Experimental Eye Research 167 (2018) 128–139

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

Omega-3 fatty acids protect retinal neurons in the DBA/2J hereditary glaucoma mouse model

Maria Kalogerou[a], Panagiotis Kolovos[a], Ekatherine Prokopiou[a], Gregory Papagregoriou[b], Constantinos Deltas[b], Stavros Malas[c], Tassos Georgiou[a,*]

[a] Ophthalmos Research and Educational Institute, Morfou 48, Egkomi, 2417 Nicosia, Cyprus
[b] Molecular Medicine Research Centre and Laboratory of Molecular and Medical Genetics, Department of Biological Sciences, University of Cyprus, Kallipoleos 75, 1678 Nicosia, Cyprus
[c] Developmental and Functional Genetics Group, The Cyprus Institute of Neurology and Genetics, 1683 Nicosia, Cyprus

ARTICLE INFO

Keywords:
DBA/2J
Glaucoma
Inflammation
Neuroprotection
Omega-3
Eicosapentaenoic acid
Retinal ganglion cell

ABSTRACT

The purpose of this study was to evaluate the neuroprotective effects of omega-3 polyunsaturated fatty acid (ω3-PUFA) supplementation, alone or in combination with timolol eye drops, in a mouse model of hereditary glaucoma. DBA/2J mice (8.5-month-old) were assigned to an ω3-PUFAs + timolol, ω3-PUFAs only, timolol only, or an untreated group. Treated mice received a daily gavage administration of eicosapentaenoic acid (EPA) and docosahexaenoic acid and/or topical instillation of timolol (0.5%) once a day for 3 months. Blood was analysed regularly to determine ω3-PUFA levels and retinas were histologically analysed. Real-time PCR and Western blot were performed for retinal pro-inflammatory cytokines and macrophages. Blood arachidonic acid/EPA ratio gradually decreased and reached the desired therapeutic range (1–1.5) after 4 weeks of daily gavage with ω3-PUFAs in the ω3-PUFAs + timolol and ω3-PUFAs only groups. Retinal ganglion cell densities were significantly higher in the ω3-PUFAs + timolol (1303.77 ± 139.62/mm^2), ω3-PUFAs only (768.40 ± 52.44/mm^2) and timolol only (910.57 ± 57.28/mm^2) groups than in the untreated group (323.39 ± 95.18/mm^2). ω3-PUFA supplementation alone or timolol alone, significantly increased protein expression levels of M1 macrophage-secreted inducible nitric oxide synthase and M2 macrophage-secreted arginase-1 in the retina, which led to significant decreases in the expression levels of tumour necrosis factor-α (TNF-α). ω3-PUFA supplementation alone also resulted in significantly reduced expression of interleukin-18 (IL-18). ω3-PUFA + timolol treatment had no effect on the expression level of any of the aforementioned mediators in the retina. Supplementation with ω3-PUFAs has neuroprotective effect in the retinas of DBA/2J mice that is enhanced when combined with timolol eye drops. The continued inflammation following ω3-PUFAs + timolol treatment suggests that downregulation of IL-18 and TNF-α may not be the only factors involved in ω3-PUFA-mediated neuroprotection in the retina.

1. Introduction

Glaucoma is a neurodegenerative disease characterized by the progressive death of retinal ganglion cells (RGCs) and excavation of the optic nerve head (ONH) (Quigley, 1993, 1999). Glaucoma is the second leading cause of irreversible blindness, and affects 60 million people worldwide. The number of patients with glaucoma is predicted to increase to 79.6 million by the year 2020 (Quigley and Broman, 2006). Pigmentary glaucoma is a common type of secondary glaucoma characterized by the disruption of the posterior iris-pigment epithelium and dispersion of the pigment throughout the anterior segment and the trabecular meshwork, resulting in a decrease in aqueous outflow and a subsequent increase in intraocular pressure (IOP) (Campbell and Schertzer, 1995; Sowka, 2004). Several risk factors, especially age and elevated IOP, play crucial roles in glaucoma development (Gordon et al., 2002; Ahmed et al., 2004). However, the exact relationship between IOP and glaucoma remains unclear; not all people with elevated IOP develop glaucoma, while even those with normal IOP can develop the disease (Heijl et al., 2002).

Low-grade inflammation is involved in the pathogenesis, development and progression of glaucoma, and is directly associated with RGC degeneration (Yan et al., 2000; Yuan and Neufeld, 2000; Tezel et al.,

* Corresponding author. Ophthalmos Research and Educational Institute, Morfou 48, Engomi, Nicosia, 2417, Cyprus.
E-mail addresses: kalogerou.maria@ucy.ac.cy (M. Kalogerou), pankoaua@hotmail.com (P. Kolovos), prokopiou.katerina@ucy.ac.cy (E. Prokopiou), papagregoriou@ucy.ac.cy (G. Papagregoriou), deltas@ucy.ac.cy (C. Deltas), smalas@cing.ac.cy (S. Malas), tassosgeorgiou@hotmail.com (T. Georgiou).

https://doi.org/10.1016/j.exer.2017.12.005
Received 20 April 2017; Received in revised form 15 November 2017; Accepted 11 December 2017
Available online 16 December 2017

2001; Zhou et al., 2005; Nakazawa et al., 2006; Vohra et al., 2013; Chi et al., 2014). Numerous studies have indicated that the immune system is involved even before the normal signs of glaucoma begin, and have suggested that elevated IOP might act later as an additional factor that aggravates disease progression (Zhou et al., 2005; Fan et al., 2010). These findings suggest that pro-inflammatory tumour necrosis factor-α (TNF-α)-, interleukin-18 (IL-18)- and interleukin-1β-mediated cell death is involved in the neurodegeneration process, and suppression of inflammation may provide a novel therapeutic target for neuroprotection in glaucoma treatment.

To date, the only therapies that can improve or halt the deterioration of vision in patients with glaucoma are those that reduce IOP via topical instillation of anti-glaucoma eye drops, laser therapy or invasive surgery (Conlon et al., 2017). Currently, the most common anti-glaucoma eye drops are topical prostaglandin analogues and beta-adrenoreceptor antagonists such as timolol (Gupta et al., 2008). Therapies that lower IOP, within normal limits, have been estimated to slow RGC degeneration by 50–60% (Quigley, 2012). Neuroprotection of RGCs is thought to be a promising therapeutic strategy directed at preventing the pathophysiology contributing to RGC death (McKinnon, 1997; Levin, 2003; Vasudevan et al., 2011; Tian et al., 2015; Levin et al., 2016; Sena and Lindsley, 2017).

Omega-3 polyunsaturated fatty acids (ω3-PUFAs) have been gaining interest of researchers for the prevention of inflammation and are considered anti-inflammatory molecules (Meydani et al., 1991; Wallace et al., 2000; Calder, 2006; Schnebelen et al., 2009, 2011). The resolution of inflammation is an active process that is primarily driven by anti-inflammatory and pro-resolving mediators derived from the ω3-PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Serhan et al., 2004; Nowak, 2010). In contrast, the omega-6 PUFA arachidonic acid (AA) functions in promoting inflammatory responses, and is the source of pro-inflammatory prostaglandins and leukotrienes that induce the production of inflammatory cytokines and leukocyte chemotaxis. The balance between the pro- and anti-inflammatory mediators plays a pivotal role in disease progression and the resolution of inflammation, and can be assessed by looking at the ratio of AA to EPA in the blood (Serhan et al., 2004; Nowak, 2010; Georgiou and Prokopiou, 2015).

Several clinical and preclinical studies have shown the protective effects of ω3-PUFAs in a number of ocular diseases, including age-related macular degeneration (AMD) (Seddon et al., 2001, 2003; Ebert et al., 2009; Tuo et al., 2009; Ramkumar et al., 2013; Georgiou and Prokopiou, 2015), age-related maculopathy (Smith et al., 2000), retinitis pigmentosa (Hodge et al., 2006) and optic nerve injury (Peng et al., 2016), and their link to glaucoma has been a subject of particular interest over the past decade (Kang et al., 2004; Nguyen et al., 2007; Garcia-Medina et al., 2015; Tellez-Vazquez, 2016). Interestingly, a number of studies have demonstrated an inverse association between ω3-PUFA intake and the occurrence of several eye diseases, including glaucoma (Connor et al., 1992; Cellini et al., 1998; Ren et al., 2006; Nguyen et al., 2007; Renard et al., 2013). Ren et al. (2006) showed that levels of EPA and DHA fatty acids in the blood of patients with primary open-angle glaucoma were lower than those in the blood of their healthy siblings. Currently, the ratio of AA/EPA in the blood is considered to be a clinically relevant measurement. Previous studies have demonstrated that assessing levels of systemic biomarkers such as EPA, DHA, and AA, provide parameters for the stratification of risks associated with inflammatory events, and the AA/EPA ratio is thus a good index for disease progression and treatment efficacy (Rupp et al., 2004; Gorusupudi et al., 2016).

Our previous studies investigated the therapeutic potential of ω3-PUFAs in rodent models of anterior ischaemic optic neuropathy (rAION) (Georgiou et al., 2017), dry AMD (Prokopiou et al., 2017) and Stargardt disease (Prokopiou et al., 2017; reviewed). We recognized the importance of assessing the blood AA/EPA ratio (1–1.5) so that EPA and DHA dosages could be adjusted as needed, providing the maximum therapeutic effect. The results demonstrated that the neuroprotective effects of ω3-PUFAs included blocking RGC apoptosis and reducing inflammation, which was achieved via decreased inflammatory cell infiltration and regulation of macrophage polarization, which in turn decreased cytokine-induced injury of the optic nerve (Georgiou et al., 2017). These findings provide important information for the treatment of patients with the aforementioned ocular pathologies. However, further work is needed to examine the therapeutic effects that the ω3-PUFAs have on additional animal models of ocular pathologies that currently lack therapeutic approaches. Thus, we used the DBA/2J mouse model of human pigmentary glaucoma to study the therapeutic effects of ω3-PUFA supplementation, used alone or in combination with timolol, on RGC survival and inflammation.

2. Materials and methods

2.1. Animals

Male and female age-matched DBA/2J mice with hereditary glaucoma and normal wild-type C57BL/6 mice were used in this study. Mice were purchased from Charles River Laboratories (Italy) and raised at the Transgenic Mouse facility at the Cyprus Institute of Neurology and Genetics (Nicosia, Cyprus). Animal care and experimental procedures were performed in accordance with the Protection and Welfare of Animals Law of 1994–2013 and the Protection and Welfare of Animals (protection of animals used for scientific purposes) regulations of 2013. In addition, the Cyprus Veterinary Service of the Ministry of Agriculture, Rural Development and Environment approved all animal experiments. All manipulations performed on the animals were completed under general anaesthesia, which was achieved by intraperitoneal injection of tribromoethanol (20 μl/g bodyweight; Sigma-Aldrich, St. Louis, MO, USA). Mice had free access to food and water and were maintained in cages in an environmentally controlled room with a temperature of 21 ± 1 °C, humidity of 50% ± 5%, and a 12-h light/dark cycle.

