following direct recording of data onto hard-copy source worksheets, the study data was entered into REDCap, a secure web-based application for research data capture, management and analysis (hosted on the University of Melbourne data centre infrastructure, provided via the National eResearch Collaboration tools and Resources project).

All data collected directly by the investigators for this study was de-identified using a participant ID code, and the participant ID code was used as an identifier on all study documentation, and for the labelling of body fluid samples (i.e., blood, tears), which are processed off-site.

The following data and information was to be recorded:

- Identifiable participant information at baseline: name; contact details; study enrolment date
- A statement that written informed consent (including the date) was obtained prior to any study procedures being performed
- Participant ID number
- Dates of all study visits
- Demographic information at baseline (age, sex, date of birth, ethnicity)

- Height and weight at baseline
- Medical and ophthalmic histories at baseline
- Pre-study and concomitant medications, including insulin type, administration method and dosage
- Study procedures performed at the University of Melbourne (UoM)
 - Habitual distance refractive correction in each eye at baseline.
 - Habitual distance visual acuity in each eye at baseline, day 30 $\pm$ 14, day 90 $\pm$ 14 and day 180 $\pm$ 24.
 - EQ-5D-5L questionnaire score at baseline, day 30 $\pm$ 14, day 90 $\pm$ 14 and day 180 $\pm$ 24.
 - Ocular Surface Disease Index (OSDI) questionnaire score at baseline, day 90 $\pm$ 14 and day 180 $\pm$ 24.
 - Tear osmolarity in each eye at baseline and day 180 $\pm$ 24.
 - Corneal sensitivity thresholds in the right eye at baseline, day 90 $\pm$ 14 and day 180 $\pm$ 24.
 - Phenol red thread test score in each eye at baseline, day 90 $\pm$ 14 and day 180 $\pm$ 24.
 - Tear collection time and volume collected at baseline and day 180 $\pm$ 24.
 - Slit lamp examination gradings in each eye at baseline, day 90 $\pm$ 14 and day 180 $\pm$ 24.
 - Intraocular pressure (IOP) in each eye at baseline and day 180 $\pm$ 24.
 - Diabetic retinopathy (DR) and diabetic macular oedema grading (Wisconsin classification) (ETDRS Research Group, 1991) in each eye at baseline and day 180 $\pm$ 24.
 - Optical coherence tomography angiography (OCT-A) imaging at baseline and day 180 $\pm$ 24.
 - Corneal sub-basal nerve plexus and dendritic cell parameters, measured using *in vivo* confocal microscopy (IVCM) in the right eye at baseline, day 90 $\pm$ 14 and day 180 $\pm$ 24.
 - Systemic fatty acid concentrations at baseline and day 180 $\pm$ 24.
- Study procedures performed at St Vincent's Hospital Melbourne (SVHM)
 - Michigan Neuropathy Screening Instrument (MNSI) and Michigan Diabetic Neuropathy Score (MDNS) examination score at baseline and day 180 $\pm$ 24.
 - Nerve conduction study (NCS) parameters for the median motor, peroneal motor, tibial motor, median sensory, sural sensory, ulnar sensory nerves at baseline and day 180 $\pm$ 24.

- Cutaneous silent periods (CSP) measurements of the upper and lower limbs at baseline and day 180 ± 24 .
- Quantitative Sudomotor Axonal Reflex Test (QSART) measurements of the forearm, proximal leg, distal leg and foot at baseline and day 180 ± 24 .
- Blood test results
 - Blood pathology test results at baseline and day 180 ± 24 .
 - Systemic fatty acid profiles at baseline and day 180 ± 24 .
- Occurrence, grading and status of any adverse events, including their potential association with the investigational product or a study procedure
- Number and proportion of supplements returned for each participant
- Participant's and examiner's forced-choice guesses of the treatment allocation (omega-3 PUFAs or placebo) on day 180 ± 24 .
- The date the participant exited the study, and a notation as to whether participant completed the study,

2. Analysis Objectives

2.1 Aim 1

To evaluate the effects of a six-month supplementation period with oral long-chain omega-3 PUFA supplements on peripheral nerve health in individuals with type-1 diabetes, as measured by the change from baseline in central corneal nerve fibre length (CNFL) at day 180.

2.2 Aim 2

To evaluate the effects of a six-month supplementation period with oral long-chain omega-3 PUFA supplements in individuals with type-1 diabetes on the change from baseline at day 180 for:

Key secondary outcomes:

Other corneal nerve structure and function parameters

[2 main variables for corneal nerve structure: central corneal nerve branch density (CNBD) and central corneal nerve fibre density (CNFD)

2 main variables for corneal nerve function: central corneal sensitivity thresholds to room and cooled temperatures]

Secondary outcomes:

- Patient-reported quality of life

[2 main variables: EQ-5D-5L VAS and Index]

- Small nerve fibre function

[6 main variables: CSP onset latency and duration in the upper limb, CSP onset latency and duration in the lower limb, QSART sweat volume and latency in the foot]

- Large nerve fibre function

[2 symptom/clinical sign composite score variables: MNSI and MDNS (clinical examination) scores]

[3 main neurophysiology variables: sural SNAP amplitude, peroneal motor conduction velocity and tibial minimum F-wave]

- Median nerve excitability

[8 main variables: Depolarising threshold electrotonus (TEd) (peak), TEd (90-100 ms), hyperpolarising threshold electrotonus (Teh) (90-100 ms), TEd (10-20 ms), superexcitability, subexcitability, resting current-voltage (I/V) slope, strength duration time constant]

2.3 Aim 3

To evaluate the safety of a six-month supplementation period with oral long-chain omega-3 PUFAs in individuals with type-1 diabetes, by evaluating the incidence of adverse events, change in habitual distance visual acuity, change in intraocular pressure, change in diabetic retinopathy grading, and change in blood pathology parameters from baseline at day 180.

3. Analysis sets/Populations

The statistical analysis aims to include all available data for all participants fulfilling all of the eligibility criteria. For the analyses of efficacy variables, participants will be included in the group to which they were randomised (i.e., as-randomised), referred to as the Intention-To-Treat sample. For the analyses of safety variables, participants will be included in the group they were treated (as-treated, if different from the randomised group assignment), referred to as the Safety sample.

3.1 Inclusion criteria

The following are the inclusion criteria for participant eligibility at Visit 1 (Baseline):

1. Male or female, ≥ 18 years of age;
2. MNSI > 2 ;
3. Written informed consent and documentation, in accordance with ethics requirements and ICH Good Clinical Practice (GCP), obtained prior to performing any study procedures;
4. Distance best corrected visual acuity of at least 6/12 Snellen equivalent in each eye using a standard logMAR visual acuity chart, measured at 3 metres;
5. Intraocular pressure (IOP) ≤ 21 mmHg in both eyes, measured using applanation tonometry;
6. Ability to understand and follow study instructions, with the intention of completing all required study visits.

3.2 Exclusion criteria

The following are the exclusion criteria for participant eligibility at Visit 1 (Baseline):

Systemic

1. Any uncontrolled systemic disease, other than sub-optimally controlled type-1 diabetes mellitus;
2. Confirmed neuropathy secondary to causes other than diabetes (e.g., alcohol polyneuropathy, Vitamin B-12 deficiency, folate deficiency, chronic renal failure, hypothyroidism, neurotoxic drug use including chemotherapy);
3. Any of the following general medical conditions: bipolar disorder, atrial fibrillation, implanted defibrillator, familial adenomatous polyposis, systemic immunocompromise;
4. A scheduled or planned systemic surgery over the course of the study;
5. Any known bleeding disorders;
6. Current consumption of a systemic anti-coagulant medication other than aspirin;
7. Females who are currently pregnant or breastfeeding;
8. Females of childbearing potential who are planning a pregnancy over the course of the study;
9. Inability to sit/lie supine comfortably during the examination procedures for any reason.

Ophthalmic

10. Known allergy to, or previous reaction to, any ocular agents used in the study (i.e., ocular anaesthetics, sodium fluorescein, lissamine green, ocular mydriatics);
11. Scheduled or planned ocular surgery over the course of the study;
12. Any history of rigid contact lens wear;
13. Grading of diabetic retinopathy worse than 'moderate' in either eye according to the simplified ETDRS (Wisconsin) classification (ETDRS Research Group 1991);
14. Presence of any of the following ocular conditions: active ocular infection or inflammation that in the judgment of the investigator may interfere with the interpretation of the study results;
15. Corneal abnormalities or damage that could disrupt normal corneal nerve morphology, including keratoconus, bullous keratopathy, advanced corneal dystrophies, history of neurotrophic keratopathy including herpes keratitis and severe Sjogren's associated dry eye disease;
16. History of refractive surgery or trauma within the past 12 months;
17. Use of autologous serum eye drops within the past three months, or their anticipated use during the course of the study;
18. Participant has a medical or ocular condition, or is in a situation, which in the principal investigator's opinion, may put the participant at significant risk, may confound the study results, or may interfere significantly with participation in the study.

Interventional

19. Current or previous regular consumption of any omega-3 oral supplements (>3 times/week) in the past three months;
20. Current participation in another interventional drug or device study or anticipated entry into such a study within one month of enrolment;
21. Known allergy or hypersensitivity to any components of the study supplements;
22. Cultural or religious beliefs that exclude the consumption of certain or all animal products.

4. Endpoints and Covariates

All variables are listed in Appendix 1 and the definition of the derived variables can be found in Appendix 2.

5. Handling of Missing Values and Other Data Conventions

During data collection, regular verification of the source documents was undertaken to minimise the potential for missing data. Data collected outside the specified time windows for each study visit will be set to missing (see Table 1 in Section 10). The number and percentage of missing data will be calculated for each of the outcomes listed in Appendix 1.

For outcomes analysed using the constrained longitudinal data analysis technique outlined in Section 6.2, we will use a likelihood-based approach that relies on the underlying assumption that the probability of missing outcome data is not related to the missing data, but to some of the observed measured data in the model (Missing At Random [MAR]). For outcomes analysed using the (ordered) logistic regression model, we will exclude those with missing baseline or missing post-baseline data (i.e., incomplete cases) relying on the underlying assumption that the missing outcome data is missing completely at random (MCAR).

For corneal nerve sensitivity thresholds, the instrument has a lowest measurable threshold (i.e., floor) of 0.1 mbar. All measured values that were recorded to be below this lower limit will be replaced by a value of the lower limit divided by two (i.e., 0.05 mbar) (Beal 2001).

6. Statistical Methodology

6.1 Baseline characteristics

See Table 1 in Appendix 4.

Participant demographic and baseline variables will be summarised and presented in a table (see Table 1 in Appendix 4) by treatment group using: frequencies and percentages (based on the non-missing sample size at baseline) for discrete variables, mean and standard deviation for continuous variables, or median and quartiles (25th to 75th percentile) for non-symmetrical continuous variables. If variables have missing data, we will report the non-missing sample size either in the table or in the footnote of the table.

6.2 Statistical procedures

See Tables 2 – 5 in Appendix 4.

