Omega-3 Fatty Acids Supplementation: Therapeutic Potential in a Mouse Model of Stargardt Disease

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METHODS. Eight-month-old mice were allocated to three groups: wild type (129S1), ABCA4−/− untreated, and ABCA4−/− o3 treated. o3 treatment lasted 3 months and comprised daily gavage administration of EPA and docosahexaenoic acid (DHA). Blood and retinal fatty acid analysis was performed using gas chromatography to adjust the blood AA/EPA ~1 to 1.5. Eyecups were histologically examined using transmission electron microscopy and confocal microscopy to evaluate lipofuscin granules and the photoreceptor layer. Retinal N-retinylidene-N-retinylethanolamine (A2E), a major component of retinal pigment epithelium lipofuscin, was quantified using liquid chromatography and tandem mass spectrometry, in addition to retinal proteomic analysis to determine changes in inflammatory proteins.

RESULTS. EPA levels increased and AA levels decreased in the blood and retinas of the treatment group. Significantly less A2E and lipofuscin granules were observed in the treatment group. The thickness of the outer nuclear layer was significantly greater in the treatment group (75.66 ± 4.80 μm) than in the wild-type (61.40 ± 1.84 μm) or untreated ABCA4−/− (56.50 ± 3.24 μm) groups. Proteomic analysis indicated lower levels of complement component 3 (C3) in the treatment group, indicative of lower complement-induced inflammatory response.

CONCLUSIONS. Three months of o3 supplementation (AA/EPA ~1–1.5) reduces A2E levels, lipofuscin granules, and C3 levels in the ABCA4−/− mouse model of Stargardt disease, consistent with slowing of the disease.

Keywords: omega-3 fatty acids, Stargardt disease, retinal pigment epithelium, photoreceptors, lipofuscin

Stargardt disease is a recessively inherited disorder that leads to macular degeneration, with an estimated prevalence of 1 in 10,000. It is characterized by a juvenile onset (within the first two decades), loss of central vision, a rapidly progressive course, and a poor visual outcome.1 Several different therapeutic approaches are being explored, including several pharmacologic agents, stem cell transplantation, and gene therapy,2,3 but the disease is currently untreatable.

We hypothesize that omega-3 (o3) polyunsaturated fatty acids (PUFAs) would be beneficial for patients with Stargardt disease. In the current study, we therefore investigated how moderate to high doses of o3 PUFAs, primarily eicosapentaenoic acid (EPA, C20:5n-3) and some docosahexaenoic acid (DHA, C22:6n-3), administered to the ABCA4−/− mouse model of Stargardt disease affect retinal pigment epithelium (RPE) cells and photoreceptors.

Stargardt disease is associated with mutations that are commonly observed in the ATP-binding cassette 4 (ABCA4) gene, a transporter protein located in the rims of photoreceptor outer segment discs in the retina.1,5 This transporter is involved in the clearance of retinaldehyde, which is released from photobleached rhodopsin and cone opsins in the outer segment discs.6 A key characteristic of the disease is the accumulation of lipofuscin, a lipid-containing fluorophore in the RPE cells, derived from the chemically modified residues of incompletely digested photoreceptor outer segments, as a result of the mutated ABCA4 transporter.5,7 The major component of lipofuscin in the mouse is N-retinylidene-N-retinylethanolamine (A2E), a vitamin A-derived pyridinium bisretinoid isomer that is a byproduct of the visual cycle and that may cause RPE damage. In turn, RPE damage may lead to retinal degeneration8,9 and eventually central blindness. However, evidence from the literature is inconsistent regarding whether lipofuscin and A2E can cause damage to the retina. In humans there is a steady age-related accumulation of lipofuscin in the normal RPE, with no sign of cell loss or structural damage.10 Recently, quantitative autofluorescence has shown that lipofuscin levels are reduced in progressive age-related macular degeneration (AMD), rather than increasing, which is contradictory to the lipofuscin toxicity theory.11,12
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al.\textsuperscript{13} reported that in the majority of patients with Stargardt disease, the eye changes determined using spectral-domain optical coherence tomography correlated well with changes on fundus autofluorescence. However, some patients (5 out of 11) demonstrated photoreceptor abnormalities without equivalent abnormality on fundus autofluorescence, suggesting that structural integrity of the photoreceptors may be affected earlier than changes in the RPE.\textsuperscript{15}

$ABCA4^{{</-}}$ mice also exhibit high levels of lipofuscin and A2E, with normal photoreceptor degeneration until 18 months.\textsuperscript{14–18} Although Charbel Issa et al.\textsuperscript{18} found small patches of autofluorescence loss in the RPE in 9-month-old $ABCA4^{{</-}}$ mice, suggestive of RPE damage before photoreceptor loss, they also concluded that high RPE lipofuscin may not adversely affect retinal structure or function over prolonged time intervals. A2E has been shown to induce the initiation of the complement cascade in RPE cells in vitro and to trigger an inflammatory response, as well as complement activation, in $ABCA4^{{</-}}$ mice.\textsuperscript{19,24} In addition, this model demonstrated delayed dark adaptation, which is consistent with what pertains in Stargardt patients.\textsuperscript{25} Thus, these mice are considered to be a good model for Stargardt disease.