2.2. Study design

The DBA/2J mice were assigned to the following treatment groups (n = 10 males and 10 females per group): ω3-PUFAs + timolol, ω3-PUFAs, timolol and untreated. All treatments were initiated at the age of 8.5 months and continued until 11.5 months of age. The mice received a daily gavage administration of fish oil (approximately 270 mg/day, K-D pharma, Germany) and/or topical instillation of timolol (Nyolol® 0.5% eye drops, Alcon Laboratories, UK) once a day until the end of the treatment (Fig. 1). The fish oil consisted of purified ethyl esters rich in EPA (400 mg) and DHA (200 mg) per gram for the liquid formulation. Blood samples were collected and analysed using a gas chromatography to determine the AA/EPA ratio. Disease progression was monitored via IOP measurements and slit lamp biomicroscopy. Mice were euthanized 1 day after the last treatment by cervical dislocation. RGC density and microglial activation were evaluated by immunohistochemistry in retinal whole mounts. mRNA levels of the pro-inflammatory cytokines IL-18 and TNF-α were measured in retinal samples by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Protein levels of IL-18, TNF-α and classically activated macrophage (M1) inducible nitric oxide synthase (iNOS) and the alternatively activated macrophage (M2) arginase-1 (Arg-1) were measured in the retinal samples by Western blot analysis.

2.3. IOP measurements

IOP measurements were performed using a rebound tonometer (Icare® TonoLab, Helsinki, Finland) on both eyes. Measurements were taken from all animals between 10:00 a.m. and 11:00 a.m. before treatment (baseline) and one month later (1 h after the daily treatment

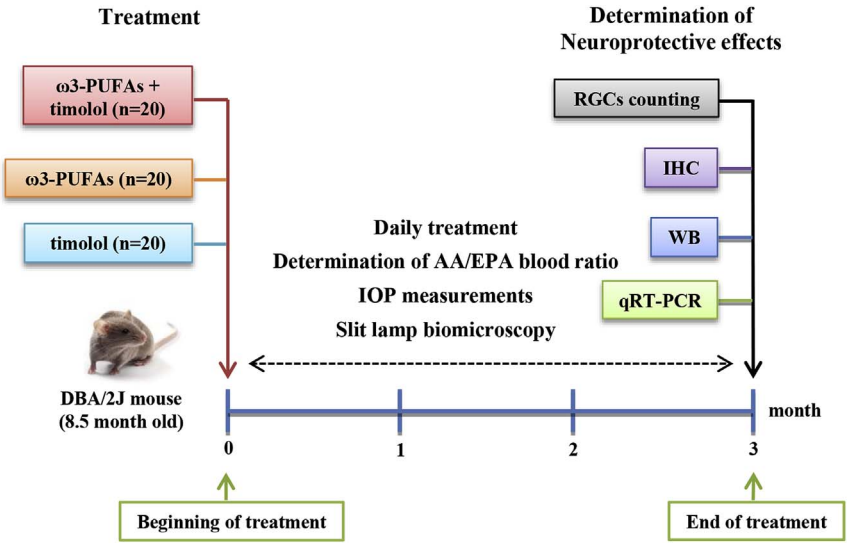

Fig. 1. Schematic illustration of the study design. ω3-PUFAs, omega-3-polyunsaturated fatty acids; AA, arachidonic acid; EPA, eicosapentaenoic acid; IOP, intraocular pressure; RGC, retinal ganglion cell; IHC, immunohistochemistry; WB, western blot; qRT-PCR, quantitative reverse transcription-polymerase chain reaction.

with ω3-PUFAs and/or timolol). Briefly, animals were anaesthetized, and their body temperature was maintained at 37 °C using a heating pad. Three IOP readings (on average) were recorded for each of the two eyes, immediately after anaesthesia was administered. Animals were allowed to recover following completion of the process. The mean IOP for each eye was averaged to give a single IOP value per animal.

2.4. Clinical slit lamp biomicroscopy

The DBA/2J mouse is a model of pigmentary glaucoma that exhibits iris atrophy, pigment dispersion, and transillumination (John et al., 1998; Chang et al., 1999; Anderson et al., 2002, 2006; Mo et al., 2003). Slit lamp biomicroscopy was performed at the beginning of the study to confirm the disease and at the end of treatment to assess any changes. Briefly, animals were anaesthetized, and their body temperature was maintained at 37 °C using a heating pad. Both eyes were examined using a Haag-Streit BQ 900® slit lamp/BQ900 with LED (USA) and photographed using a 40 × objective lens. The photographs were analysed by an investigator who was blinded to the treatment groups.

2.5. Fatty acid analysis

2.5.1. Sample preparation for analysis

To determine and maintain the optimum ω3-PUFAs dosage, blood samples from the DBA/2J mice who were given fish oil were collected at baseline and at weeks 1, 2, 4, 8, and 11 (1 h before treatment), processed for fatty acid separation, and analysed using gas chromatography (Masood et al., 2005). ω3-PUFA dosage was adjusted as needed so that the blood AA/EPA ratio was within the therapeutic range of 1–1.5. Blood samples were collected on Whatman filter paper and stored at −20 °C until the analysis. For each sample, NaOH/MeOH was added and heated for 1 min at 85 °C, and then 14% BF3/MeOH was added and heated for 7 min at 85 °C. Once the samples reached room temperature, 0.3 g NaCl was added to ensure complete migration of the total fatty acid methyl-ester fraction to the organic phase, followed by 1 mL n-hexane. When the samples were separated into the organic and the aqueous phases, the organic phase was collected, and this process was repeated three times. To remove any impurities, 0.9 g Na2S04 was added to the samples, which were then centrifuged at 3300 rpm for 5 min. The supernatants were collected and dried using an analytical evaporator at approximately 45 °C under a nitrogen stream. Once dried, the samples were redissolved in n-hexane and analysed using a gas chromatography flame-ionization detector (GC-FID).

2.5.2. GC-FID

GC-FID analysis was performed as previously described (Masood et al., 2005; Georgiou et al., 2017), using an Agilent GC-6890 system equipped with an FID. An H_2 flow rate of 35 mL/min and an air flow rate of 350 mL/min were used. The flow rate of the carrier gas (He) was set at 2.5 mL/min. The temperatures of the injection port and detector were set at 280 °C and 300 °C, respectively. The oven temperature was programmed to initiate at 160 °C for 3 min, increase to 200 °C at a rate of 20 °C/min, hold for 4 min, and, increase to 250 °C at a rate of 5 °C/min, and finally to be held at that temperature for 23 min. The injection volume was 1 μL in the split-less injection mode. A capillary column (DP-23 fused-silica capillary, 30 m × 0.25 mm I.D. × 0.25 μm film thickness; Supelco, Bellefonte, PA, USA) was employed.

2.6. Immunostaining of retinal whole-mounts

Both eyeballs from each animal were harvested upon euthanasia, fixed with 2% paraformaldehyde for 2 h at 4 °C, and rinsed three times with phosphate buffered saline (PBS). The retinas were carefully dissected (n = 16/group), permeabilized with PBS containing 1% Triton-X-100, and blocked with donkey serum diluted 1:10 in PBS for 1 h at room temperature. The retinas were then incubated with primary antibodies against either brain-specific homeobox/POU domain protein-3a (Brn-3a) or ionized calcium binding adaptor molecule-1 (Iba-1) overnight at 4 °C (Table 1). After washing, the retinas were incubated with either Alexa Fluor 488 donkey anti-goat IgG or Alexa Fluor 594 donkey anti-rabbit IgG secondary antibodies (1:2000; Invitrogen). After rinsing in PBS, the retinas were flat mounted on a glass microscope slide using Dako fluorescence-mounting medium (Dako, Denmark), and imaged using a Leica TCS SP5 confocal microscope (Leica, Germany). The images spanned the nerve fiber layer (NFL)/ganglion cell layer (GCL). Photographs of fluorescent-labeled cells were taken in 12 standardized fields of each retinal whole-mount at 40 × magnification. These fields were located, three (centre, middle, and periphery) in each retinal

Table 1
Primary antibodies used in this study.

Antigen	Host	Working Dilution	Manufacturer
Brn-3a	Goat	1:200	Santa Cruz Biotechnology, Inc., Germany
Iba-1	Rabbit	1:500	Biocare Medical, USA
IL-18	Rabbit	1:800	BioVision, Inc., USA
TNF-α	Goat	1:400	Santa Cruz Biotechnology, Inc., Germany
Arg-1	Rabbit	1:500	Thermo Fisher Scientific, USA
β-Actin	Rabbit	1:2000	Cell Signaling Technology, USA

Table 2
Specific sets of primers confirmed by qRT-PCR.

Gene names	Primer Sequences
Tnf-α	(F) GTCGTAGCAAACCACCAAGT (R) CTTTGAGATCCATGCCGTTG
Il-18	(F) GCCGACTTCACTGTACAACC (R) TCTGGTCTGGGGTTCACTGG
β-actin	(F) GCCTTCCTTCTTGGGTATGG (R) CGGATGTCAACGTCACACTT

F, forward primer; R, reverse primer.

quadrant, in both vertical and horizontal meridians. The number of Brn-3a- or Iba-1-positive cells in each field was counted using ImageJ software (https://imagej.nih.gov/ij/) by an investigator who was blinded to the experimental treatment. The total number of Brn-3a- or Iba-1-positive cells obtained in these fields was averaged to obtain a mean cell density per retina.

2.7. qRT-PCR

After retinal dissection, total RNA was isolated using a NucleoSpin® RNA/Protein kit (Macherey-Nagel, USA). The purity and quantity of each RNA sample were determined by NanoDrop™ 2000 Spectrophotometer (Nanodrop Technologies, Montchanin, DE). The total RNA sample from each animal (n = 4/group) was reversely transcribed for 1 h at 42 °C with a ProtoScript(R) II First Strand cDNA Synthesis Kit according to the manufacturer's standard protocol (New England Biolabs, UK). qRT-PCR was performed on a ViiA™ 7 Real-Time PCR System (Applied Biosystems, USA) using a Fast SYBR® Green Master Mix (Thermo Fisher Scientific, USA). The gene-specific forward and reverse primers used in this study are listed in Table 2. The expression levels of each target gene were normalized to those of the housekeeping gene *β*-actin (bACTN). Data are reported in relation to untreated controls.