All outcomes will be summarised by visit and treatment group using descriptive statistics. Depending on the distribution, one of the following models will be fitted:

- Continuous repeated measures data collected at more than one visit will be analysed using a constrained longitudinal data analysis technique (Liang 2000). The outcome (dependent variable) will consist of the baseline and post-baseline values. The model will assume a common baseline mean across the two treatment arms due to random allocation. It will incorporate time point (study visit) as a categorical variable, thus it will not assume a specific trajectory over time. In addition to study visit, the model will include treatment as the main factor and the treatment by study visit interaction. The variance–covariance among the repeated measurements will be defined as unstructured. In the case of non-convergence, we will consider alternative structures (first-order autoregressive, Toeplitz, compound symmetry). Using this model, study participants with a missing baseline or a missing post-baseline value will be included in the analysis but study participants with no outcome values will be excluded. This model will yield unbiased results when the outcome data are Missing At Random. We will report the estimate and two-sided confidence interval of the absolute difference between two treatment groups in the change from baseline to 3 months and 6 months post-baseline (if applicable). Before fitting this model, the distribution of the outcome will be explored. In addition, we will examine if there are potential violations of underlying model assumptions (e.g. residuals). As a result, we may apply an appropriate transformation (e.g. log-transformation) before fitting the final model.
- Binary data will be analysed using a logistic regression model. We will report the estimate and two-sided confidence interval of the odds ratio between the two treatment groups.
- Categorical data will be analysed using an ordinal logistic regression model. We will report the estimate and two-sided confidence interval of the odds ratio between the treatment groups.

Details of which model will be used for each of the primary, key secondary, and secondary outcomes can be found in Appendix 3.

6.3 Compliance

See Table 4 in Appendix 4.

The compliance outcomes (such as the percentage of omega-3 and omega-6 fatty acids present in erythrocytes (determined from dried blood spot tests

[analysed by Waite Lipid Analysis Services, Australia]) at the study endpoint (day 180) will be analysed using a linear regression model, adjusted for baseline. Before fitting this model, the distribution of the outcome will be explored. In addition, we will examine if there are potential violations of underlying model assumptions (e.g., residuals). As a result, we may apply an appropriate transformation (e.g., log-transformation) before fitting the final model. The proportion of returned capsules (counted by an independent, masked reconciler) will be summarised by treatment group.

Compliance (%) will be derived as 100 times [(actual number of capsules taken over the study period) divided by (total number of capsules that should have been taken over the study period)], with study period based on actual trial participation duration. The actual number of capsules taken over the study period is derived as the number of capsules dispensed minus the number of capsules returned. Participants with compliance less than 75% or above 125% will be considered as non-compliant to study drug intake (AusPAR2011; NCT01797185). A sensitivity analysis will be performed for the primary outcome to examine the treatment effect when non-compliant cases are excluded from the analysis.

6.4 Concomitant medications

Concomitant medications will be categorised by their therapeutic class, according to the United States Pharmacopeia Drug Classification system. The number and percentage of participants taking concomitant medications in each class, for each participant group, will be tabulated and reported in the supplementary information.

6.5 Adverse events

Information about adverse events will be collated, summarised and provided in the supplementary information. The incidence of participants with adverse events at each visit, type of event, event severity, and a judgement regarding its association with the investigational product or a study procedure, will be tabulated and reported in the supplementary information.

7. Measures to Adjust for Multiplicity, Confounders, Heterogeneity

7.1 Adjustments for confounders

In addition to the models specified above that only include treatment group, we will fit two adjusted models to the outcomes linked to the primary, key secondary, and secondary aims that adjusts for:

Adjusted model 1:

- Age (years)
- Diabetes duration (years)
- HbA1c (mmol/mol)

Adjusted model 2:

- Age (years)
- Diabetes duration (years)
- HbA1c (mmol/mol)
- Baseline CNFL (mm/mm²) (continuous) if it is imbalanced between groups at baseline
- Baseline omega-3 index (%) (continuous) if it is imbalanced between groups at baseline.

Results will be compared with the unadjusted analyses and discussed in the main study report.

7.2 Subgroup analyses

Exploratory subgroup analyses will be performed for the primary outcome for the following subgroups:

- CNFL at baseline (above 12.5mm/mm² and below or equal to 12.5 mm/mm²), as the threshold considered diagnostic for diabetic sensorimotor polyneuropathy in type-1 diabetes (Perkins et al., 2018).
- Presence of small fibre neuropathy (yes/no) at baseline (physician-diagnosed, using results from abnormal QSART or CSP) (Kamel et al., 2015).

Subgroup (main effect) and the subgroup-by-treatment-by-visit interaction (as well as subgroup-by-treatment and subgroup-by-visit interaction) terms will be added to the unadjusted model to evaluate

whether the treatment effect differs between subgroup categories at the different time points. Results of the subgroup analyses will be summarised using Forest plots, presenting the estimate and two-sided 95% confidence interval of the treatment effect within each subgroup level along with corresponding p-value and including the p-value for heterogeneity of the treatment effect between the subgroups.

Analyses of any other subgroups will be considered as *post hoc* exploratory analyses.

7.2.1 Multiple testing

The primary outcome at day 180 will be evaluated using the two-sided 5% level of significance.

Multiple testing for the key secondary outcomes related to corneal nerve parameters at day 180 will be assessed using the Benjamini-Hochberg stepwise method with a false discovery rate of 5% for: corneal nerve fibre density (CNFD), corneal nerve branch density (CNBD), central corneal sensitivity thresholds to room temperature, and central corneal sensitivity thresholds to cooled temperature. For these key secondary outcomes at day 180, we will report the multiplicity adjusted p-values.

For all other post-baseline time points and outcomes, we will report multiplicity unadjusted p-values. Given the large number of statistical tests, the probability of finding at least one false positive finding will be close to 100%. For this reason, we will exercise caution and in addition to p-values, associations will be interpreted based on the magnitude and direction of the association and width of the 95% confidence intervals.

All tests will be performed two-sided, and we will report two-sided 95% confidence intervals.

8. Sensitivity Analyses

Additional sensitivity analyses will be performed for the outcomes linked to the primary and key secondary aims for the following dataset:

- Dataset excluding participants deemed non-compliant (see definition in Section 6.3).

9. QC Plans

Source data verification of the data entered on REDCap was performed by the Principal Investigator (PI). Data was manually checked for accuracy from the source worksheets/original digital data record, for all participants, for the primary outcome (CNFL) and key secondary outcomes (CNFD, CNBD, central corneal sensitivity thresholds to room temperature, and central corneal sensitivity thresholds to cooled temperature). In addition, a subset of data comprising at least 20% of the full dataset, was randomly selected by the PI for verification, to ensure that the observations and findings are recorded correctly and completely. Data found as incorrectly entered were verified against the original source documents, corrected on REDCap and recorded accordingly.

10. Programming Plans

A list of all planned tables and figures and their templates can be found in Appendix 4.

Tolerance windows will be used for converting visit dates into visit numbers according to the study visit schedule before summarising or analysing the data. The relative day of testing will be derived as the visit date minus the supplementation start date. The visit windows in Table 1 will be applied to the testing dates.

Table 1. Visit windows

Visit	Target day	Lower limit (incl.)	Upper limit (incl.)
Day 1	Supplement start date (Day 0)	Day -35	1
Day 30	Day 30	Day 14	Day 44
Day 90	Day 90	Day 76	Day 104
Day 180	Day 180	Day 156	Day 204

Assessments performed outside of these visit windows will not be included in the analyses. If a participant has more than one assessment performed within the same visit window, then the assessment closest to the target date will be used. The visit windows will be applied to the study variables described in Appendix 1.

11. REFERENCES

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12. Appendix 1

VARIABLES IN THE DATA SET

	<u>Variable label</u>	<u>Unit</u>	<u>Variable in data set</u>
Baseline			
R	Sex	0 = Female 1 = Male	<sex>
R	Age	Years	<age>
R	Diabetes duration (year(s) since diagnosis)	Years	<hx_dm_duration>
D	Body mass index	Kg/m ²	<bmi>
R	Insulin intake method	0 = pump 1 = multiple daily injections	< hx_insulin_type>
R	Concomitant medications	0 = no 1 = yes	<hx_concomitant_medications>
R	Regular soft contact lens wear (at least once per week)	0 = No 1 = Yes	<ohx_scl>
R	History of ophthalmic surgery	0 = No 1 = Yes	<ohx_surgeries>
D	Diagnosed as having an abnormal small fibre function using either QSART or CSP	0 = Normal 1 = Abnormal	<sfn_qsart_csp>
R	Completed study	0 = No 1 = Yes	<completed_study>
Primary efficacy			
R	Central Corneal nerve fibre length [right eye]	mm/mm ²	<ivcm_cnfl_c>
Secondary and exploratory efficacy outcomes			
Corneal nerve parameters: main secondary			
R	Central corneal nerve fibre density [right eye]	nerves/mm ²	<ivcm_cnfd_c>
R	Central corneal nerve branch density [right eye]	branches/mm ²	<ivcm_cnbd_c>
R	Central corneal sensitivity threshold to room temperature [right eye]	mBar	<ncca_rm_c>
R	Central corneal sensitivity threshold to cooled temperature [right eye]	mBar	<ncca_cd_c>
Corneal nerve parameters: exploratory			
R	Central corneal dendritic cell density [right eye]	cells/mm ²	<ivcm_dcc_c_total>
R	Peripheral corneal nerve fibre length [right eye]	mm/mm ²	<ivcm_cnfl_p>
R	Peripheral corneal dendritic cell density [right eye]	cells/mm ²	<ivcm_dcc_p_total>
R	Peripheral corneal sensitivity threshold to room temperature [right eye]	mBar	<ncca_rm_p>
R	Peripheral corneal sensitivity threshold to cooled temperature [right eye]	mBar	<ncca_cd_p>
Quality of life: secondary			
D	Change in health state: EQ-5D-5L index score	Range: -0.569 to 1	<eq5d5l_index>

	Variable label	Unit	Variable in data set
R	Change in health state: EQ-5D-5L VAS score	Range: 0 to 100	<eq5d5l_vas>
	Small fibre function: secondary		
R	Cutaneous silent period - upper limb latency	ms	<csp_upper_lat>
R	Cutaneous silent period - upper limb duration	ms	<csp_upper_duration>
R	Cutaneous silent period - lower limb latency	ms	<csp_lower_lat>
R	Cutaneous silent period - lower limb duration	ms	<csp_lower_duration>
R	QSART sweat volume - measured at the foot	µL	<qsart_foot_vol>
R	QSART sweat latency - measured at the foot	s	<qsart_foot_lat>
	Small fibre function: exploratory		
D	QSART neuropathy grading (neuropathy/ length-dependent)	0 = None 1 = Length- dependent SFN 2 = Non-length dependent SFN	<qsart_neuropathy>
R	QSART: forearm volume	uL	<qsart_forearm_vol>
R	QSART: forearm latency	s	<qsart_forearm_lat>
R	QSART: proximal leg volume	uL	<qsart_proxleg_vol>
R	QSART: proximal leg latency	s	<qsart_proxleg_lat>
R	QSART: distal leg volume	uL	<qsart_distleg_vol>
R	QSART: distal leg latency	s	<qsart_distleg_lat>
	Large fibre function: secondary		
R	Michigan Neuropathy Screening Instrument (MNSI) score	Range: 0 to 23	<mnsi_total>
R	Michigan Diabetic Neuropathy Score (MDNS) composite score: clinical examination	4 Levels: 0 = Class 0 1 = Class 1 2 = Class 2 3 = Class 3	<mdns_clin_class>
R	Nerve conduction studies: Sural SNAP amplitude (Mid. lower leg-Lat. malleolus)	uV	<sensory_sural_amp>
R	Nerve conduction studies: Peroneal CMAP velocity (Bl. knee – ankle)	m/s	<motor_peroneal_vel>
R	Nerve conduction studies: Tibial minimum F-wave latency (Ankle – Abd hal)	ms	<motor_tibial_fminlat>
	Large fibre function: exploratory		
R	Michigan Diabetic Neuropathy Score (MDNS) composite score: neurophysiology composite score (NCS)	4 Levels: 0 = Class 0 1 = Class 1 2 = Class 2 3 = Class 3	<mdns_neurophys_class>
R	NCS: median motor latency (wrist-APB)	ms	<motor_median_lat1>
R	NCS: median motor amplitude (wrist-APB)	mV	<motor_median_amp1>
R	NCS: median motor latency (elbow-wrist)	ms	<motor_median_lat2>
R	NCS: median motor amplitude (elbow-wrist)	mV	<motor_median_amp2>

	<u>Variable label</u>	<u>Unit</u>	<u>Variable in data set</u>
R	NCS: median motor velocity (elbow-wrist)	m/s	<motor_median_vel>
R	NCS: peroneal motor latency (ankle-EDB)	ms	<motor_peroneal_lat1>
R	NCS: peroneal motor amplitude (ankle-EDB)	mV	<motor_peroneal_amp1>
R	NCS: peroneal motor latency (Bl. knee-Ankle)	ms	<motor_peroneal_lat2>
R	NCS: peroneal motor amplitude (Bl. knee-Ankle)	mV	<motor_peroneal_amp2>
R	NCS: tibial motor latency (Ankle-Abd hal)	ms	<motor_tibial_lat>
R	NCS: tibial motor amplitude (Ankle-Abd hal)	mV	<motor_tibial_amp>
R	NCS: tibial motor velocity (Ankle-Abd hal)	m/s	<motor_tibial_vel>
R	NCS: Median sensory latency (Dig II-Wrist; orthodromic)	ms	<sensory_median_lat1>
R	NCS: Median sensory amplitude (Dig II-Wrist; orthodromic)	uV	<sensory_median_amp1>
R	NCS: Median sensory velocity (Dig II-Wrist; orthodromic)	m/s	<sensory_median_vel1>
R	NCS: Median sensory latency (Wrist-Dig II; antidromic)	ms	<sensory_median_lat2>
R	NCS: Median sensory amplitude (Wrist-Dig II; antidromic)	uV	<sensory_median_amp2>
R	NCS: Median sensory velocity (Wrist-Dig II; antidromic)	m/s	<sensory_median_vel2>
R	NCS: Sural sensory latency (Mid. lower leg-Lat. malleolus)	ms	<sensory_sural_lat>
R	NCS: Sural sensory velocity (Mid. lower leg-Lat. malleolus)	m/s	<sensory_sural_vel>
R	NCS: ulnar sensory latency (Dig V-Wrist; orthodromic)	ms	<sensory_ulnar_lat1>
R	NCS: ulnar sensory amplitude (Dig V-Wrist; orthodromic)	uV	<sensory_ulnar_amp1>
R	NCS: ulnar sensory velocity (Dig V-Wrist; orthodromic)	m/s	<sensory_ulnar_vel1>
R	NCS: ulnar sensory latency (Wrist – Dig V; antidromic)	ms	<sensory_ulnar_lat2>
R	NCS: ulnar sensory amplitude (Wrist – Dig V; antidromic)	uV	<sensory_ulnar_amp2>
R	NCS: ulnar sensory velocity (Wrist – Dig V; antidromic)	m/s	<sensory_ulnar_vel2>
Nerve excitability: secondary			
R	Median motor nerve excitability profile: TE _d (peak)	-	<nes_22_ted_peak>
R	Median motor nerve excitability profile: TE _d (90-100 ms)	-	<nes_18_ted_90_100>
R	Median motor nerve excitability profile: TE _h (90-100 ms)	-	<nes_10_teh_90_100>
R	Median motor nerve excitability profile: TE _d (10-20 ms)	-	<nes_11_ted_10_20>
R	Median motor nerve excitability profile: Superexcitability	%	<nes_12_superexcitability>

	Variable label	Unit	Variable in data set
R	Median motor nerve excitability profile: Subexcitability	%	<nes_13_subexcitability>
R	Median motor nerve excitability profile: Resting current-voltage (I/V) slope	-	<nes_6_resting_iv>
R	Median motor nerve excitability profile: strength duration time constant	ms	<nes_2_sdtc>
	Nerve excitability: exploratory		
R	Nerve excitability: Stimulus (mA) for 50% max response	mA	<nes_1_stimulus_50_max>
R	Nerve excitability: Rheobase (mA)	mA	<nes_3_rheobase>
R	Nerve excitability: Stimulus-response\slope	-	<nes_4_stimulus_response_slope>
R	Nerve excitability: Peak response (mV)	mV	<nes_5_peak_response>
R	Nerve excitability: Minimum I/V slope	-	<nes_7_min_iv>
R	Nerve excitability: Temperature (°C)	C	<nes_8_temp>
R	Nerve excitability: RRP (ms)	ms	<nes_9_rrp>
R	Nerve excitability: Nerve excitability: Polarizing current	% threshold	<nes_14_pol_current_threshold>
R	Nerve excitability: Polarizing current\ (mA)	mA	<nes_15_pol_current_ma>
R	Nerve excitability: Latency (ms)	ms	<nes_16_latency>
R	Nerve excitability: TE _d (40-60ms)	-	<nes_17_ted_40_60>
R	Nerve excitability: TE _h (10-20ms)	-	<nes_19_teh_10_20>
R	Nerve excitability: TE _d (undershoot)	-	<nes_20_ted_undershoot>
R	Nerve excitability: TE _h (overshoot)	-	<nes_21_teh_overshoot>
R	Nerve excitability: S ₂ accommodation	-	<nes_23_s2_accom>
R	Nerve excitability: Accommodation half-time (ms)	ms	<nes_24_accom_half_time>
R	Nerve excitability: Hyperpol. I/V slope	-	<nes_25_hyperbol_iv_slope>
R	Nerve excitability: Refractoriness at 2.5ms (%)	%	<nes_26_refractoriness_2.5ms>
R	Nerve excitability: TE _h (20-40ms)	-	<nes_27_teh_20_40>
R	Nerve excitability: TE _h (slope 101-140ms)	-	<nes_27_teh_20_40>
R	Nerve excitability: Refractoriness at 2 ms (%)	%	<nes_29_refractoriness_2ms>
R	Nerve excitability: Superexcitability at 7 ms (%)	%	<nes_30_refractoriness_7ms>
R	Nerve excitability: Superexcitability at 5 ms (%)	%	<nes_31_refractoriness_5>
R	Nerve excitability: TE _d 20(peak)	-	<nes_32_ted20_peak>
	Signs of dry eye disease: exploratory		
D	Ocular Surface Disease Index score	score 0 - 100	<ocul_osdi>
R	Tear osmolarity (highest of the two eyes)	mOsmol/L	<ocul_osmol_highest>

	<u>Variable label</u>	<u>Unit</u>	<u>Variable in data set</u>
R	Sodium fluorescein tear break-up time in the right eye (average of three repeat measurements)	s	<ocul_nafl_tbut_r>
R	Phenol red thread test (average of both eyes)	mm	<ocul_prt_ave>
R	Anterior blepharitis grading (average of both eyes)	Efron scale score 0-4 (continuous)	<ocul_bleph_ave>
R	Meibomian gland dysfunction grading (average of both eyes)	Efron scale score 0-4 (continuous)	<ocul_mgd_ave>
R	Conjunctival redness grading (average of both eyes)	Efron scale score 0-4 (continuous)	<ocul_conj_red_ave>
R	Limbal redness grading (average of both eyes)	Efron scale score 0-4 (continuous)	<ocul_limbal_red_ave>
R	Corneal sodium fluorescein staining score (average of both eyes)	Oxford Score 0-4 (continuous)	<ocul_nafl_stain_ave>
Indirect healthcare costs: exploratory			
D	iMTA iPCQ - loss of productivity due to absences from paid work	hours	<imta_absenteeism>
D	iMTA iPCQ - loss of productivity due to presenteeism	hours	<imta_presenteeism>
D	iMTA iPCQ - loss of productivity of unpaid work	hours	<imta_unpaid>
Safety outcomes			
R	Habitual distance visual acuity measured using a standard eye chart - Right eye	LogMAR	<ocul_va_r_logmar>
R	Habitual distance visual acuity measured using a standard eye chart - Left eye	LogMAR	<ocul_va_l_logmar>
R	Intraocular pressure - Right eye	mmHg	<ocul_iop_r>
R	Intraocular pressure - Left eye	mmHg	<ocul_iop_l>
R	Diabetic retinopathy grading - Worst eye retinopathy grading	6 Grades: 0 = None 1 = Minimal 2 = Mild 3 = Moderate 4 = Severe 5 = Proliferative	<dr_retinopathy>
R	Diabetic macular oedema grading - Worst eye macular oedema grading	3 Grades: 0 = None 1 = Macular oedema 2 = Clinically significant macular oedema	<dr_dme>
R	Bloods: HbA _{1c} , mmol/mol	mmol/mol	<bt_hbaic_mmol>
R	Bloods: cholesterol	mg/dL	<bt_cholesterol>
R	Bloods: HDL	mmol/L	<bt_hdl>
R	Bloods: LDL	mmol/L	<bt_ldl>
R	Bloods: folate	nmol/L	<bt_folate>

	Variable label	Unit	Variable in data set
R	Bloods: vitamin B-12	pmol/L	<bt_b12>
R	Bloods: creatinine	umol/L	<bt_creatinine>
R	Adverse events	0 = None 1 = AE 2 = SAE	<adverse_events>
R	Bloods: haemoglobin (Hb)	g/L	<bt_hb>
R	Bloods: platelets	$\times 10^9$ /L	<bt_platelets>
R	Bloods: white blood cells (WBC)	$\times 10^9$ /L	<bt_wbc>
R	Bloods: HbA1C, Percentage (%)	%	<bt_hbaic_percent>
R	Bloods: triglycerides	mmol/L	<bt_triglycerides>
R	Bloods: ALT	U/L	<bt_alt>
R	Bloods: T-Bil	umol/L	<bt_tbil>
R	Bloods: ALP	U/L	<bt_alp>
R	Bloods: GGT	U/L	<bt_ggt>
R	Bloods: Na+	mmol/L	<bt_sodium>
R	Bloods: K+	mmol/L	<bt_potassium>
R	Bloods: CO ₂	mmol/L	<bt_bicarb>
R	Bloods: TSH	mU/L	<bt_tsh>
R	Bloods: eGFR	mL/min/1.73m ² Categorical: 0 = >90 1 = <90	<bt_egfr>
Compliance outcomes			
R	Omega-3 index	%	<pufa_n3_index>
R	Omega-3: EPA concentration	%	<pufa_n3_epa>
R	Omega-3: DHA concentration	%	<pufa_n3_dha>
R	Omega-6 concentration	%	<pufa_n6_total>
R	Omega-9 concentration	%	<pufa_n9_total>
R	Omega-6:Omega-3 ratio	-	<pufa_n6_n3_ratio>
R	Compliance: capsule count	%	<capsules_taken>
Efficacy of masking			
R	Intervention guess: participant	0 = Placebo 1 = Omega-3	<guess_participant>
R	Intervention guess: outcome assessor	0 = Placebo 1 = Omega-3	<guess_examiner>

D = derived outcome; R = raw outcome. SFN = small fibre neuropathy
 Colour codes: orange = primary outcome; green = main secondary outcomes; white = exploratory outcomes.