2.8. Western blot analysis

The total protein was isolated from the dissected retinas using a NucleoSpin® RNA/Protein kit (Macherey-Nagel, USA). The quantity of each protein sample was determined by using the Qubit® protein assay kit (Thermo Fisher Scientific, USA). A 15-μg protein aliquot from each animal (n = 4/group) was electrophoresed on 10% or 12% polyacrylamide gels and transferred to polyvinylidene-difluoride (PVDF) membranes. The PVDF membranes were blocked with either milk or bovine serum albumin (5%) in PBS/0.1% Tween-20 at room temperature for 1 h, and then incubated overnight at 4 °C with primary antibodies against IL-18, TNF-α, iNOS, ARG-1, and *β*-actin (Table 1). The blots were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies-either goat anti-rabbit IgG-HRP antibody (1:2000; Cell Signaling Technology, USA) or donkey anti-goat IgG-HRP antibody (1:2000; Santa Cruz Biotechnology, Inc., Germany) for 1 h at room temperature. Protein signals were visualized with an Enhanced Chemiluminescence Plus Blotting Detection system (Amersham Biosciences, Buckinghamshire, UK) on a ChemiDoc XRS + imager (Bio-Rad, USA). Immunoblots were re-probed with primary antibody against *β*-actin to confirm that equal amounts of protein were loaded on the membrane. Density of bands was defined using ImageJ analysis software. Data are reported in relation to untreated controls.

2.9. Statistical analysis

All data in this study are expressed as the mean ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism software (version 5.0; GraphPad Software Inc., La Jolla, CA, USA). Comparisons between experimental groups were made using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test. Student's *t*-tests were used for comparisons between two groups. Statistical significance was set at $p < .05$.

3. Results

3.1. The blood AA/EPA ratio decreased after treatment with ω3-PUFAs

To confirm that the AA/EPA ratios stayed within the desired therapeutic range, we analysed the blood using gas chromatography at set intervals. The blood AA/EPA ratio gradually decreased and reached the desired therapeutic range after 4 weeks of daily gavage with ω3-PUFAs in the ω3-PUFAs + timolol (4.83 ± 0.26 → 1.33 ± 0.04, $p = .0001$) and ω3-PUFAs (4.27 ± 0.17 → 1.21 ± 0.02, $p = .0001$) groups (Fig. 2). In contrast, the blood AA/EPA ratio was similar before and throughout the treatment in the group that received timolol and the untreated group (mean AA/EPA ratio: 5.08 ± 0.14 and 5.13 ± 0.13, respectively).

3.2. Treatment with ω3-PUFAs may not delay disease progression in the anterior part of the eye

To monitor disease progression, we measured the IOP at the beginning of treatment and one month later. We found that IOP decreased in both groups that received timolol (ω3-PUFAs + timolol: 27.03 ± 0.69 mmHg → 23.75 ± 0.57 mmHg, $p < .001$; timolol: 27.56 ± 0.79 mmHg → 23.00 ± 0.59 mmHg, $p < .001$). In contrast, IOP was similar before treatment and at the one-month follow-up in the group that received ω3-PUFAs only and the untreated group (ω3-PUFAs: 26.53 ± 0.73 mmHg → 27.13 ± 0.61 mmHg, $p = .53$; untreated: 27.11 ± 0.81 mmHg → 27.47 ± 0.65 mmHg, $p = .7354$). Additionally, we monitored mouse body weight once a week. Initially,

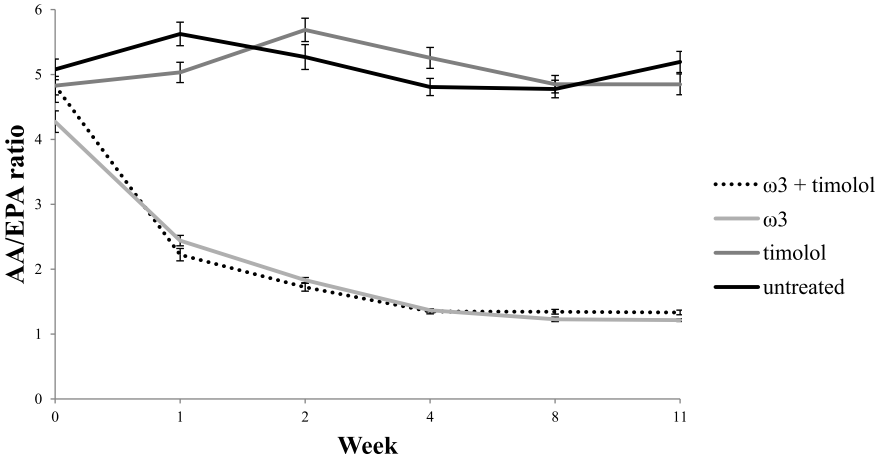

Fig. 2. Blood AA/EPA levels. Levels of AA and EPA were determined by gas chromatographic analysis (mean ± SEM). N = 20 for each treatment. The AA/EPA ratio gradually decreased from Week 0 to Week 11 after daily gavage with ω3-PUFAs. AA, arachidonic acid; EPA, eicosapentaenoic acid; SEM, standard error of the mean; ω3-PUFAs, omega-3-polyunsaturated fatty acids.

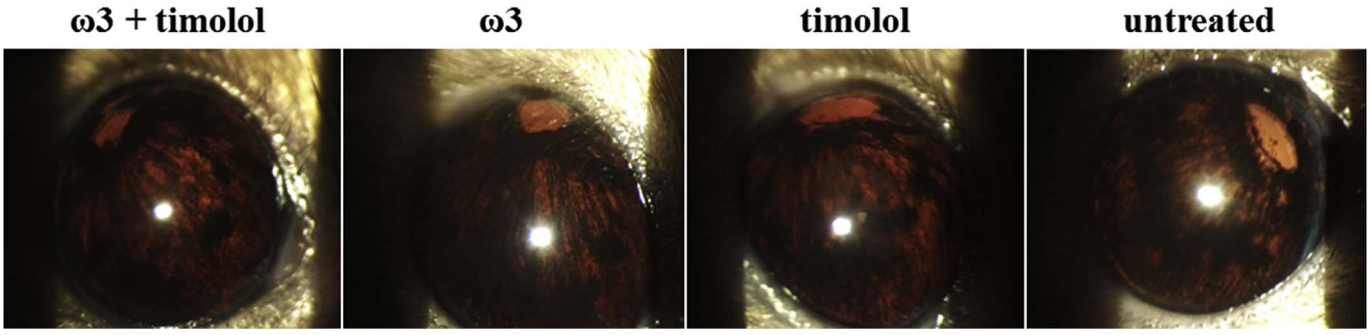

Fig. 3. Glaucomatous progression in the DBA/2J mice was not affected by ω3-PUFAs + timolol, ω3-PUFAs, or timolol treatment. Representative photographs of iris pigment disease and transillumination defects on the iris surface of DBA/2J mice treated with ω3-PUFAs + timolol, ω3-PUFAs, timolol and untreated. Defects became evident in all treatment groups. ω3-PUFAs, omega-3-polyunsaturated fatty acids.

body weight did not differ significantly between the four groups ($p = .99$). However, minor weight loss was observed in both groups that received ω3-PUFAs (ω3-PUFAs + timolol: 28.2 ± 0.79 g → 27.2 ± 0.72 g, $p = .3541$; ω3-PUFAs only: 28.2 ± 0.72 g → 27.4 ± 0.62 g, $p = .4063$). Slit lamp biomicroscopy indicated that iris morphology at the end of treatment did not differ across the four groups (Fig. 3). All DBA/2J mice exhibited severe iris atrophy, pigment dispersion, and transillumination. Taken together, these results suggest that 3-month treatment with ω3-PUFAs may not delay disease progression in the anterior part of the eye.

3.3. All treatments promoted RGC survival

To investigate RGC survival in the DBA/2J mice after treatment with ω3-PUFAs + timolol, ω3-PUFAs, or timolol, we evaluated the presence of Brn-3a in whole retinal flatmounts (Fig. 4A). Densities of Brn-3a-positive cells (RGCs) in the ω3-PUFAs + timolol (1303.77 ± 139.62/mm^2), ω3-PUFAs (768.40 ± 52.44/mm^2), and timolol (910.57 ± 57.28/mm^2) groups were significantly higher (4, 2.6, and 2.8 times, respectively; $p < .01$) than those in the untreated group (323.39 ± 95.18/mm^2) (Fig. 4B). Subsequent analysis indicated a significantly higher density of surviving RGCs in the ω3-PUFAs + timolol group than in either of the other treatment groups (69% vs. ω3-PUFAs, $p < .05$; 43% vs. timolol, $p < .05$). Further, age-matched wild-type C57BL/6 mice (1500.99 ± 156.75/mm^2) showed a significantly higher density of surviving RGCs than the untreated ($p < .001$), ω3-PUFAs ($p < .01$), or timolol ($p < .05$) DBA/2J mice. In contrast, RGC density in the age-matched C57BL/6 mice did not differ from that in the ω3-PUFAs + timolol group ($p = .38$). Taken together, these results suggest that treatment with ω3-PUFAs and timolol, administered alone or in combination, preserves RGC density. Interestingly, treatment with ω3-PUFAs + timolol restores RGC density to wild-type levels.

3.4. None of the treatments affected retinal microglial activation

To investigate the potential effect of the treatments on retinal microglial activation, we evaluated the presence of Iba-1 (a retinal microglia-specific calcium adaptor protein linked to activation) in NFL/GCL of whole retinal flatmounts (Fig. 5A). Iba-1-labeled cells were evenly distributed throughout the retina in a mosaic-like fashion without overlap between neighboring cells. Iba-1-labeled microglia showed diverse morphologic complexity from finely ramified to round somata with sturdy and simplified processes. In addition to microglial cells, components of the NFL, including hyalocytes, were labeled and recognized as round cells without ramification. Quantitative analysis of Iba-1-positive cells showed that the density of hyalocyte/microglial cells did not differ across groups ($p = .82$) (Fig. 5B). These data suggest that none of the treatments suppress retinal microglial activation; however, we cannot argue for sure to whether there is activation based on cell morphology.

3.5. Treatment with either ω3-PUFAs or timolol reduced the expression of pro-inflammatory cytokines in the retina

To determine how the treatments affected pro-inflammatory responses, mRNA and protein-expression levels of IL-18 and TNF-α were evaluated in the retinal samples using qRT-PCR and Western blot analysis, respectively. qRT-PCR indicated no significant differences in mRNA levels for *Il-18* ($p = .68$) or *Tnf-α* ($p = .11$) across the four groups (Fig. 6A). In contrast, Western blot analysis indicated that IL-18 protein-expression levels were significantly lower (1.7-times) in the ω3-PUFAs group than in the untreated group ($p < .05$) (Fig. 6B). Additionally, TNF-α levels were significantly lower in the ω3-PUFAs and the timolol (2.3 and 2 times, respectively) groups than in the untreated group ($p < .001$ and $p < .001$, respectively). Significant increases in IL-18 and TNF-α protein-expression levels were observed in the ω3-PUFAs + timolol group compared with those in the ω3-PUFAs (IL-18: $p < .0001$; TNF-α: $p < .001$) and timolol (IL-18: $p < .01$; TNF-α: $p < .001$) groups. Taken together, these results indicate that treatment with either ω3-PUFAs or timolol suppresses the expression of pro-inflammatory cytokines in the retina. However, ω3-PUFA + timolol treatment has no effect on the expression level of any of the aforementioned cytokines in the retina.