13. Appendix 2

DEFINITIONS OF DERIVED VARIABLES IN THE DATA SET

Derived variable	Range	Derived from
Body mass index		Weight/(height) ²
OSDI score derived as (sum of scores for all questions answered) × 100/[(total number of questions answered) × 4]	0 to 100	Derived using the OSDI scoring sheet
Change in health state: EQ-5D-5L index score	-0.569 to 1	Derived using the UK index value set
MDNS – class of neuropathy (clinical examination)	Classes 0 to 3	Derived using MDNS scoring sheet
MDNS – class of neuropathy (electrophysiology)	Classes 0 to 3	Derived using MDNS scoring sheet
Evidence of neuropathy using QSART	0 = None 1 = Length dependent SFN 2 = Non-length dependent SFN	Clinically derived using QSART scores at forearm, foot, proximal leg and distal leg
Evidence of small fibre neuropathy using abnormal QSART or CSP	0 = None 1 = SFN	Clinically derived using an abnormal QSART or CSP score
iMTA iPCQ - loss of productivity due to absence from paid work		Method derived from iPCQ Manual
iMTA iPCQ - loss of productivity due to presenteeism		Method derived from iPCQ Manual
iMTA iPCQ - loss of productivity of unpaid work		Method derived from iPCQ Manual

SFN = small fibre neuropathy

14. Appendix 3

LIST OF ANALYSES FOR MAIN OUTCOMES

The distribution column of this table will be completed after unmasking, based on an examination of the distribution along with potential violation of the model assumptions to inform the need for a transformation and which transformation is most appropriate.

Outcome	Distribution/Transformation	Days	Analysis model
Primary efficacy			
Central Corneal nerve fibre length [right eye]		1, 90, 180	Repeated measures model.
Key secondary efficacy			
Corneal nerve parameters			
Central corneal nerve fibre density [right eye]		1, 90, 180	Repeated measures model.
Central corneal nerve branch density [right eye]			
Central corneal sensitivity threshold to room temperature [right eye]		1, 90, 180	Repeated measures model.
Central corneal sensitivity threshold to cooled temperature [right eye]			
Secondary efficacy			
Quality of life			
Change in health state: EQ-5D-5L index score		1, 90, 180	Repeated measures model.
Change in health state: EQ-5D-5L VAS score			
Small nerve fibre function			
Cutaneous silent period - upper limb onset		1, 180	Repeated measures model.
Cutaneous silent period - upper limb duration			
Cutaneous silent period - lower limb onset			
Cutaneous silent period - lower limb duration			
QSART sweat volume -measured at the foot		1, 180	Repeated measures model.
QSART sweat latency -measured at the foot			
Large nerve fibre function			

Outcome	Distribution/Transformation	Days	Analysis model
Michigan Neuropathy Screening Instrument (MNSI) score		1, 180	Repeated measures model.
Michigan Diabetic Neuropathy Score (MDNS) composite score: clinical examination class	Categorical variables with 4 ordinal levels	1, 180	Ordered logistic regression model
Nerve conduction studies: Sural SNAP amplitude (Mid. lower leg-Lat. malleolus)		1, 180	Repeated measures model.
Nerve conduction studies: Peroneal CMAP velocity (Bl. knee – ankle)			
Nerve conduction studies: Tibial minimum F-wave latency (Ankle – Abd hal)			
Nerve excitability			
Median motor nerve excitability profile: TE _d (10-20 ms)		1, 180	Repeated measures model.
Median motor nerve excitability profile: TE _d (peak)			
Median motor nerve excitability profile: TE _d (90-100 ms)			
Median motor nerve excitability profile: TE _h (90-100 ms)			
Median motor nerve excitability profile: Superexcitability			
Median motor nerve excitability profile: Subexcitability			
Median motor nerve excitability profile: Resting I/V slope			
Median motor nerve excitability profile: strength duration time constant			
Safety			
Habitual visual acuity measured using a standard eye chart - Right eye		1, 30, 90, 180	Repeated measures model.
Habitual visual measured using a standard eye chart - Left eye		1, 30, 90, 180	
Intraocular pressure - Right eye		1, 180	Repeated measures model.
Intraocular pressure - Right eye		1, 180	
Diabetic retinopathy grading - Worst eye retinopathy grading	Categorical variable with 4 ordinal levels	1, 180	Ordered logistic regression model
Diabetic retinopathy grading - Worst eye macular oedema grading	Categorical variable with 3 ordinal levels.		
Bloods: HbA _{1c} (mmol/mol)		1, 180	Repeated measures model.

Outcome	Distribution/Transformation	Days	Analysis model
Bloods: cholesterol			
Bloods: HDL			
Bloods: LDL			
Bloods: folate			
Bloods: vitamin B-12			
Bloods: creatinine			
Compliance			
Omega-3 index		1, 180	Repeated measures model.
Omega-3: EPA concentration			
Omega-3: DHA concentration			
Omega-6 concentration			
Omega-9 concentration			
Omega-6:Omega-3 ratio			

15. Appendix 4

LIST OF TABLES/FIGURES

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* These tables will consist of summary statistics for all visits (thus not limited to baseline and day 180) along with the analysis for all post-baseline time-points.

Example of Table 1: Baseline Participant Characteristics (Intention-To-Treat sample)

Characteristic	Omega-3 PUFAs (N=XX)	Placebo (N=XX)
Male sex, n (%)	XX (XX.X)	XX (XX.X)
Age (years), median (IQR)	X.X (X.X)	X.X (X.X)
Duration of type-1 diabetes (years), median (IQR)	X.X (X.X)	X.X (X.X)
Body mass index (kg/m ²), median (IQR)	X.X (X.X)	X.X (X.X)
Insulin pump, n (%)	XX (XX.X)	XX (XX.X)
Contact lens wear, n (%)	XX (XX.X)	XX (XX.X)
History of (any) ophthalmic surgery, n (%)	XX (XX.X)	XX (XX.X)
Presence of small fibre neuropathy,* n (%)	XX (XX.X)	XX (XX.X)

*Abnormal small fibre function defined as having abnormal QSART or abnormal CSP at baseline (Kamel, 2015)

Example of Table 2: Central corneal nerve efficacy outcomes (Intention-To-Treat sample)

	Omega-3 PUFAs			Placebo			Omega-3 PUFAs vs Placebo	
	Baseline [*] (N=XX)	Day 180 [*] (N=XX)	Change from baseline [*] (N=XX)	Baseline [*] (N=XX)	Day 180 [*] (N=XX)	Change from baseline [*] (N=XX)	Estimate (95% CI)	P-value [†]
Primary outcome								
CNFL (mm/mm ²)								
Key secondary outcomes								
CNFD (nerves/mm ²)								
CNBD (branches/mm ²)								
Central corneal sensitivity thresholds (mbar) - Room temperature - Cooled temperature								

^{*} Mean and standard deviation, unless stated otherwise.

[†] Multiplicity-adjusted p-value for the key secondary outcomes.

Abbreviations: CNBD; corneal nerve branch density CNFD, corneal nerve fibre density; CNFL, corneal nerve fibre length

Example of Table 3: Clinical neurophysiology outcomes (Intention-To-Treat sample)

	Omega-3 PUFAs			Placebo			Omega-3 PUFAs vs Placebo	
	Baseline* (N=XX)	Day 180* (N=XX)	Change from baseline* (N=XX)	Baseline* (N=XX)	Day 180* (N=XX)	Change from baseline* (N=XX)	Estimate (95% CI)	p-value†
Quality of life - EQ 5D-5L Index - EQ 5D-5L VAS								
Small nerve fibre function								
Cutaneous silent periods - Upper limb latency onset (ms) - Upper limb duration (ms) - Lower limb latency onset (ms) - Lower limb duration (ms)								
Quantitative Sudomotor Axonal Reflex Test at the foot - Sweat Volume (μL) - Response Latency (s)								
Large nerve fibre function								
Neuropathy score - MNSI (out of 13) - MDNS clinical examination (out of 46)								
Nerve conduction studies - Sural sensory amplitude (μV) - Peroneal motor velocity (ms ⁻¹) - Tibial minimum F-wave latency (s)								
Nerve excitability - TE _d (10–20 ms) - TE _d (peak) - TE _d (90–100 ms) - TE _h (90–100 ms) - Superexcitability (%) - Subexcitability (%) - Resting I/V slope - SDTC, ms								

* Mean and standard deviation, unless stated otherwise.

† p-values are not adjusted for multiple testing.

Example of Table 4: Compliance (Safety sample)

	Omega-3 PUFAs			Placebo			Omega-3 PUFAs vs Placebo	
	Baseline* (N=XX)	Day 180* (N=XX)	Change from baseline* (N=XX)	Baseline* (N=XX)	Day 180* (N=XX)	Change from baseline* (N=XX)	Estimate (95% CI) (N=XX)	P-value [†]
Omega-3 PUFAs, % - Omega-3 index - EPA - DHA								
Total omega-3, %								
Total omega-6, %								
Total omega-9, %								
Omega-6:Omega-3 ratio								

* Mean and standard deviation, unless stated otherwise.

† p-values are not adjusted for multiple testing.

Abbreviations: MDNS, Michigan Diabetic Neuropathy score; MNSI, Michigan Neuropathy Screening Index; TSH, thyroid stimulating hormone;

Example of Table 5: Safety outcomes and blood biochemistry (Safety sample)

	Omega-3 PUFAs			Placebo			Omega-3 PUFAs vs Placebo	
	Baseline* (N=XX)	Day 180* (N=XX)	Change from baseline* (N=XX)	Baseline* (N=XX)	Day 180* (N=XX)	Change from baseline* (N=XX)	Estimate (95% CI) (N=XX)	p-value†
Adverse events - At least one AE or SAE, n (%) - At least one AE, n (%) - At least one SAE, n (%)								
Habitual distance visual acuity (LogMAR) - Right eye - Left eye								
Intraocular pressure (mmHg) - Right eye - Left eye								
Diabetic retinopathy in the worse eye, n (%) - None - Mild - Moderate - Severe - Proliferative								
Diabetic macular oedema in the worse eye, n (%) - None - Macular oedema - Clinically significant macular oedema								
Blood biochemistry								
HbA1C (mmol/mol)								
Cholesterol (mmol/L)								
HDL (mmol/L)								
LDL (mmol/L)								
Creatinine (µmol/L)								
Vitamin B12 (pmol/L)								
Folate (nmol/L)								

* Mean and standard deviation unless stated otherwise.

† p-values are not adjusted for multiple testing.

Abbreviations: HbA1C, haemoglobin A1c; HDL, high-density lipoproteins; LDL, low density lipoproteins.

Appendix H. Chapter 7: Supplementary materials [3 of 3]

nPROOFS₁ results of exploratory outcomes

Results of the exploratory outcomes for the randomised controlled clinical trial:

“Investigating the neuroPRotective effect of Oral Omega-3 Fatty acid Supplementation in type 1 diabetes (nPROOFS₁): a randomised, placebo-controlled trial”

Changes from the planned analysis in the Statistical Analysis Plan

1. A sensitivity analysis was performed for outcomes with substantial imbalance at baseline either in the mean (continuous outcomes) or geometric mean (log base e transformed outcomes) between groups. The imposed constraint of balance at baseline of the constrained longitudinal data analysis model was removed by adjusting the primary model for the interaction between baseline and treatment in addition to the factors already part of the longitudinal data analysis model.
 2. We had intended to analyse categorical and binary variables using (ordinal) logistic regression analysis. However, this model could not be fitted due to the distribution of the data within and between groups. Instead, for categorical and binary variables, change from baseline at the study endpoint for each participant was categorised as negative change, positive change, or no change. Fisher's exact test was used to compare the proportions of participants between treatment groups. For MDNS scores, in changing from analysing this parameter as a continuous variable to a categorical variable, the class of neuropathy (class 0–3 as defined by the MDNS) was derived as the highest score from the clinical scoring or neurophysiology component.
 3. Between-group comparisons of dry eye symptom scores using the exploratory outcome of Ocular Surface Disease Index (OSDI) (appendix 3) could not be performed using the prespecified repeated measures model due to the number of scores with the value of zero. For the OSDI outcome, comparison of change between the omega-3 PUFA and placebo groups was tested using the non-parametric Wilcoxon rank sum test. The estimate and 95% confidence intervals between-groups were obtained using the Hodges-Lehman non-parametric estimator.
 4. Total omega-3 concentrations (%) are not additionally analysed as EPA and DHA concentrations have been reported.
 5. Exploratory outcomes for indirect health care were not analysed.
-

Table S1. Summary of concomitant systemic medications (Intention-To-Treat sample)

Medication category	Omega-3 PUFAs (n=21)	Placebo (n=22)
None	5 [24%]	7 [32%]
At least one concomitant medication	16 [76%]	15 [68%]
Blood Products and Modifiers	1 [5%]	1 [5%]
Analgesics	2 [10%]	3 [14%]
Antibacterials	1 [5%]	1 [5%]
Anticonvulsants	0 [0%]	2 [9%]
Antidepressants	1 [5%]	3 [14%]
Antiparasitics	0 [0%]	1 [5%]
Blood Glucose Regulators	1 [5%]	0 [0%]
Cardiovascular agents	15 [71%]	13 [59%]
Contraceptives	2 [10%]	1 [5%]
Dermatological agents	1 [5%]	3 [59%]
Electrolytes/Minerals/Metals/ Vitamins	12 [57%]	6 [27%]
Vitamins	7 [33%]	5 [23%]
Electrolyte/Mineral Replacement	5 [24%]	1 [5%]
Gastrointestinal Agents	3 [14%]	0 [0%]
Genetic or Enzyme Disorder: Replacement, Modifiers, Treatment	1 [5%]	1 [5%]
Hormonal Agents, Stimulant/Replacement/ Modifying (Thyroid)	1 [5%]	2 [9%]
Immunological Agents	1 [5%]	1 [5%]
Inflammatory Bowel Disease Agents	0 [0%]	1 [5%]
Respiratory Tract/ Pulmonary Agents	2 [10%]	1 [5%]
Sleep Disorder Agents	1 [5%]	0 [0%]

Data reported as the number of participants with the medications. Concomitant medications are categorised using the United States Pharmacopeia (USP; United States Pharmacopeial Convention) Drug Classifications 2019, published on <https://www.usp.org/>

Table S2. Safety outcome: Summary of adverse events (Safety sample)

	Omega-3 PUFAs (n=21)	Placebo (n=22)	Omega-3 PUFAs vs Placebo p-value
Summary of all adverse events*			
At least one AE or SAE, n [%]	9 [43%]	7 [32%]	0.54
At least one AE, n [%]	9 [43%]	6 [27%]	0.35
At least one SAE, n [%]	0 [0%]	1 [5%]	1.0
Unrelated adverse events†			
Haematologic	0	1	-
Ocular			-
Posterior segment/retinal condition (not associated with diabetes)	0	2	
Light sensitivity/headache	1	2	-
Respiratory symptoms	4	2	-
Gastrointestinal (unrelated SAE)	0	1	-
Musculoskeletal pain/cramping	1	1	-
Change in blood glucose control	0	1	-
Dermatological: rash or skin changes	2	0	-
Cardiovascular: heart palpitations	1	0	-
Immunologic: unrelated allergic reaction	1	0	-
Potentially-related adverse events‡			
Gastrointestinal: nausea‡	2	1	-

AE=adverse event. SAE=serious adverse event. PUFA=polyunsaturated fatty acid.

*Data are reported as number of individuals with at least one AEs.

†Data are reported as the number of adverse events.

‡Gastrointestinal effects were deemed to be possibly related to the treatment due to biological plausibility.

Table S3. Corneal structure (*in vivo* confocal microscopy) and function (non-contact aesthesiometry) outcomes baseline, day 90, and day 180 (Intention-To-Treat sample)

	Omega-3 PUFAs					Placebo					Omega-3 PUFAs vs Placebo			
	Baseline (n=21)	Day 90 (n=19)	Change from baseline at day 90 (n=19)	Day 180 (n=21)	Change from baseline at day 180 (n=21)	Baseline (n=22)	Day 90 (n=18)	Change from baseline at day 90 (n=18)	Day 180 (n=19)	Change from baseline at day 180 (n=19)	Estimate (95% CI) Day 90 (n=43)	p- value	Estimate (95% CI) Day 180 (n=43)	p-value [†]
Central CNFL (mm/mm ²)	11.49 (3.34)	13.09 (2.45)	1.62 (1.92)	13.55 (3.58)	2.06 (1.73)	12.38 (3.21)	12.30 (3.79)	-0.15 (1.12)	11.41 (3.66)	-0.72 (1.68)	1.80 (0.83, 2.78)	<0.001	2.70 (1.64, 3.75)	<0.001
Central CNFD (nerves/mm ²)	19.93 (7.72)	22.45 (5.84)	2.78 (3.99)	23.41 (8.08)	3.48 (4.15)	20.27 (7.36)	20.92 (8.32)	0.49 (3.19)	18.45 (7.57)	-1.57 (4.02)	2.41 (0.26, 4.56)	0.028	4.98 (2.51, 7.44)	<0.001
Central CNBD (branches/mm ²)	19.69 (10.76)	24.92 (7.46)	5.65 (8.23)	27.06 (13.59)	7.37 (6.34)	23.48 (10.27)	21.58 (11.98)	-2.84 (6.39)	19.54 (12.64)	-3.68 (7.35)	7.31 (3.01, 11.61)	0.001	11.23 (7.01, 15.45)	<0.001
Peripheral CNFL (mm/mm ²)	5.09 (2.82)	4.40 (2.34)	-0.92 (2.19)	4.83 (2.23)	-0.26 (2.58)	5.20 (2.56)	4.47 (2.42)	-0.52 (2.09)	3.39 (2.49)	-1.90 (2.65)	-0.20 (-1.35, 0.94)	0.73	1.51 (0.24, 2.77)	0.020
Central corneal sensitivity threshold to room temperature (mbar), median (IQR) [†]	0.47 (0.38 - 0.75)	0.47 (0.25 - 0.80)	-0.15 (-0.20 - 0.12)	0.38 (0.25 - 0.60)	-0.12 (-0.20 - -0.00)	0.30 (0.28 - 0.45)	0.36 (0.25 - 0.57)	0.01 (- 0.00 - 0.20)	0.30 (0.22 - 0.62)	0.12 (-0.03 - 0.20)	0.73 (0.50, 1.04) ^{†‡}	0.084 [‡]	0.68 (0.45, 1.02) ^{†‡}	0.061 [‡]
Central corneal sensitivity threshold to cooled temperature (mbar), median (IQR) [†]	0.43 (0.30 - 0.70)	0.40 (0.22 - 0.68)	-0.07 (-0.22 - 0.05)	0.35 (0.22 - 0.60)	-0.07 (-0.20 - 0.03)	0.32 (0.15 - 0.47)	0.36 (0.20 - 0.60)	0.09 (0.00 - 0.20)	0.40 (0.20 - 0.52)	0.05 (-0.00 - 0.20)	0.73 (0.48, 1.12) ^{†‡}	0.15 [‡]	0.68 (0.45, 1.04) ^{†‡}	0.077 [‡]

Central corneal dendritic cell density (cells/mm ²), median (IQR) [†]	19.79 (10.42 - 34.38)	31.25 (17.19 - 45.83)	6.77 (-6.77 - 15.63)	30.21 (16.15 - 44.79)	7.81 (-3.65 - 19.79)	37.76 (18.23 - 88.02)	34.64 (15.10 - 71.35)	-8.07 (-20.31 - -0.52)	21.88 (16.67 - 55.73)	-2.08 (-11.46 - 15.10)	2.22 (1.32, 3.75) ^{†‡}	0.003 0 [‡]	2.65 (0.90, 7.79) ^{†‡}	0.077 [‡]
Peripheral corneal dendritic cell density (cells/mm ²), median (IQR) [†]	69.53 (43.75 - 124.22)	64.84 (46.09 - 119.53)	5.47 (-8.59 - 18.75)	66.41 (42.19 - 82.03)	-2.34 (-46.88 - 6.25)	105.08 (76.56 - 187.50)	94.14 (45.31 - 151.56)	-11.33 (-41.41 - 17.97)	75.00 (60.94 - 135.16)	-10.16 (-40.62 - 10.94)	2.18 (0.78, 6.06) ^{†‡}	0.14 [‡]	2.03 (0.73, 5.62) ^{†‡}	0.18 [‡]
Peripheral corneal sensitivity threshold to room temperature (mbar), median (IQR) [†]	0.62 (0.35 - 0.95)	0.55 (0.43 - 0.80)	-0.05 (-0.20 - 0.10)	0.50 (0.25 - 0.88)	-0.08 (-0.38 - 0.20)	0.44 (0.28 - 0.52)	0.50 (0.30 - 0.65)	0.07 (-0.03 - 0.18)	0.45 (0.28 - 0.75)	0.23 (-0.17 - 0.28)	0.73 (0.46, 1.18) ^{†‡}	0.20 [‡]	0.75 (0.46, 1.21) ^{†‡}	0.23 [‡]
Peripheral corneal sensitivity threshold to cooled temperature (mbar), median (IQR) [†]	0.52 (0.28 - 0.70)	0.45 (0.40 - 0.73)	-0.05 (-0.15 - -0.02)	0.32 (0.25 - 0.65)	-0.10 (-0.27 - 0.13)	0.36 (0.28 - 0.45)	0.43 (0.25 - 0.65)	0.03 (-0.12 - 0.33)	0.35 (0.30 - 0.55)	0.02 (-0.08 - 0.12)	0.96 (0.56, 1.63) ^{†‡}	0.87 [‡]	0.87 (0.58, 1.32) ^{†‡}	0.51 [‡]

Mean and standard deviation, unless stated otherwise. All values are derived from examination of the right eye of all participants. CNBD=corneal nerve branch density. CNFD=corneal nerve fibre density. CNFL=corneal nerve fibre length. PUFA=polyunsaturated fatty acid.