3.6. Treatment with either ω3-PUFAs or timolol reduced the expression of M1 and M2 macrophages in the retina

The protein levels of the pro-inflammatory M1 and pro-resolving M2 macrophage markers iNOS and ARG-1 in the retinal samples were evaluated by Western blot (Fig. 7). Compared with the untreated group, protein-expression levels of iNOS and ARG-1 were significantly greater in the ω3-PUFAs group (iNOS: 7.5 times, $p < .01$; ARG-1: 5.3 times, $p < .01$) and in the timolol group (iNOS: 7 times, $p < .05$; ARG-1: 3.9 times, $p < .05$). However, protein-expression levels of iNOS and ARG-1 were significantly lower in the ω3-PUFAs + timolol group than in the ω3-PUFAs (iNOS: $p < .01$; ARG-1: $p < .01$) and timolol (iNOS: $p < .05$; ARG-1: $p < .05$) groups. These data suggest that treatment with either ω3-PUFAs or timolol reduces the expression of M1 and M2 macrophages in the retina; although, ω3-PUFA + timolol treatment has no effect.

4. Discussion

Our present findings suggest that the administration of ω3-PUFAs, alone or in combination with anti-glaucoma timolol eye drops, has a neuroprotective effect in the DBA/2J mouse model of hereditary glaucoma (when the blood AA/EPA ratio is 1–1.5), as demonstrated by the RGC density analysis. Administering ω3-PUFAs with timolol, or

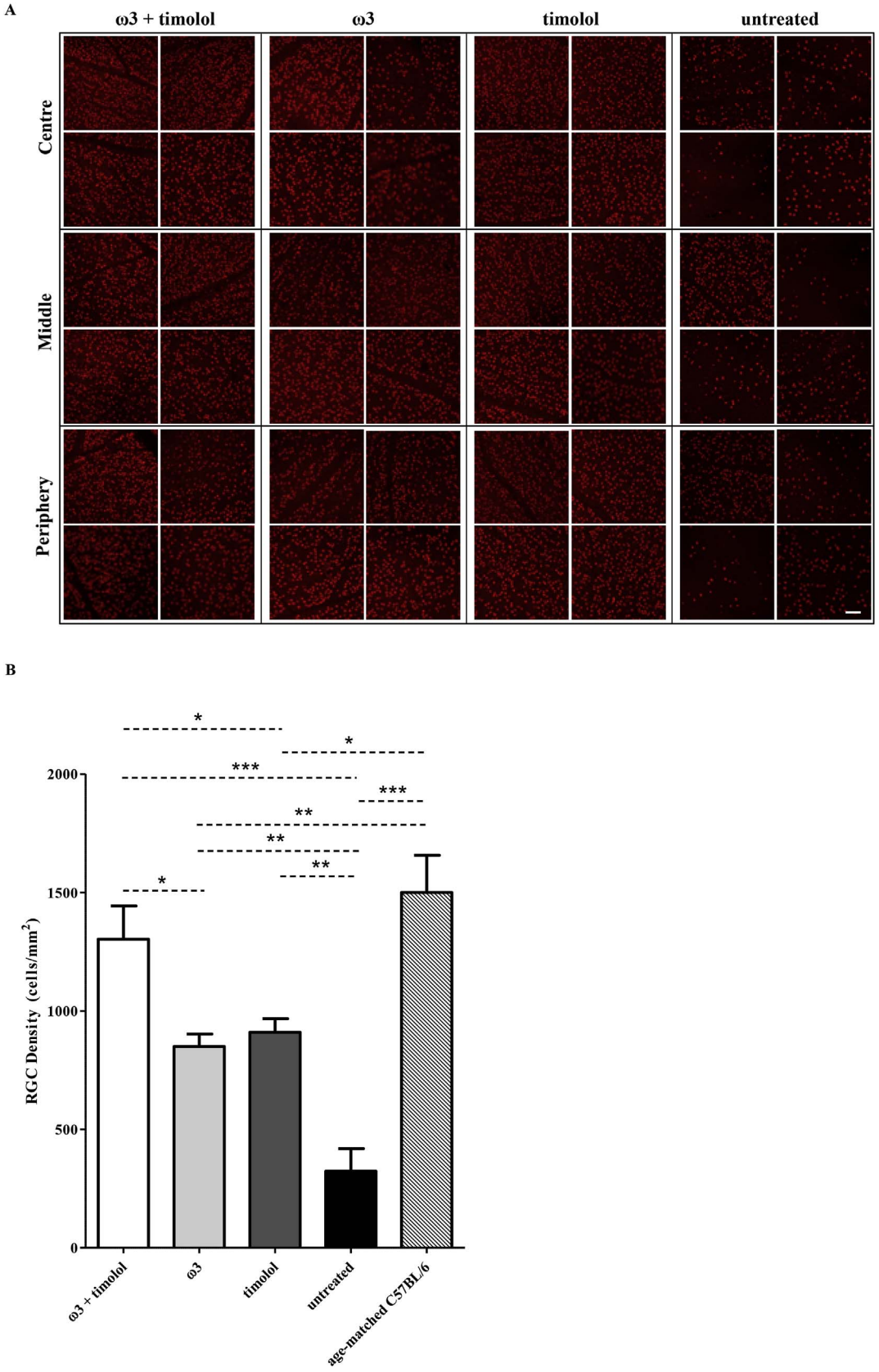

Fig. 4. RGC survival in the DBA/2J mice after treatment with ω3-PUFAs + timolol, ω3-PUFAs, or timolol. (A) Representative photomicrographs that were analysed, indicate the characteristics of Brn-3a-positive RGCs (red) in flat-mounted central, middle and peripheral retina from 11.5-month-old DBA/2J mice treated with ω3-PUFAs + timolol, ω3-PUFAs, timolol, and untreated. (B) Quantification of RGC density (no. cells/mm^2, n = 8/group). *P < .05, **P < .01, ***P < .001. Scale bar: 190 μm. RGC, retinal ganglion cell; ω3-PUFAs, omega-3-polyunsaturated fatty acids; Brn-3a, brain-specific homeobox/POU domain protein-3a.

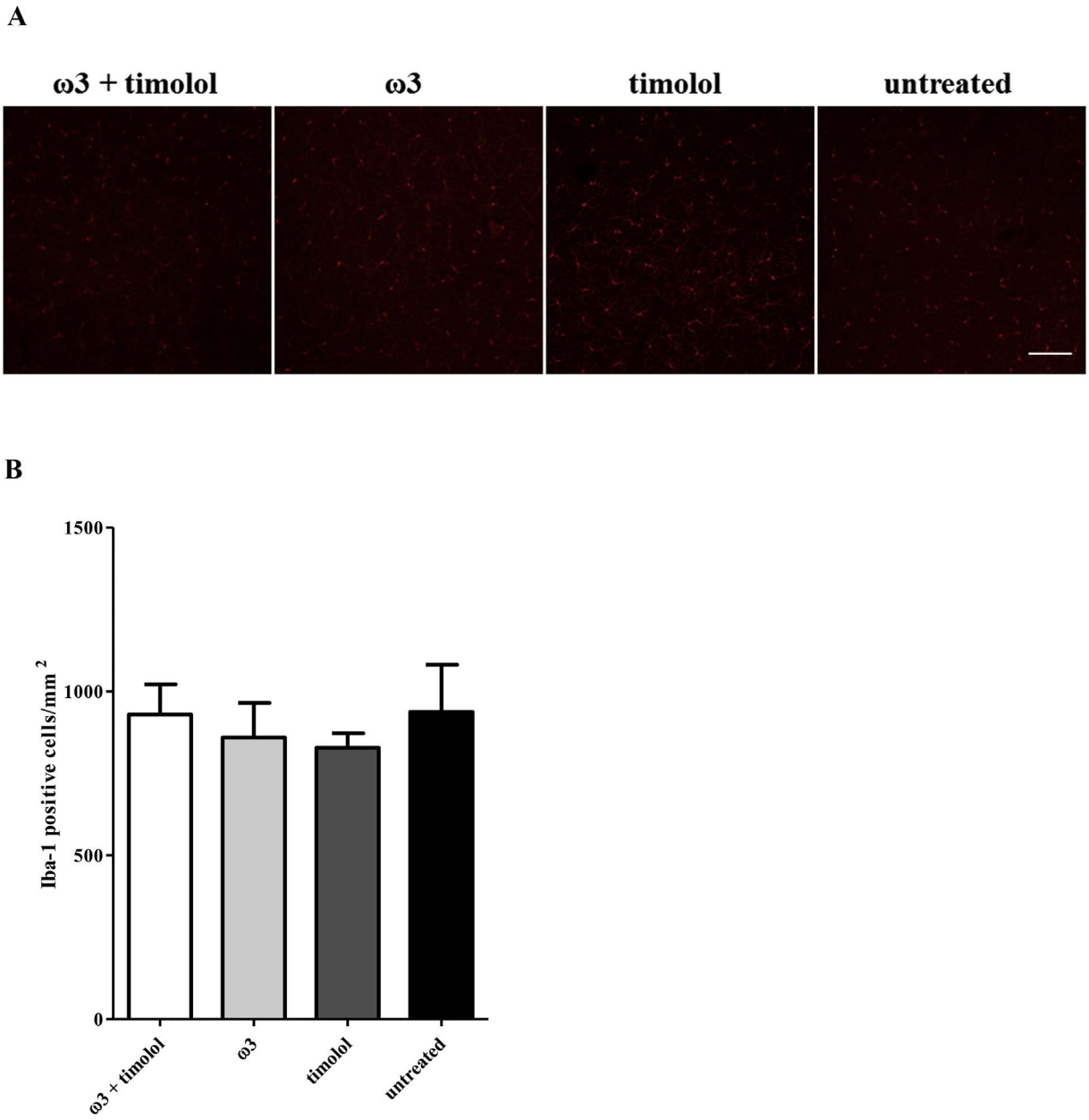

Fig. 5. Retinal microglia activation in the DBA/2J mice after treatment with ω3-PUFAs + timolol, ω3-PUFAs, or timolol. (A) Representative photomicrographs show Iba-1-positive cells (red) in retinal NFL/GCL (hyalocytes and microglial cells) from 11.5-month-old DBA/2J mice treated with ω3-PUFAs + timolol, ω3-PUFAs, timolol, and untreated. (B) Quantitative analysis of Iba-1-positive cells/mm^2 (n = 8/group) indicated no significant differences between the treatment groups. Scale bar: 190 μm ω3-PUFAs, omega-3-polyunsaturated fatty acids; Iba-1, ionized calcium-binding adaptor molecule-1; NFL, nerve fiber layer; GCL, ganglion cell layer.

either treatment separately, promoted RGC survival.

Diet enriched with ω3-PUFAs has beneficial effects across the retina, with the greatest improvement occurring in RGC function (Nguyen et al., 2008). For example, increased production of ω3-PUFAs enhanced RGC survival after optic nerve crush injury in mice (Peng et al., 2016). Additionally, our previous study demonstrated that ω3-PUFAs have neuroprotective effects on a rAION model through the dual actions of blocking RGC apoptosis and reducing inflammation in the optic nerve (Georgiou et al., 2017). In another preclinical study, Nguyen et al. (2013) investigated the effects of ω3-PUFA deficiency and IOP stress on retinal function and reported that these factors cause RGC dysfunction, suggesting that a diet sufficient in ω3-PUFAs will improve RGC function and make them less susceptible to elevated IOP. In our study, combined treatment with ω3-PUFAs and timolol had a better neuroprotective effect than either therapy alone. There was 69% and 43% more RGC survival in the ω3-PUFAs + timolol group compared to the ω3-PUFAs and timolol groups, respectively. It does not escape our attention that the retinal degeneration in DBA/2J mice has been reported to be diffuse, sectorial and asymmetric (Danias et al., 2003; Jakobs et al., 2005; Filippopoulos et al., 2006; Schlamp et al., 2006; Pérez de Lara et al., 2014). Thus, a functional and morphological analysis using pattern electroretinogram and an automatic quantification of the entire RGC population per retina, respectively, would allow a more reliable and accurate assessment of disease progression and treatment efficiency (Salinas-Navarro et al., 2009; Vidal-Sanz et al., 2012; Galindo-Romero et al., 2013).

Microglia become activated early in the glaucomatous process and accumulate throughout the retina and the ONH, producing pro-inflammatory cytokines, reactive oxygen species, neurotoxic matrix metalloproteinases, and neurotrophic factors. A reduction in the level of pro-inflammatory cytokines can prevent RGC degeneration in glaucoma (Neufeld, 1999; Tezel et al., 2003; Wang et al., 2016). Our present

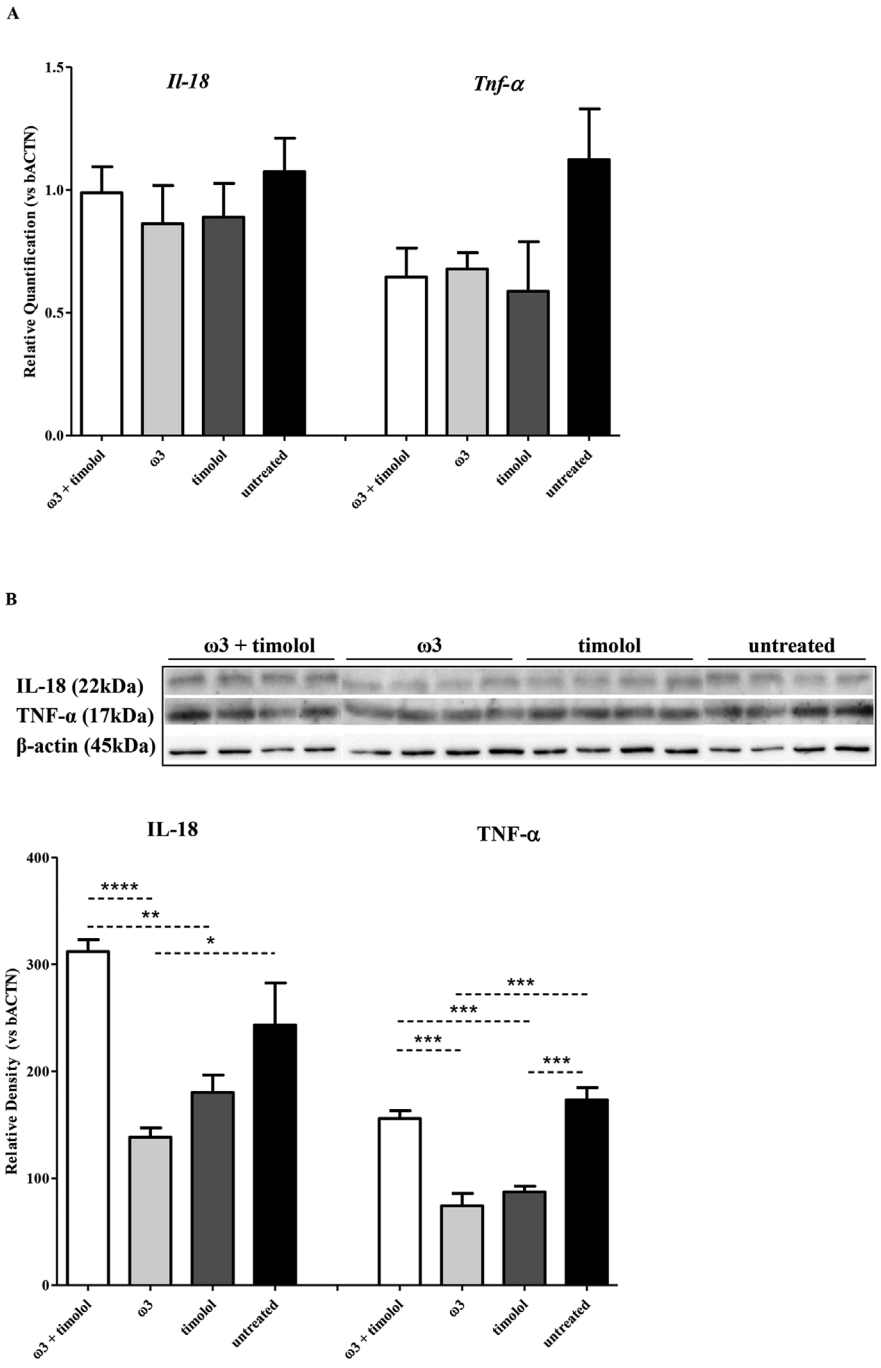

Fig. 6. Changes in IL-18 and TNF-α expression levels in the retina of DBA/2J mice treated with ω3-PUFAs + timolol, ω3-PUFAs, or timolol. (A) qRT-PCR analysis showed no significant differences in *Il-18* and *Tnf-α* expression levels in the retinas of DBA/2J mice treated with either ω3-PUFAs + timolol, ω3-PUFAs, timolol, or untreated (n = 4/group). (B) Western blot analysis shows lower IL-18 expression levels in the retinas of the ω3-PUFAs group and lower TNF-α expression levels in the retinas of the ω3-PUFAs and timolol groups. IL-18 and TNF-α expression levels were higher in the retinas of the ω3-PUFAs + timolol group than in the ω3-PUFAs and timolol groups (n = 4/group). $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$. IL-18, interleukin-18; TNF-α, tumour necrosis factor-α; ω3-PUFAs, omega-3-polyunsaturated fatty acids.

results showed that either ω3-PUFAs or timolol suppresses the production of pro-inflammatory cytokines in the retinas of DBA/2J mice, whereas there is no evidence of reduction of retinal microglial cell densities; although, there might be a trend. Given that microglia become less ramified and increase their soma size with activation, any changes in cell morphology are indicative of changes in cell activation state (Bosco et al., 2008). The fact that either ω3-PUFAs or timolol has not clear effect on microglial cell densities makes a future morphometric cell analysis essential in order to investigate whether these treatments induce any changes in retinal microglial cells.

The anti-inflammatory properties of ω3-PUFA supplementation in Parkinson's disease, stroke, prostate cancer, and depression are closely related to the downregulation of the pro-inflammatory M1 macrophage-secreted factor iNOS (Liang et al., 2016; Nobre et al., 2016; Shen et al., 2016; Mori et al., 2017; Dang et al., 2017). Additionally, ω3-PUFAs have been shown to promote the resolution of inflammation by inducing the polarization of macrophages from the inflammatory M1 to the pro-resolving M2 phenotype (Titos et al., 2011; Dalli and Serhan, 2012; Chang et al., 2015). However, our observations demonstrated that treatment with ω3-PUFAs, used alone, induces expression of iNOS and the M2 macrophage-secreted factor ARG-1 with a subsequent decrease in the production of TNF-α and IL-18 in the retina. In contrast, the combined treatment of ω3-PUFAs and timolol had no effect on iNOS and ARG-1 expression, leading to higher levels of retinal TNF-α and IL-18. This contradictory effect is accepted with difficulty following the robust protection that the same combined treatment had on RGC survival. Overall, these findings suggest that downregulation of IL-18 and TNF-α may not be the only factor involved in ω3-PUFA-mediated neuroprotection in the retina. It is also evident that ω3-PUFA supplementation alone had no effect on IOP, indicating that reduced IOP

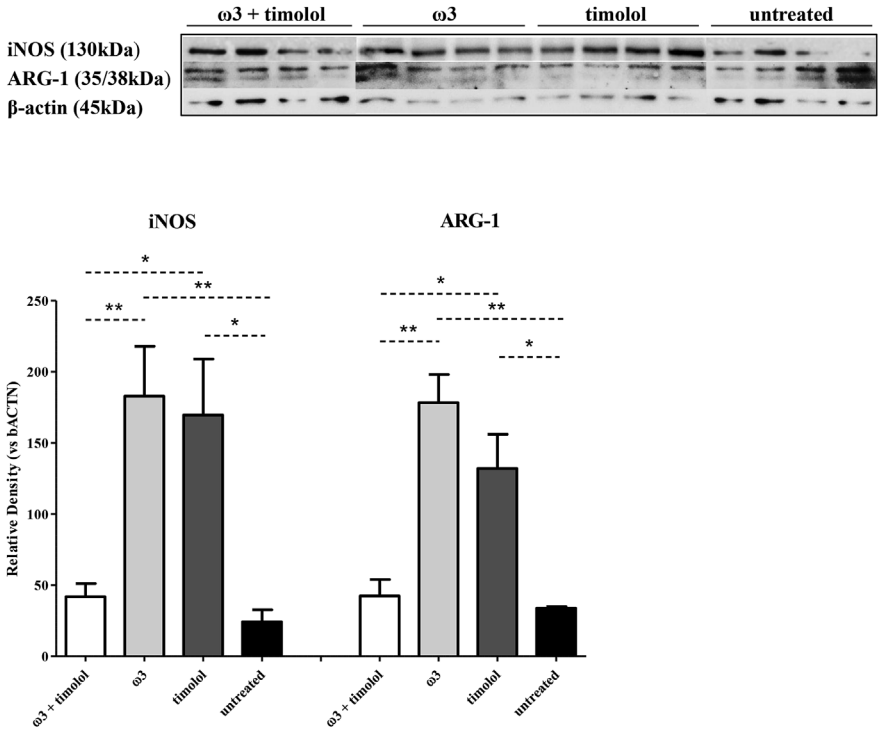

Fig. 7. Comparison of iNOS and ARG-1 expression levels in the retinas of DBA/2J mice treated with ω3-PUFAs + timolol, ω3-PUFAs, or timolol. Western blot analysis shows higher iNOS and ARG-1 expression levels in the retinas of the ω3-PUFAs and timolol groups. Expression levels of iNOS and ARG-1 were lower in the retinas of the ω3-PUFAs + timolol group than in the ω3-PUFAs and timolol groups (n = 4/group). $*P < .05$, $**P < .01$. iNOS, inducible nitric oxide synthase; ARG-1, arginase-1; ω3-PUFAs, omega-3-polyunsaturated fatty acids.

cannot explain the ω3-PUFA-mediated neuroprotection. Further research is needed to investigate how ω3-PUFAs might affect ocular blood flow in the DBA/2J mice, which might contribute to the neuroprotection of their RGCs in DBA/2J mice.