* p-values are not adjusted for multiple testing.

[†] Descriptive data are presented as median and interquartile range; estimates are presented as geometric mean ratio of the change from baseline in Omega-3 PUFAs vs Placebo at day 180

[‡] Estimate and 95% CI after accounting for imbalance at baseline in the statistical analysis model.

Table S4A: Exploratory outcome: large nerve fibre function (motor nerve conduction studies) (Intention-To-Treat sample)

	Omega-3 PUFAs			Placebo			Omega-3 PUFAs vs Placebo	
	Baseline (n=20) [§]	Day 180 (n=20) [§]	Change from baseline at day 180 (n=20)	Baseline (n=22)	Day 180 (n=19)	Change from baseline at day 180 (n=19)	Estimate (95% CI) Day 180 (n=42)	p- value*
Median motor latency, Wrist-APB (ms)	3.55 (0.52)	3.45 (0.44)	-0.10 (0.16)	3.83 (0.80)	3.88 (0.77)	0.01 (0.35)	-0.16 (-0.32, -0.01)	0.043
Median motor latency, Elbow-APB (ms)	8.03 (0.73)	7.83 (0.68)	-0.20 (0.28)	8.52 (1.32)	8.50 (1.33)	-0.06 (0.49)	-0.19 (-0.43, 0.06)	0.13
Median motor amplitude, Wrist stimulation (mV)	7.93 (1.96)	8.59 (1.91)	0.66 (0.77)	7.61 (2.65)	7.88 (2.84)	0.49 (1.08)	0.20 (-0.38, 0.78)	0.50
Median motor amplitude, Elbow stimulation (mV)	7.54 (1.96)	8.20 (1.83)	0.66 (0.69)	7.33 (2.59)	7.56 (2.79)	0.46 (1.06)	0.22 (-0.34, 0.77)	0.44
Median motor velocity, Elbow-wrist (ms ⁻¹)	54.04 (3.50)	54.68 (4.52)	0.63 (3.01)	51.16 (4.89)	51.43 (5.76)	0.47 (2.69)	0.21 (-1.58, 2.00)	0.82
Peroneal motor latency, Ankle-EDB (ms), median (IQR) [†]	3.83 (3.38 - 4.53)	3.72 (3.31 - 4.34)	-0.10 (-0.56 - 0.26)	3.72 (3.42 - 4.56)	3.80 (3.55 - 4.12)	0.09 (-0.50 - 0.25)	0.98 (0.92, 1.03)	0.40
Peroneal motor latency, Below knee-EDB (ms), median (IQR) [†]	11.90 (11.15 - 12.75)	11.40 (11.05 - 12.60)	-0.10 (-1.25 - 0.30)	12.15 (11.10 - 13.40)	11.90 (11.20 - 13.00)	-0.10 (-0.30 - 0.00)	0.99 (0.96, 1.03)	0.68
Peroneal motor amplitude, Ankle stimulation (mV)	4.19 (2.62)	4.63 (2.53)	0.44 (1.11)	3.89 (1.97)	4.27 (2.16)	0.29 (0.64)	0.17 (-0.40, 0.73)	0.56
Peroneal motor amplitude, Below knee stimulation (mV)	3.51 (2.44)	3.78 (2.24)	0.27 (0.98)	3.26 (1.79)	3.59 (1.91)	0.27 (0.64)	0.02 (-0.48, 0.52)	0.94
Peroneal motor velocity, Below. knee-ankle (ms ⁻¹)	42.32 (3.86)	42.04 (5.16)	-0.28 (4.46)	41.04 (5.16)	41.14 (4.92)	0.08 (3.73)	0.01 (-2.43, 2.46)	0.99
Tibial motor latency, Ankle-Abd hal (ms), median (IQR) ^{††}	3.38 (3.04 - 4.15)	3.58 (3.10 - 4.39)	0.20 (-0.34 - 0.51)	3.46 (3.15 - 4.00)	3.96 (3.34 - 4.32)	0.08 (-0.30 - 0.63)	0.96 (0.87, 1.06)	0.3

Tibial motor amplitude, Ankle– abductor hallucis (mV) ‡	11.59 (5.25)	10.94 (4.21)	-0.61 (1.98)	10.33 (5.26)	10.28 (5.55)	-0.06 (1.73)	-0.39 (-1.51, 0.73)	0.50
Tibial motor velocity, Ankle– Abd hal (ms ⁻¹) [‡]	56.07 (5.97)	55.49 (5.54)	-0.25 (2.13)	54.24 (6.10)	54.10 (5.48)	-0.09 (1.82)	0.04 (-1.19, 1.27)	0.95

Mean and standard deviation, unless stated otherwise. Abd hal= abductor hallucis. APB= Abductor Pollicis Brevis. EDB= extensor digitorum brevis

*p-values are not adjusted for multiple testing.

[†]Descriptive data are presented as median and interquartile range; estimates are presented as geometric mean ratio of the change from baseline in Omega-3 PUFAs vs Placebo at day 180

[‡] For tibial motor nerve conduction, number of participants analysed in the treatment group was n=19 at baseline and n=20 at day 180; number analysed in the placebo group was n=22 at baseline and n=18 at day 180.

[§] One participant in the omega-3 PUFA group completed the study but did not undergo procedures relating to small or large nerve fibre function in the limbs.

Table S4B. Exploratory outcome: large nerve fibre function (sensory nerve conduction studies) (Intention-To-Treat sample)

	Omega-3 PUFAs			Placebo			Omega-3 PUFAs vs Placebo	
	Baseline (n=20) [†]	Day 180 (n=20) [†]	Change from baseline at day 180 (n=20)	Baseline (n=22)	Day 180 (n=19)	Change from baseline at day 180 (n=19)	Estimate (95% CI) Day 180 (n=42)	p- value *
Median sensory latency, orthodromic (ms)	3.03 (0.43) (n=20)	3.01 (0.41) (n=20)	-0.02 (0.16) (n=20)	3.09 (0.47) (n=20)	3.10 (0.48) (n=17)	-0.05 (0.31) (n=17)	0.01 (-0.14, 0.16) (n=40)	0.89
Median sensory amplitude, orthodromic (µV)	13.53 (6.62) (n=20)	13.00 (5.63) (n=20)	-0.53 (3.87) (n=20)	12.94 (7.83) (n=22)	12.53 (7.42) (n=19)	-0.37 (4.08) (n=19)	0.01 (-2.16, 2.18) (n=42)	1.0
Median sensory velocity, orthodromic (ms ⁻¹)	54.93 (8.87) (n=20)	54.60 (7.88) (n=20)	-0.33 (3.17) (n=20)	53.44 (7.72) (n=20)	53.33 (8.24) (n=16)	-0.03 (3.99) (n=16)	-0.14 (-2.40, 2.13) (n=40)	0.91
Median sensory latency, antidromic (ms)	3.27 (0.48) (n=19)	3.20 (0.48) (n=20)	-0.06 (0.17) (n=19)	3.33 (0.67) (n=21)	3.35 (0.60) (n=18)	-0.04 (0.41) (n=18)	-0.05 (-0.23, 0.13) (n=40)	0.60

Median sensory amplitude, antidromic (μV)	35.72 (18.82) (n=19)	33.61 (18.20) (n=20)	-1.12 (4.02) (n=19)	34.45 (21.36) (n=22)	31.08 (21.82) (n=19)	-3.35 (7.27) (n=19)	2.25 (-1.36, 5.85) (n=41)	0.22
Median sensory velocity, antidromic (ms^{-1})	53.28 (8.71) (n=19)	53.75 (8.27) (n=20)	0.30 (3.61) (n=19)	52.15 (8.87) (n=21)	51.94 (8.87) (n=17)	-0.29 (4.69) (n=17)	0.75 (-1.89, 3.39) (n=40)	0.58
Ulnar sensory latency, orthodromic (ms)	2.49 (0.21) (n=20)	2.44 (0.22) (n=20)	-0.05 (0.15) (n=20)	2.51 (0.37) (n=21)	2.56 (0.35) (n=19)	-0.00 (0.19) (n=18)	-0.07 (-0.16, 0.03) (n=41)	0.15
Ulnar sensory amplitude, orthodromic (μV)	9.18 (4.67) (n=20)	8.61 (4.18) (n=20)	-0.57 (2.30) (n=20)	7.15 (4.83) (n=22)	7.58 (4.78) (n=19)	0.53 (1.37) (n=19)	-0.81 (-1.93, 0.31) (n=42)	0.16
Ulnar sensory velocity, orthodromic (ms^{-1})	57.98 (5.45) (n=20)	57.94 (5.21) (n=20)	-0.04 (3.31) (n=20)	57.40 (7.98) (n=21)	56.29 (7.01) (n=18)	-0.69 (4.59) (n=17)	0.95 (-1.40, 3.29) (n=41)	0.43
Ulnar sensory latency, antidromic (ms)	31.66 (16.04) (n=19)	29.87 (14.66) (n=20)	-0.82 (5.51) (n=19)	28.80 (17.45) (n=22)	29.02 (19.19) (n=19)	0.47 (9.55) (n=19)	-1.19 (-6.04, 3.66) (n=41)	0.63
Ulnar sensory amplitude, antidromic (μV)	55.75 (6.15) (n=19)	56.13 (4.83) (n=20)	0.34 (5.12) (n=19)	54.15 (7.51) (n=22)	55.62 (8.67) (n=18)	1.10 (6.09) (n=18)	-0.35 (-3.73, 3.02) (n=41)	0.84
Ulnar sensory velocity, antidromic (ms^{-1})	2.74 (0.28) (n=19)	2.64 (0.25) (n=20)	-0.09 (0.24) (n=19)	2.85 (0.62) (n=22)	2.71 (0.30) (n=19)	-0.16 (0.50) (n=19)	-0.02 (-0.16, 0.11) (n=41)	0.73
Sural sensory latency, orthodromic (ms)	3.72 (0.27) (n=20)	3.70 (0.37) (n=20)	-0.02 (0.32) (n=20)	3.67 (0.48) (n=21)	3.72 (0.55) (n=19)	-0.01 (0.42) (n=18)	-0.02 (-0.25, 0.21) (n=41)	0.87
Sural sensory amplitude, orthodromic (μV)	11.93 (7.11) (n=20)	11.44 (6.70) (n=20)	-0.49 (3.88) (n=20)	12.26 (7.80) (n=22)	12.27 (8.25) (n=19)	-0.37 (2.84) (n=19)	-0.20 (-2.26, 1.86) (n=42)	0.85
Sural sensory velocity, orthodromic (ms^{-1})	47.79 (3.90) (n=20)	48.24 (5.06) (n=20)	0.45 (3.54) (n=20)	48.25 (7.50) (n=21)	70.91 (99.31) (n=18)	24.29 (102.69) (n=17)	-22.98 (-66.48, 20.51) (n=41)	0.30

Mean and standard deviation, unless stated otherwise. IQR=interquartile range. PUFAs=polyunsaturated fatty acids.