Our results suggest that maintaining a low AA/EPA ratio in the blood may help improve several retinopathies and optic neuropathies. This hypothesis is supported by several recent studies (Georgiou and Prokopiou, 2015; Georgiou et al., 2017). For instance, Georgiou and Prokopiou (2015) conducted an observational study in which patients with dry AMD exhibited significant improvement in vision after receiving ω3-PUFAs (5–7.5 g/day) supplements for up to 6 months while maintaining a blood AA/EPA ratio less than 2. In contrast, a two-year follow-up study of oral antioxidant supplementation with ω3-PUFAs revealed that ω3-PUFAs did not appear useful as an adjuvant treatment for mild/moderate primary open-angle glaucoma (Garcia-Medina et al., 2015). This negative result can be explained by the extremely low dosage of ω3-PUFAs (181 mg/day), which was not likely sufficient to reduce the blood AA/EPA ratio. In the current study, a key factor that likely led to the positive results was the care we took to regularly assess the blood AA/EPA ratio, and to adjust the ω3-PUFAs dosage such that the ratio remained between 1 and 1.5.

As demonstrated in this study, reducing IOP with timolol, increases expression of iNOS and ARG-1 in macrophages, reduces expression of TNF-α and subsequently prevents ganglion cell loss in the retina of DBA/2J mice. This effect is consistent with previous studies in which timolol not only enhanced the survival of RGCs but also restored visual function in DBA/2J mice (Schuettauf et al., 2002; Wong and Brown, 2012). Previous studies have demonstrated that retinal and choroidal blood flow are lower in DBA/2J mice than in wild-type C57BL/6 mice, which suggests a possible ischaemic contribution to the optic neuropathy and visual dysfunction that occur in older DBA/2J mice (Saleh et al., 2007; Calkins et al., 2008). Timolol might protect RGCs as a result of increased blood flow in the retina and choroid following IOP reduction. However, further research is necessary to investigate the detailed mechanisms of timolol-mediated neuroprotection in the retina.

In conclusion, our study showed that ω3-PUFA supplementation, either alone or in combination with timolol, prevents deterioration of RGCs in the DBA/2J mouse model of hereditary glaucoma. The neuroprotection is enhanced when the ω3-PUFAs are combined with timolol than either therapy alone. These data provide insights into the role of inflammation in the pathogenesis of glaucoma, and indicate that ω3-PUFA administration could be beneficial to some extent for controlling inflammation in the retina. As inflammation was not blocked by the combined treatment of ω3-PUFAs and timolol, downregulation of IL-18 and TNF-α might not be the only factor involved in ω3-PUFA-mediated neuroprotection in the retina. In resolution, all lipid-derived specialized pro-resolving mediators (SPMs) are reported to halt polymorphonuclear neutrophil recruitment and to promote macrophage phagocytosis and clearance of apoptotic cells (Serhan, 2014; Spite et al., 2014). Notably, E-series resolvins, derived from EPA, and D-series resolvins/protectins/maresins, derived from DHA, are known as novel autacoids that not only resolve inflammation, but also protect organs and stimulate tissue regeneration (Serhan and Chiang, 2013; Serhan, 2014; Spite et al., 2014). The aforementioned SPMs might also protect RGCs as a result of increased blood flow in the retina and choroid through an unknown mechanism. It would be interesting to identify further how SPMs, regulate discrete processes in tissue-specific resolution program(s) and tissue regeneration. Nevertheless, these novel data might indicate a turning point in the current treatment approach for patients with glaucoma. ω3-PUFAs could be considered a potential therapeutic regimen in combination with current anti-glaucoma medical treatments that lower IOP. Potentially, early administration with ω3-PUFAs or even an optimal dosage of ω3-PUFAs in the DBA/2J mice might improve the neuroprotective effects on the RGCs. Undoubtedly, further studies are needed to investigate the detailed mechanisms underlying the neuroprotective effect that ω3-PUFAs have on RGCs and to determine the best AA/EPA blood ratio for the maximum therapeutic effect.

Acknowledgements

The authors thank Dr. Jose Manuel Romero (School of Medicine, Dentistry and Biochemical Sciences, Queen's University Belfast, Ireland) for providing training and guidance regarding to the experimental protocols.

Abbreviations

AA	arachidonic acid
AMD	age-related macular degeneration
Arg-1	arginase-1
Brn-3a	brain-specific homeobox/POU domain protein-3a
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
GC-FID	gas chromatography-flame ionization detector
GCL	ganglion cell layer
HRP	horseradish peroxidase
Iba-1	ionized calcium binding adaptor molecule-1
IL-18	interleukin-18
iNOS	inducible nitric oxide synthase
IOP	intraocular pressure
NFL	nerve fiber layer
ONH	optic nerve head
PBS	phosphate buffered saline
PUFA	polyunsaturated fatty acid
PVDF	polyvinylidene difluoride
qRT-PCR	quantitative reverse transcription-polymerase chain reaction
rAION	rat model of anterior ischaemic optic neuropathy
RGC	retinal ganglion cell
SEM	standard error of the mean
SPM	specialized pro-resolving mediator
TNF-α	tumour necrosis factor-alpha
ω3	omega-3

Conflicts of interest

Dr Tassos Georgiou has patent on the use of omega-3 fatty acids for glaucoma. There are no other financial interests to disclose.

Data availability

The datasets produced and/or analysed during the current study are available from the corresponding author upon reasonable request.

Funding statement

This research was supported by Ophthalmos Research and Educational Institute (Nicosia, Cyprus) and did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Authors' contributions

MK and TG conceived and designed the experiments. MK, PK, and EP performed the experiments. MK and GP analysed the data. TG, CD and SM contributed the reagents/materials/analysis tools. MK wrote the paper. All contributing authors have read and approved the final version of the manuscript.

References

Ahmed, F., Brown, K.M., Stephan, D.A., Morrison, J.C., Johnson, E.C., Tomarev, S.I., 2004. Microarray analysis of changes in mRNA levels in the rat retina after experimental elevation of intraocular pressure. Invest. Ophthalmol. Vis. Sci. 45, 1247–1258. http://dx.doi.org/10.1167/iovs.03-1123.

Anderson, M.G., Libby, R.T., Mao, M., Cosma, I.M., Wilson, L.A., Smith, R.S., John, S.W., 2006. Genetic context determines susceptibility to intraocular pressure elevation in a mouse pigmentary glaucoma. BMC Biol. 4, 20. http://dx.doi.org/10.1186/1741-7007-4-20.

Anderson, M.G., Smith, R.S., Hawes, N.L., Zabaleta, A., Chang, B., Wiggs, J.L., John, S.W., 2002. Mutations in genes encoding melanosomal proteins cause pigmentary glaucoma in DBA/2J mice. Nat. Genet. 30, 81–85. http://dx.doi.org/10.1038/ng794.

Bosco, A., Inman, D.M., Steele, M.R., et al., 2008. Reduced retina microglial activation and improved optic nerve integrity with minocycline treatment in the DBA/2J mouse model of glaucoma. Invest. Ophthalmol. Vis. Sci. 49, 1437–1446. http://dx.doi.org/10.1167/iovs.07-1337.

Calder, P.C., 2006. n-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. Am. J. Clin. Nutr. 83, 1505S–1519S.

Calkins, D.J., Horner, P.J., Roberts, R., Gradianu, M., Berkowitz, B.A., 2008. Manganese-enhanced MRI of the DBA/2J mouse model of hereditary glaucoma. Invest. Ophthalmol. Vis. Sci. 49, 5083–5088. http://dx.doi.org/10.1167/iovs.08-2205.

Campbell, D.G., Schertzer, R.M., 1995. Pathophysiology of pigment dispersion syndrome and pigmentary glaucoma. Curr. Opin. Ophthalmol. 6, 96–101.

Cellini, M., Caramazza, N., Mangiafico, P., Possati, G.L., Caramazza, R., 1998. Fatty acid use in glaucomatous optic neuropathy treatment. Acta Ophthalmol. Scand. Suppl. 76, 41–42. http://dx.doi.org/10.1111/j.1600-0420.1998.tb00880.x.

Chang, B., Smith, R.S., Hawes, N.L., et al., 1999. Interacting loci cause severe iris atrophy and glaucoma in DBA/2J mice. Nat. Genet. 21, 405–409. http://dx.doi.org/10.1038/7741.

Chang, H.Y., Lee, H.N., Kim, W., Surh, Y.J., 2015. Docosahexaenoic acid induces M2 macrophage polarization through peroxisome proliferator-activated receptor gamma activation. Life Sci. 120, 39–47. http://dx.doi.org/10.1016/j.lfs.2014.10.014.

Chi, W., Li, F., Chen, H., 2014. Caspase-8 promotes NLRP1/NLRP3 inflammasome activation and IL-1beta production in acute glaucoma. Proc. Natl. Acad. Sci. U. S. A. 111, 11181–11186. http://dx.doi.org/10.1073/pnas.1402819111.

Conlon, R., Saheb, H., Ahmed II, , 2017. Glaucoma treatment trends: a review. Can. J. Ophthalmol. 52, 114–124. http://dx.doi.org/10.1016/j.jcjo.2016.07.013.

Connor, W.E., Neuringer, M., Reisbick, S., 1992. Essential fatty acids: the importance of n-3 fatty acids in the retina and brain. Nutr. Rev. 50, 21–29. http://dx.doi.org/10.1111/j.1753-4887.1992.tb01286.x.

Dalli, J., Serhan, C.N., 2012. Specific lipid mediator signatures of human phagocytes: microparticles stimulate macrophage efferocytosis and pro-resolving mediators. Blood 120, e60–72. http://dx.doi.org/10.1182/blood-2012-04-423525.

Dang, R., Zhou, X., Tang, M., Xu, P., Gong, X., Liu, Y., Jiao, H., Jiang, P., 2017. Fish oil supplementation attenuates neuroinflammation and alleviates depressive-like behavior in rats submitted to repeated lipopolysaccharide. Eur. J. Nutr. http://dx.doi.org/10.1007/s00394-016-1373-z.