*p-values are not adjusted for multiple testing.

† One participant in the omega-3 PUFA group completed the study but did not undergo procedures relating to small or large nerve fibre function in the limbs.

Table S5. Exploratory outcome: small nerve fibre function (quantitative sudomotor axonal reflex testing) (Intention-To-Treat sample)

	Omega-3 PUFAs			Placebo			Omega-3 PUFAs vs Placebo	
	Baseline (n=20)	Day 180 (n=20)	Change from baseline at day 180 (n=20)	Baseline (n=22)	Day 180 (n=17)	Change from baseline at day 180 (n=17)	Estimate (95% CI) Day 180 (n=42)	P- value*
Forearm: sweat volume (μL) [†]	1.74 (1.35)	1.50 (1.52)	-0.24 (0.97)	1.49 (0.79)	2.02 (1.36)	0.54 (1.07)	-0.76 (-1.42, -0.11)	0.022
Forearm: response latency (s) [†]	104.35 (44.98)	121.65 (38.71)	17.30 (63.91)	113.14 (37.11)	101.24 (43.56)	-10.76 (38.61)	21.66 (-4.47, 47.79)	0.10
Proximal leg: sweat volume (μL) [‡]	1.04 (0.49)	1.05 (0.46)	0.01 (0.42)	1.19 (0.51)	0.93 (0.56)	-0.16 (0.47)	0.16 (-0.16, 0.47)	0.33
Proximal leg: response latency (s) [‡]	118.71 (41.65)	120.79 (42.23)	1.00 (55.71)	127.36 (46.45)	129.69 (43.89)	10.56 (59.44)	-7.67 (-39.21, 23.86)	0.63
Distal leg: sweat volume (μL) [§]	1.52 (1.06)	1.44 (1.17)	-0.06 (0.88)	1.72 (1.03)	1.81 (1.32)	0.22 (1.31)	-0.31 (-1.00, 0.38)	0.38
Distal leg: response latency (s) [§]	105.95 (52.50)	119.11 (34.47)	15.61 (56.51)	117.27 (49.42)	124.33 (64.15)	4.83 (87.00)	-5.38 (-39.02, 28.26)	0.75

Mean and standard deviation, unless stated otherwise. PUFAs=polyunsaturated fatty acids.

* p-values are not adjusted for multiple testing.

† For QSART in the forearm, number of participants analysed in the treatment group was n=20 at baseline and at day 180; number analysed in the placebo group was n=22 at baseline and n=17 at day 180.

‡ For QSART in the proximal leg, number of participants analysed in the treatment group was n=17 at baseline and n=14 at day 180; number analysed in the placebo group was n=14 at baseline and n=12 at day 180. Estimates for between-group change derived from n=31.

§ For QSART in the distal leg, number of participants analysed in the treatment group was n=20 at baseline and n=18 at day 180; number analysed in the placebo group was n=22 at baseline and n=18 at day 180.

^{||} One participant in the omega-3 PUFA group completed the study but did not undergo procedures relating to small or large nerve fibre function in the limbs.

Table S6. Exploratory outcome: nerve excitability (Intention-To-Treat sample)

	Omega-3 PUFAs			Placebo			Omega-3 PUFAs vs Placebo	
	Baseline (n=18) [§]	Day 180 (n=16) [§]	Change from baseline at day 180 (n=16)	Baseline (n=22)	Day 180 (n=18)	Change from baseline at day 180 (n=18)	Estimate (95% CI) Day 180 (n=40)	P- value*
TEd peak (%)	70.24 (4.24)	69.45 (4.69)	-0.80 (3.42)	67.58 (4.48)	69.39 (9.02)	1.37 (9.81)	-2.43 (-7.24, 2.39) [‡]	0.32
TEd 90-100 (%)	45.08 (3.09)	44.94 (3.92)	-0.18 (4.21)	44.12 (4.89)	47.49 (13.85)	4.34 (15.21)	-2.90 (-9.87, 4.07)	0.42
TEh 90-100 (%)	-117.02 (17.34)	-126.35 (21.27)	-6.56 (12.56)	-117.77 (19.66)	-115.24 (19.16)	1.61 (14.66)	-9.33 (-18.41, -0.25)	0.044
TEd 10-20 (%)	70.52 (4.60)	70.27 (4.71)	-0.24 (3.78)	67.99 (4.30)	68.00 (4.71)	-0.74 (4.25)	1.09 (-1.42, 3.60)	0.40
Superexcitability (%)	-23.45 (5.11)	-23.48 (5.35)	0.15 (3.14)	-21.14 (6.79)	-21.60 (6.73)	-0.10 (4.70)	-0.19 (-2.73, 2.34)	0.88
Subexcitability (%)	13.61 (2.78)	13.78 (3.04)	0.08 (2.60)	12.75 (4.10)	12.51 (3.29)	-0.43 (4.07)	0.95 (-0.88, 2.78)	0.31
SDTC (ms)	0.49 (0.10)	0.48 (0.09)	-0.02 (0.08)	0.50 (0.11)	0.49 (0.09)	-0.01 (0.13)	-0.01 (-0.07, 0.05)	0.74
Resting I/V slope, median (IQR) [†]	0.61 (0.54 - 0.65)	0.58 (0.56 - 0.63)	0.01 (-0.03 - 0.04)	0.62 (0.56 - 0.71)	0.63 (0.56 - 0.76)	0.08 (-0.07 - 0.14)	0.96 (0.77, 1.19) [†]	0.73
Stimulus (mA) for 50% max response, median (IQR) [†]	4.00 (3.63 - 4.41)	4.86 (4.20 - 5.82)	0.72 (0.31 - 1.58)	4.20 (3.34 - 5.92)	4.44 (4.28 - 5.41)	0.41 (-0.21 - 1.10)	1.11 (0.96, 1.28) [†]	0.16
Rheobase (mA), median (IQR) [†]	2.68 (2.03 - 2.82)	3.02 (2.73 - 3.88)	0.69 (0.17 - 1.21)	2.60 (2.10 - 3.40)	3.08 (2.50 - 3.64)	0.29 (-0.12 - 0.83)	1.10 (0.92, 1.32) [†]	0.29
Stimulus-response slope	4.68 (1.53)	4.64 (1.51)	-0.00 (0.65)	4.73 (1.12)	4.90 (1.06)	0.18 (0.92)	-0.20 (-0.70, 0.30)	0.44
Peak response (mV)	73.22 (91.26)	98.92 (94.33)	25.30 (81.73)	48.79 (62.38)	62.60 (87.98)	12.77 (106.27)	25.82 (-29.82, 81.45)	0.36
Minimum I/V slope	0.24 (0.05)	0.23 (0.04)	-0.01 (0.04)	0.24 (0.04)	0.24 (0.04)	-0.00 (0.03)	-0.01 (-0.03, 0.01)	0.41
RRP (ms)	3.26 (0.32)	3.07 (0.31)	-0.20 (0.33)	3.42 (0.51)	3.24 (0.53)	-0.20 (0.39)	-0.06 (-0.27, 0.16)	0.59
Latency (ms)	7.14 (0.64)	6.97 (0.60)	-0.15 (0.29)	7.33 (0.81)	7.38 (0.86)	0.00 (0.53)	-0.19 (-0.47, 0.09)	0.18

TEd 40-60 (%)	53.63 (2.86)	51.76 (3.60)	-1.96 (3.28)	53.25 (7.51)	55.32 (11.98)	1.80 (14.30)	-3.59 (-9.65, 2.47)	0.25
TEh 10-20 (%)	-75.53 (6.75)	-77.25 (5.52)	-0.69 (3.63)	-76.58 (6.72)	-74.57 (4.83)	2.41 (5.62)	-2.90 (-5.33, -0.47)	0.019
TEd undershoot (%)	-20.89 (3.54)	-20.59 (3.89)	0.34 (2.79)	-20.58 (3.95)	-19.37 (5.09)	1.78 (4.93)	-1.30 (-3.86, 1.25)	0.32
TEh overshoot (%)	17.11 (4.88)	17.16 (3.92)	-0.33 (3.14)	16.88 (3.89)	15.99 (4.29)	-1.16 (3.03)	0.95 (-0.93, 2.83)	0.32
S2 accommodation	23.85 (6.70)	24.50 (2.70)	-0.62 (2.61)	21.62 (6.28)	21.90 (7.03)	0.24 (10.19)	2.48 (-1.19, 6.14)	0.19
Accommodation half-time (ms)	43.57 (7.21)	40.12 (4.05)	-1.96 (3.92)	44.84 (9.80)	43.97 (6.16)	-1.28 (10.44)	-3.21 (-6.52, 0.10)	0.058
Hyperpolarizing I/V slope	0.32 (0.07)	0.33 (0.06)	0.01 (0.10)	0.32 (0.05)	0.33 (0.05)	0.02 (0.05)	-0.01 (-0.05, 0.03)	0.68
Refractoriness at 2.5 ms (%)	37.35 (20.08)	24.67 (14.49)	-13.93 (18.51)	37.41 (20.44)	28.03 (20.14)	-9.92 (19.20)	-3.65 (-13.55, 6.25)	0.47
TEh 20-40 (%)	-93.61 (9.98)	-96.72 (9.91)	-1.37 (5.24)	-93.57 (10.15)	-91.53 (7.74)	2.42 (6.80)	-4.24 (-7.78, -0.70)	0.019
TEh slope 101-140 (%)	2.07 (0.39)	2.22 (0.44)	0.08 (0.26)	2.05 (0.36)	2.00 (0.44)	-0.06 (0.22)	0.15 (-0.01, 0.31)	0.071
Refractoriness at 2 ms (%), median (IQR) [†]	84.75 (68.47 - 126.47)	70.52 (51.73 - 104.75)	-27.64 (-45.77 - 6.64)	84.82 (69.84 - 114.53)	73.74 (57.64 - 86.94)	-18.74 (-34.35 - -3.02)	0.97 (0.74, 1.29) [†]	0.86
Superexcitability at 7 ms (%)	-23.38 (5.28)	-22.50 (5.40)	1.04 (2.74)	-20.92 (6.66)	-21.21 (6.08)	0.08 (5.47)	0.28 (-2.36, 2.93)	0.83
Superexcitability at 5 ms (%)	-22.90 (4.97)	-24.73 (5.52)	-1.71 (4.46)	-20.51 (7.69)	-21.34 (7.91)	-0.64 (4.26)	-1.48 (-4.27, 1.30)	0.30
TEd 20 peak (%)	39.52 (3.31)	39.58 (3.80)	0.04 (2.48)	37.73 (3.00)	37.63 (3.38)	-0.89 (3.04)	0.62 (-1.31, 2.56) [‡]	0.24

Mean and standard deviation, unless stated otherwise. IQR=interquartile range. I/V=current/voltage. RRP= relative refractory period. PUFAs=polyunsaturated fatty acids. SDTC=strength duration time constant, TEd=depolarising threshold electrotonus. TEh= hyperpolarising threshold electrotonus.

* p-values are not adjusted for multiple testing.