Danias, J., Lee, K.C., Zamora, M.F., et al., 2003. Quantitative analysis of retinal ganglion cell (RGC) loss in aging DBA/2NNia glaucomatous mice: comparison with RGC loss in aging C57/BL6 mice. Invest. Ophthalmol. Vis. Sci. 44, 5151–5162. http://dx.doi.org/10.1167/iovs.02-1101.

Ebert, S., Weigelt, K., Walczak, Y., Drobnik, W., Mauerer, R., Hume, D.A., Weber, B.H., Langmann, T., 2009. Docosahexaenoic acid attenuates microglial activation and delays early retinal degeneration. J. Neurochem. 110, 1863–1875. http://dx.doi.org/10.1111/j.1471-4159.2009.06286.x.

Fan, W., Li, X., Wang, W., Mo, J.S., Kaplan, H., Cooper, N.G., 2010. Early involvement of immune/inflammatory response genes in retinal degeneration in DBA/2J mice. Ophthalmol. Eye Dis. 1, 23–41.

Filippopoulos, T., Danias, J., Chen, B., Podos, S.M., Mittag, T.W., 2006. Topographic and morphologic analyses of retinal ganglion cell loss in old DBA/2NNia mice. Invest. Ophthalmol. Vis. Sci. 47, 1968–1974. http://dx.doi.org/10.1167/iovs.05-0955.

Galindo-Romero, C., Valiente-Soriano, F.J., Jiménez-López, M., García-Ayuso, D., Villegas-Pérez, M.P., Vidal-Sanz, M., Agudo-Barriuso, M., 2013. Effect of brain-derived neurotrophic factor on mouse axotomized retinal ganglion cells and phagocytic microglia. Invest. Ophthalmol. Vis. Sci. 54, 974–985. http://dx.doi.org/10.1167/iovs.12-11207.

Garcia-Medina, J.J., Garcia-Medina, M., Garrido-Fernandez, P., Galvan-Espinosa, J., Garcia-Maturana, C., Zanon-Moreno, V., Pinazo-Duran, M.D., 2015. A two-year follow-up of oral antioxidant supplementation in primary open-angle glaucoma: an open-label, randomized, controlled trial. Acta Ophthalmol. 93, 546–554. http://dx.doi.org/10.1111/aos.12629.

Georgiou, T., Prokopiou, E., 2015. The new era of Omega-3 fatty acids supplementation: therapeutic effects on dry age-related macular degeneration. J. Stem Cell. 10, 205–215.

Georgiou, T., Wen, Y.T., Chang, C.H., et al., 2017. Neuroprotective effects of omega-3 polyunsaturated fatty acids in a rat model of anterior ischemic optic neuropathy. Invest. Ophthalmol. Vis. Sci. 58, 1603–1611. http://dx.doi.org/10.1167/iovs.16-20979.

Gordon, M.O., Beiser, J.A., Brandt, J.D., et al., 2002. The Ocular Hypertension Treatment Study: baseline factors that predict the onset of primary open-angle glaucoma. Arch. Ophthalmol. 120, 714–720. http://dx.doi.org/10.1001/archopht.120.6.714.

Gorusupudi, A., Liu, A., Hageman, G.S., Bernstein, P.S., 2016. Associations of human retinal very long-chain polyunsaturated fatty acids with dietary lipid biomarkers. J. Lipid Res. 57, 499–508. http://dx.doi.org/10.1194/jlr.P065540.

Gupta, S.K., Niranjan, D.G., Agrawal, S.S., Srivastava, S., Saxena, R., 2008. Recent advances in pharmacotherapy of glaucoma. Indian J. Pharmacol. 40, 197–208. http://dx.doi.org/10.4103/0253-7613.44151.

Heijl, A., Leske, M.C., Bengtsson, B., Hyman, L., Bengtsson, B., Hussein, M., Early Manifest Glaucoma Trial, G., 2002. Reduction of intraocular pressure and glaucoma progression: results from the Early Manifest Glaucoma Trial. Arch. Ophthalmol. 120, 1268–1279. http://dx.doi.org/10.1001/archopht.120.10.1268.

Hodge, W.G., Barnes, D., Schachter, H.M., 2006. The evidence for efficacy of omega-3 fatty acids in preventing or slowing the progression of retinitis pigmentosa: a systematic review. Can. J. Ophthalmol. 41, 481–490. http://dx.doi.org/10.1016/S0008-4182(06)80012-8.

Jakobs, T.C., Libby, R.T., Ben, Y., John, S.W., Masland, R.H., 2005. Retinal ganglion cell degeneration is topological but not cell type specific in DBA/2J mice. J. Cell Biol. 171, 313–325. http://dx.doi.org/10.1083/jcb.200506099.

John, S.W., Smith, R.S., Savinova, O.V., et al., 1998. Essential iris atrophy, pigment dispersion, and glaucoma in DBA/2J mice. Invest. Ophthalmol. Vis. Sci. 39, 951–962.

Kang, J.H., Pasquale, L.R., Willett, W.C., Rosner, B.A., Egan, K.M., Faberowski, N.,