[†] Descriptive data are presented as median and interquartile range; estimates are presented as geometric mean ratio of the change from baseline in Omega-3 PUFAs vs Placebo at day 180

[‡] Estimate and 95% CI after accounting for imbalance at baseline in the statistical analysis model

[§] In the omega-3 PUFA group, one person completed the study but did not undergo procedures relating to small or large nerve fibre function in the limbs; two people could not tolerate the procedure.

Table S7. Exploratory outcomes: anterior ocular examination at baseline, day 90, and day 180 (Intention-To-Treat sample)

	Omega-3 PUFAs					Placebo					Omega-3 PUFAs vs Placebo			
	Baseline (n=21)	Day 90 (n=19)	Change from baseline at day 90 (n=19)	Day 180 (n=21)	Change from baseline at day 180 (n=21)	Baseline (n=22)	Day 90 (n=18)	Change from baseline at day 90 (n=18)	Day 180 (n=19)	Change from baseline at day 180 (n=19)	Estimate (95% CI) Day 90 (n=43)	p-value [*]	Estimate (95% CI) Day 180 (n=43)	p-value [*]
Ocular Surface Disease Index score (0-100), median (IQR) [‡]	6.3 (2.1 - 12.5)	6.3 (2.1 - 13.9)	0.0 (-4.1 - 2.1)	6.8 (2.1 - 10.4)	0.0 (-2.1 - 2.1)	5.3 (2.1 - 8.3)	4.2 (0.0 - 6.3)	-0.0 (-2.8 - 0.0)	4.2 (0.0 - 12.5)	0.0 (-4.2 - 4.2)	0 (-2.8, 2.2) [‡]	0.59	0 (-3, 4.2) [‡]	0.74
Phenol red thread test (mm) [§]	14.1 (4.3)	14.8 (2.8)	1.0 (4.0)	15.0 (4.4)	0.6 (5.3)	13.9 (4.7)	13.1 (4.1)	-1.0 (4.7)	13.2 (4.7)	-0.2 (4.0)	1.8 (-0.3, 3.8)	0.087	1.3 (-1.4, 4.0)	0.33
Anterior blepharitis grading [‡]	1.48 (0.81)	1.24 (0.56)	-0.35 (0.71)	0.98 (0.61)	-0.50 (0.70)	1.08 (0.82)	1.26 (0.85)	0.21 (0.61)	1.36 (0.81)	0.21 (0.71)	-0.33 (-0.69, 0.02)	0.066	-0.54 (-0.90, -0.18)	0.0030
Meibomian gland dysfunction grading [‡]	1.63 (0.84)	1.35 (0.57)	-0.35 (0.78)	1.23 (0.68)	-0.39 (0.72)	1.00 (0.76)	1.26 (0.85)	0.24 (0.59)	1.31 (0.77)	0.26 (0.72)	-0.56 (-0.98, -0.13)	0.011	-0.67 (-1.11, -0.23)	0.0030
Conjunctival redness grading [‡]	0.88 (0.36)	0.85 (0.30)	-0.07 (0.29)	0.82 (0.40)	-0.06 (0.26)	0.79 (0.32)	0.88 (0.33)	0.14 (0.29)	0.82 (0.30)	0.05 (0.35)	-0.13 (-0.29, 0.03)	0.099	-0.07 (-0.24, 0.11)	0.47
Limbal redness grading [‡]	0.69 (0.55)	0.65 (0.50)	-0.07 (0.36)	0.61 (0.53)	-0.07 (0.37)	0.52 (0.37)	0.71 (0.50)	0.23 (0.40)	0.52 (0.30)	0.03 (0.29)	-0.24 (-0.47, -0.01)	0.040	-0.04 (-0.23, 0.15)	0.67
Sodium fluorescein tear break-up time (s) [right eye], median (IQR) [†]	7.18 (5.49 - 9.61)	10.23 (7.09 - 13.88)	2.52 (0.45 - 5.31)	10.77 (7.91 - 12.93)	4.79 (-0.49 - 7.16)	9.99 (7.40 - 18.83)	11.30 (7.54 - 16.05)	-0.44 (-5.95 - 4.55)	10.44 (7.71 - 14.22)	-1.26 (-4.43 - 1.75)	1.39 (1.04, 1.86) [†]	0.028	1.64 (1.20, 2.23) [†]	0.0020

Osmolarity (mOsm) [highest of two eyes]	307.0 (12.4)	-	-	309.1 (10.4)	2.1 (14.3)	301.2 (13.6)	-	-	305.21(1.0)	3.5 (16.9)	-	-	3.2 (-3.4, 9.7)	0.34
Ocular surface staining, n [%]														
<0.5	14 [67%]	14 [74%]	-	12 [57%]	-	16 [73%]	11 [61%]	-	16 [84%]	-				
0.5 - <1.0	3 [14%]	4 [21%]	-	5 [24%]	-	3 [14%]	4 [22%]	-	2 [11%]	-				
1.0-<1.5	1 [5%]	0 [0%]	-	3 [14%]	-	1 [5%]	3 [17%]	-	0 [0%]	-				
1.5-<2.0	1 [5%]	1 [5%]	-	1 [5%]	-	1 [5%]	0 [0%]	-	1 [5%]	-	-	0.057	-	0.16
2.0 - 2.5	1 [5%]	0 [0%]	-	0 [0%]	-	1 [5%]	0 [0%]	-	0 [0%]	-				
2.5-<3.0	1 [5%]	0 [0%]	-	0 [0%]	-	0 [0%]	0 [0%]	-	0 [0%]	-				
>3.0	0 [0%]	0 [0%]	-	0 [0%]	-	0 [0%]	0 [0%]	-	0 [0%]	-				

Mean and standard deviation, unless stated otherwise. All values are derived from the average value of two eyes, unless otherwise indicated.

IQR=interquartile range. PUFAs=polyunsaturated fatty acids.

* p-values are not adjusted for multiple testing.

† Estimates are presented as geometric mean ratio of the change from baseline in Omega-3 PUFAs vs Placebo at day 180

‡ p-value for treatment comparison in change from baseline values was derived using the Wilcoxon rank sum test. Estimates and 95% confidence intervals at days 90 and 180 were derived using Hodges-Lehman non-parametric estimator.

§ Number of participants analysed for phenol red thread test in the treatment group was n=21 at baseline, n=19 at day 90 and n=19 at day 180; number analysed in the placebo group was n=22 at baseline, n=18 at day 90 and n=17 at day 180. Estimates for between-group change derived from n=43

‡ Using the Efron grading scale

|| Estimate and 95% CI after accounting for imbalance at baseline in the statistical analysis model.

Table S8. Safety outcome: blood pathology parameters at baseline and day 180 (Safety sample)

	Omega-3 PUFAs			Placebo			Omega-3 PUFAs vs Placebo	
	Baseline (n=21)	Day 180 (n=20)	Change from baseline at day 180 (n=20)	Baseline (n=19)	Day 180 (n=16)	Change from baseline at day 180 (n=16)	Estimate (95% CI) Day 180 (n=40)	P- value*
Haemoglobin (HB) (g/L)	140.5 (9.3)	139.8 (11.4)	-0.7 (5.9)	141.7 (10.8)	139.7 (15.3)	-1.3 (7.9)	1.0 (-3.5, 5.4)	0.67
Platelets (x10 ⁹ /L)	259.2 (40.3)	257.0 (47.8)	-2.3 (20.1)	244.5 (57.3)	251.6 (64.5)	9.6 (30.5)	-10.2 (-26.6, 6.3)	0.23
HbA1c (%)	7.34 (0.81)	7.44 (0.79)	0.10 (0.51)	7.82 (1.11)	7.81 (0.94)	0.13 (0.54)	-0.11 (-0.42, 0.21)	0.50
Alkaline phosphatase (ALP) (U/L)	75.3 (18.0)	76.6 (19.6)	1.2 (7.4)	79.1 (26.0)	83.4 (33.3)	6.5 (11.3)	-4.9 (-10.9, 1.1)	0.11
Sodium (mmol/L)	140.2 (2.0)	140.1 (2.2)	-0.1 (2.1)	139.4 (3.0)	140.7 (3.2)	0.9 (2.9)	-0.9 (-2.4, 0.6)	0.22
Potassium (mmol/L)	4.46 (0.28)	4.35 (0.29)	-0.11 (0.31)	4.32 (0.36)	4.52 (0.36)	0.15 (0.40)	-0.20 (-0.39, -0.00)	0.045
Carbon dioxide (mmol/L)	27.3 (2.7)	27.2 (2.0)	-0.10 (2.5)	26.7 (2.2)	27.1 (3.1)	0.3 (2.2)	-0.1 (-1.5, 1.2)	0.85
Thyroid stimulating hormone (TSH)(mU/L)	1.88 (0.86)	1.70 (0.65)	-0.23 (0.56)	1.65 (1.34)	1.96 (1.90)	0.39 (0.73)	-0.64 (-1.06, -0.23)	0.0020
White blood cells (x10 ⁹ /L), median (IQR) [†]	5.60 (4.60 - 6.50)	5.60 (4.50 - 6.60)	-0.20 (-0.60 - 0.60)	5.55 (4.85 - 7.65)	5.90 (5.05 - 7.45)	0.20 (-0.90 - 1.00)	0.99 (0.84, 1.17) [†]	0.94

Triglycerides (mmol/L), median (IQR) [†]	0.80 (0.60 - 0.80)	0.60 (0.50 - 0.90)	-0.15 (-0.25 - -0.05)	0.70 (0.60 - 1.00)	0.65 (0.55 - 0.80)	-0.10 (-0.30 - 0.10)	0.99 (0.84, 1.16) [†]	0.87
Alanine transaminase (ALT) (U/L), median (IQR) [†]	19.0 (14.0 - 24.0)	20.0 (16.0 - 26.0)	1.0 (-2.0 - 4.0)	17.0 (12.0 - 25.0)	18.0 (15.0 - 27.0)	1.0 (-1.0 - 5.0)	1.1 (0.9, 1.3) [†]	0.62
Total bilirubin (T-Bil) (umol/L), median (IQR) [†]	13.0 (10.0 - 17.0)	14.0 (10.5 - 16.5)	0.0 (-2.0 - 3.5)	13.0 (8.0 - 16.0)	9.50 (8.5 - 14.5)	-2.0 (-5.0 - 0.0)	1.2 (1.0, 1.6) [†]	0.097
Gamma-glutamyl transferase (GGT) (U/L), median (IQR) [†]	15.0 (11.0 - 27.0)	15.0 (13.0 - 21.0)	1.0 (0.0 - 5.0)	15.0 (10.0 - 17.0)	16.0 (12.5 - 19.5)	2.0 (-2.0 - 3.0)	1.1 (0.9, 1.3) [†]	0.32
Estimated glomerular filtration rate (eGFR), n [%]								
>90	13 [62%]	13 [62%]	-	11 [73%]	11 [73%]	-	-	1.0
<90	8 [38%]	8 [38%]	-	4 [7%]	4 [7%]	-	-	

Mean and standard deviation, unless stated otherwise. IQR=interquartile range. PUFAs=polyunsaturated fatty acids.

*p-values are not adjusted for multiple testing.

[†]Descriptive data are presented as median and interquartile range; estimates are presented as geometric mean ratio of the change from baseline in Omega-3 PUFAs vs Placebo at day 180