Hankinson, S.E., 2004. Dietary fat consumption and primary open-angle glaucoma. Am. J. Clin. Nutr. 79, 755–764.
Levin, L.A., 2003. Retinal ganglion cells and neuroprotection for glaucoma. Surv. Ophthalmol. 48, S21–S24. http://dx.doi.org/10.1016/S0039-6257(03)00007-9.
Levin, L.A., Crowe, M.E., Quigley, H.A., Lasker II, o A., Glaucomatous Neurodegeneration, P., 2016. Neuroprotection for glaucoma: Requirements for clinical translation. Exp. Eye Res. http://dx.doi.org/10.1016/j.exer.2016.12.005. pii: S0014-4835(16)30530-9.
Liang, P., Henning, S.M., Schokrpur, S., et al., 2016. Effect of dietary Omega-3 fatty acids on tumor-associated macrophages and prostate cancer progression. Prostate 76, 1293–1302. http://dx.doi.org/10.1002/pros.23218.
Masood, A., Stark, K.D., Salem Jr., N., 2005. A simplified and efficient method for the analysis of fatty acid methyl esters suitable for large clinical studies. J. Lipid Res. 46, 2299–2305. http://dx.doi.org/10.1194/jlr.D500022-JLR200.
McKinnon, S.J., 1997. Glaucoma, apoptosis, and neuroprotection. Curr. Opin. Ophthalmol. 8, 28–37.
Meydani, S.N., Endres, S., Woods, M.M., Goldin, B.R., Soo, C., Morrill-Labrode, A., Dinarello, C.A., Gorbach, S.L., 1991. Oral (n-3) fatty acid supplementation suppresses cytokine production and lymphocyte proliferation: comparison between young and older women. J. Nutr. 121, 547–555.
Mo, J.S., Anderson, M.G., Gregory, M., et al., 2003. By altering ocular immune privilege, bone marrow-derived cells pathogenically contribute to DBA/2J pigmentary glaucoma. J. Exp. Med. 197, 1335–1344. http://dx.doi.org/10.1084/jem.20022041.
Mori, M.A., Delattre, A.M., Carabelli, B., et al., 2017. Neuroprotective effect of omega-3 polyunsaturated fatty acids in the 6-OHDA model of Parkinson's disease is mediated by a reduction of inducible nitric oxide synthase. Nutr. Neurosci. 1-11. http://dx.doi.org/10.1080/1028415X.2017.1290928.
Nakazawa, T., Nakazawa, C., Matsubara, A., et al., 2006. Tumor necrosis factor-alpha mediates oligodendrocyte death and delayed retinal ganglion cell loss in a mouse model of glaucoma. J. Neurosci. 26, 12633–12641. http://dx.doi.org/10.1523/JNEUROSCI.2801-06.2006.
Neufeld, A.H., 1999. Microglia in the optic nerve head and the region of parapapillary chorioretinal atrophy in glaucoma. Arch. Ophthalmol. 117, 1050–1056. http://dx.doi.org/10.1001/archopht.117.8.1050.
Nguyen, C.T., Bui, B.V., Sinclair, A.J., Vingrys, A.J., 2007. Dietary omega 3 fatty acids decrease intraocular pressure with age by increasing aqueous outflow. Invest. Ophthalmol. Vis. Sci. 48, 756–762. http://dx.doi.org/10.1167/iovs.06-0585.
Nguyen, C.T., Vingrys, A.J., Bui, B.V., 2008. Dietary omega-3 fatty acids and ganglion cell function. Invest. Ophthalmol. Vis. Sci. 49, 3586–3594. http://dx.doi.org/10.1167/iovs.08-1735.
Nguyen, C.T., Vingrys, A.J., Bui, B.V., 2013. Dietary omega-3 deficiency and IOP insult are additive risk factors for ganglion cell dysfunction. J. Glaucoma 22, 269–277. http://dx.doi.org/10.1097/IJG.0b013e318237cac7.
Nobre, M.E., Correia, A.O., Mendonca, F.N., et al., 2016. Omega-3 fatty acids: possible neuroprotective mechanisms in the model of global ischemia in rats. J. Nutr. Med. 2016, 6462120. http://dx.doi.org/10.1155/2016/6462120.
Nowak, J.Z., 2010. Anti-inflammatory pro-resolving derivatives of omega-3 and omega-6 polyunsaturated fatty acids. Postepy. Hig. Med. Dosw. (Online) 64, 115–132.
Peng, S., Shi, Z., Su, H., So, K.F., Cui, Q., 2016. Increased production of omega-3 fatty acids protects retinal ganglion cells after optic nerve injury in mice. Exp. Eye Res. 148, 90–96. http://dx.doi.org/10.1016/j.exer.2016.06.001.
Pérez de Lara, M.J., Santano, C., Guzmán-Aránguez, A., Valiente-Soriano, F.J., Avilés-Trigueros, M., Vidal-Sanz, M., de la Villa, P., Pintor, J., 2014. Assessment of inner retina dysfunction and progressive ganglion cell loss in a mouse model of glaucoma. Exp. Eye Res. 122, 40–49. http://dx.doi.org/10.1016/j.exer.2014.02.022.
Prokopiou, E., Kolovos, P., Kalogerou, M., Neokleous, A., Papagregoriou, G., Deltas, C., Malas, S., Georgiou, T., 2017. Therapeutic potential of omega-3 fatty acids supplementation in a mouse model of dry macular degeneration. BMJ Open Ophth. 1, e000056. http://dx.doi.org/10.1136/bmjophth-2016–20000056.
Quigley, H.A., 1993. Open-angle glaucoma. N. Engl. J. Med. 328, 1097–1106. http://dx.doi.org/10.1056/NEJM199304153281507.
Quigley, H.A., 1999. Neuronal death in glaucoma. Prog. Retin. Eye Res. 18, 39–57.
Quigley, H.A., 2012. Clinical trials for glaucoma neuroprotection are not impossible. Curr. Opin. Ophthalmol. 23, 144–154. http://dx.doi.org/10.1097/ICU.0b013e32834ff490.
Quigley, H.A., Broman, A.T., 2006. The number of people with glaucoma worldwide in 2010 and 2020. Br. J. Ophthalmol. 90, 262–267. http://dx.doi.org/10.1136/bjo.2005.081224.
Ramkumar, H.L., Tuo, J., Shen, D.F., Zhang, J., Cao, X., Chew, E.Y., Chan, C.C., 2013. Nutrient supplementation with n3 polyunsaturated fatty acids, lutein, and zeaxanthin decrease A2E accumulation and VEGF expression in the retinas of Ccl2/Cx3cr1-deficient mice on Crb1rd8 background. J. Nutr. 143, 1129–1135. http://dx.doi.org/10.3945/jn.112.169649.
Ren, H., Magulike, N., Ghebremeskel, K., Crawford, M., 2006. Primary open-angle glaucoma patients have reduced levels of blood docosahexaenoic and eicosapentaenoic acids. Prostagl. Leukot. Essent. Fat. Acids 74, 157–163. http://dx.doi.org/10.1016/j.plefa.2005.11.007.
Renard, J.P., Rouland, J.F., Bron, A., et al., 2013. Nutritional, lifestyle and environmental factors in ocular hypertension and primary open-angle glaucoma: an exploratory case-control study. Acta Ophthalmol. 91, 505–513. http://dx.doi.org/10.1111/j.1755-3768.2011.02356.x.
Rupp, H., Wagner, D., Rupp, T., Schulte, L.M., Maisch, B., 2004. Risk stratification by the "EPA + DHA level" and the "EPA/AA ratio" focus on anti-inflammatory and antiarrhythmogenic effects of long-chain omega-3 fatty acids. Herz 29, 673–685. http://dx.doi.org/10.1007/s00059-004-2602-4.
Saleh, M., Nagaraju, M., Porciatti, V., 2007. Longitudinal evaluation of retinal ganglion cell function and IOP in the DBA/2J mouse model of glaucoma. Invest. Ophthalmol. Vis. Sci. 48, 4564–4572. http://dx.doi.org/10.1167/iovs.07-0483.
Salinas-Navarro, M., Mayor-Torroglosa, S., Jiménez-López, M., Avilés-Trigueros, M., Holmes, T.M., Lund, R.D., Villegas-Pérez, M.P., Vidal-Sanz, M., 2009. A computerized analysis of the entire retinal ganglion cell population and its spatial distribution in adult rats. Vis. Res. 49, 115–126. http://dx.doi.org/10.1016/j.visres.2008.09.029.
Schlamp, C.L., Li, Y., Dietz, J.A., Janssen, K.T., Nickells, R.W., 2006. Progressive ganglion cell loss and optic nerve degeneration in DBA/2J mice is variable and asymmetric. BMC Neurosci. 7 (66). http://dx.doi.org/10.1186/1471-2202-7-66.
Schnebelen, C., Fourgeux, C., Pasquis, B., Creuzot-Garcher, C.P., Bron, A.M., Bretillon, L., Acar, N., 2011. Dietary polyunsaturated fatty acids reduce retinal stress induced by an elevation of intraocular pressure in rats. Nutr. Res. 31, 286–295. http://dx.doi.org/10.1016/j.nutres.2011.03.011.
Schnebelen, C., Pasquis, B., Salinas-Navarro, M., et al., 2009. A dietary combination of omega-3 and omega-6 polyunsaturated fatty acids is more efficient than single supplementations in the prevention of retinal damage induced by elevation of intraocular pressure in rats. Graefes Arch. Clin. Exp. Ophthalmol. 247, 1191–1203. http://dx.doi.org/10.1007/s00417-009-1094-6.
Schuettauf, F., Quinto, K., Naskar, R., Zurakowski, D., 2002. Effects of anti-glaucoma medications on ganglion cell survival: the DBA/2J mouse model. Vis. Res. 42, 2333–2337.
Seddon, J.M., Cote, J., Rosner, B., 2003. Progression of age-related macular degeneration: association with dietary fat, transunsaturated fat, nuts, and fish intake. Arch. Ophthalmol. 121, 1728–1737. http://dx.doi.org/10.1001/archopht.121.12.1728.
Seddon, J.M., Rosner, B., Sperduto, R.D., Yannuzzi, L., Haller, J.A., Blair, N.P., Willett, W., 2001. Dietary fat and risk for advanced age-related macular degeneration. Arch. Ophthalmol. 119, 1191–1199.
Sena, D.F., Lindsley, K., 2017. Neuroprotection for treatment of glaucoma in adults. Cochrane Database Syst. Rev. 1, CD006539. http://dx.doi.org/10.1002/14651858.CD006539.pub4.
Serhan, C.N., 2014. Pro-resolving lipid mediators are leads for resolution physiology. Nature 510, 92–101. http://dx.doi.org/10.1038/nature13479.
Serhan, C.N., Chiang, N., 2013. Resolution phase lipid mediators of inflammation: agonists of resolution. Curr. Opin. Pharmacol. 13, 632–640. http://dx.doi.org/10.1016/j.coph.2013.05.012.
Serhan, C.N., Arita, M., Hong, S., Gotlinger, K., 2004. Resolvins, docosatrienes, and neuroprotectins, novel omega-3-derived mediators, and their endogenous aspirin-triggered epimers. Lipids 39, 1125–1132. http://dx.doi.org/10.1007/s11745-004-1339-7.
Shen, J., Hafeez, A., Stevenson, J., et al., 2016. Omega-3 fatty acid supplement prevents development of intracranial atherosclerosis. Neuroscience 334, 226–235. http://dx.doi.org/10.1016/j.neuroscience.2016.08.013.
Smith, W., Mitchell, P., Leeder, S.R., 2000. Dietary fat and fish intake and age-related maculopathy. Arch. Ophthalmol. 118, 401–404. http://dx.doi.org/10.1001/archopht.118.3.401.
Sowka, J., 2004. Pigment dispersion syndrome and pigmentary glaucoma. Optometry 75, 115–122. http://dx.doi.org/10.1016/S1529-1839(04)70023-8.
Spite, M., Clària, J., Serhan, C.N., 2014. Resolvins, specialized proresolving lipid mediators, and their potential roles in metabolic diseases. Cell Metabol. 19, 21–36. http://dx.doi.org/10.1016/j.cmet.2013.10.006.
Tellez-Vazquez, J., 2016. Omega-3 fatty acid supplementation improves dry eye symptoms in patients with glaucoma: results of a prospective multicenter study. Clin. Ophthalmol. 10, 617–626. http://dx.doi.org/10.2147/OPTH.S96433.
Tezel, G., Chauhan, B.C., LeBlanc, R.P., Wax, M.B., 2003. Immunohistochemical assessment of the glial mitogen-activated protein kinase activation in glaucoma. Invest. Ophthalmol. Vis. Sci. 44, 3025–3033. http://dx.doi.org/10.1167/iovs.02-1136.
Tezel, G., Li, L.Y., Patil, R.V., Wax, M.B., 2001. TNF-alpha and TNF-alpha receptor-1 in the retina of normal and glaucomatous eyes. Invest. Ophthalmol. Vis. Sci. 42, 1787–1794.
Tian, K., Shibata-Germanos, S., Pahlitzsch, M., Cordeiro, M.F., 2015. Current perspective of neuroprotection and glaucoma. Clin. Ophthalmol. 9, 2109–2118. http://dx.doi.org/10.2147/OPTH.S80445.
Titos, E., Rius, B., Gonzalez-Periz, A., Lopez-Vicario, C., Moran-Salvador, E., Martinez-Clemente, M., Arroyo, V., Claria, J., 2011. Resolvin D1 and its precursor docosahexaenoic acid promote resolution of adipose tissue inflammation by eliciting macrophage polarization toward an M2-like phenotype. J. Immunol. 187, 5408–5418. http://dx.doi.org/10.4049/jimmunol.1100225.
Tuo, J., Ross, R.J., Herzlich, A.A., et al., 2009. A high omega-3 fatty acid diet reduces retinal lesions in a murine model of macular degeneration. Am. J. Pathol. 175, 799–807. http://dx.doi.org/10.2353/ajpath.2009.090089.
Vasudevan, S.K., Gupta, V., Crowston, J.G., 2011. Neuroprotection in glaucoma. Indian J. Ophthalmol. 59, S102–S113. http://dx.doi.org/10.4103/0301-4738.73700.
Vidal-Sanz, M., Salinas-Navarro, M., Nadal-Nicolás, F.M., et al., 2012. Understanding glaucomatous damage: anatomical and functional data from ocular hypertensive rodent retinas. Prog. Retin. Eye Res. 31, 1–27. http://dx.doi.org/10.1016/j.preteyeres.2011.08.001.
Vohra, R., Tsai, J.C., Kolko, M., 2013. The role of inflammation in the pathogenesis of glaucoma. Surv. Ophthalmol. 58, 311–320. http://dx.doi.org/10.1016/j.survophthal.2012.08.010.
Wallace, F.A., Miles, E.A., Calder, P.C., 2000. Activation state alters the effect of dietary fatty acids on pro-inflammatory mediator production by murine macrophages. Cytokine 12, 1374–1379. http://dx.doi.org/10.1006/cyto.2000.0735.
Wang, J.W., Chen, S.D., Zhang, X.L., Jonas, J.B., 2016. Retinal microglia in glaucoma. J. Glaucoma 25, 459–465. http://dx.doi.org/10.1097/IJG.0000000000000200.
Wong, A.A., Brown, R.E., 2012. A neurobehavioral analysis of the prevention of visual impairment in the DBA/2J mouse model of glaucoma. Invest. Ophthalmol. Vis. Sci.

53, 5956–5966. http://dx.doi.org/10.1167/iovs.12-10020.
Yan, X., Tezel, G., Wax, M.B., Edward, D.P., 2000. Matrix metalloproteinases and tumor necrosis factor alpha in glaucomatous optic nerve head. Arch. Ophthalmol. 118, 666–673. http://dx.doi.org/10.1001/archopht.118.5.666.
Yuan, L., Neufeld, A.H., 2000. Tumor necrosis factor-alpha: a potentially neurodestructive cytokine produced by glia in the human glaucomatous optic nerve head. Glia 32, 42–50. http://dx.doi.org/10.1002/1098-1136(200010)32. 1 < 42::AID-GLIA40 > 3.0.CO;2–3.
Zhou, X., Li, F., Kong, L., Tomita, H., Li, C., Cao, W., 2005. Involvement of inflammation, degradation, and apoptosis in a mouse model of glaucoma. J. Biol. Chem. 280, 31240–31248. http://dx.doi.org/10.1074/jbc.M502641200.