$o3$ PUFAs have recently been shown to protect the eye from retinal damage in several disease states and are known to reduce inflammation, primarily through resolvins that are derived from two $o3$ PUFAs: EPA and DHA. Additionally, proinflammatory eicosanoids, including prostaglandins (PGs) and leukotrienes that are involved in leukocyte chemotaxis and inflammatory cytokine production, are generated from the omega-6 ($o6$) PUFAs arachidonic acid (AA, C20:4n-6).\textsuperscript{30} A balance between the mediators produced by the $o3$ and $o6$ PUFAs plays a key role in the resolution of inflammatory responses and therefore possibly in Stargardt disease progression.

The complement cascade is primarily involved in the detection and removal of foreign pathogens, which leads to inflammation, opsonization, phagocytosis, and cell death.\textsuperscript{27} Among the three complement pathways (classical, lectin, and alternative), genetic variants particularly in the alternative pathway have been associated with an increased risk of a late-stage macular degeneration.\textsuperscript{28} Compromised components of these pathways might eventually lead to retinal cell death due to genetic risk factors in the complement cascade.\textsuperscript{29} A2E-induced complement dysregulation and oxidative stress may involve a chronic inflammatory response that may result in retinal degeneration as observed in the $ABCA4^{{</-}}$ animal model.\textsuperscript{34}

Several studies have demonstrated that $o3$ PUFAs might protect against vascular and neural retinal pathology associated with inflammation, ischemia, light, oxygen, and age.\textsuperscript{35–36} A promising effect has also been observed in animal models related to retinal degeneration treated with $o3$ PUFAs.\textsuperscript{51,52} Our research team has demonstrated the neuroprotective effect of $o3$ PUFAs in several animal models. In particular, therapeutic efficacy of $o3$ was tested in a rat model of anterior ischemic optic neuropathy. Our findings showed that the effect is mediated by several different actions, including preventing the apoptosis of retinal ganglion cells, decreasing inflammatory cell infiltration, and regulating macrophage polarization, which decreases cytokine-induced injury of the optic nerve.\textsuperscript{35} Similarly, Prokopiou et al.\textsuperscript{54} reported the therapeutic potential of $o3$ PUFAs in the $CCL2^{/-}$ mouse model of retinal degeneration. A protective effect, perhaps with regenerative potential, was observed in the $o3$-treated group, with a pronounced increase in the size of the photoreceptor outer nuclear layer (ONL) and a reduction in interleukin-18 (IL-18), a specific inflammatory marker.\textsuperscript{54} Recently, Kalogerou et al.\textsuperscript{55} demonstrated the effectiveness of $o3$ in the DBA/2J glaucoma model, where retinal ganglion cells were protected against cell death. Importantly, in all of our studies, the levels of particular PUFAs, including EPA and AA, were examined during treatment, which allowed treatment dosage to be adjusted in order to achieve and maintain an AA/EPA blood ratio level $\sim1$ to 1.5.

In addition to preclinical studies, the role of dietary $o3$ PUFAs has been investigated in relation to the severity of Stargardt disease in individual patients. Red blood cell membrane and adipose-lipid EPA and DHA levels were inversely correlated with the phenotypic severity of vision loss based on the best-corrected visual acuity, dilated fundus examination, and fundus photography.\textsuperscript{56} Querques et al.\textsuperscript{57} reported a study where 840 mg/day DHA was given for 6 months to 20 patients with late-onset Stargardt disease. A complete ophthalmologic examination was performed before and after treatment. The study demonstrated that even though DHA influenced some functional parameters, it did not result in any significant short-term benefit.\textsuperscript{57} Similarly, the Age-Related Eye Disease Study 2 (AREDS2) and another study performed by Souied et al.\textsuperscript{58} did not show evidence of therapeutic potential of $o3$ PUFAs in patients with AMD.\textsuperscript{38,39}

We believe that higher doses of EPA are required in order to observe a beneficial clinical effect and EPA is the main PUFA that might have this potential and not DHA. Therefore, Georgiou and Prokopiou\textsuperscript{59} performed observational studies in which patients with dry AMD were given supplements with high doses of EPA and DHA for up to 6 months. Our study demonstrated significant improvement in vision ($\geq 15$ letters gain) when the AA/EPA was maintained at $\sim1$ to 2.\textsuperscript{40}

In the current study, we evaluated the role of $o3$ PUFAs, EPA/DHA (5:1), in the $ABCA4^{{</-}}$ mouse model of Stargardt disease. Our hypothesis was based on the fact that higher doses of $o3$ PUFAs (than those previously published) are apparently required in order to achieve benefit in Stargardt disease.

**Materials and Methods**

**Animals**

Age-matched (8-month-old) $ABCA4^{{</-}}$ (1295-Abca4<tm1Ght>/J Jackson Laboratory, Bar Harbor, ME, USA) and 129/Svlmj (Jackson Laboratory) wild-type mice of both sexes were used in this study and kept under standard housing conditions with a 12-hour/12-hour dark/light cycle and with food and water provided ad libitum. Animal care and experimental procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the Cyprus Government’s Chief Veterinary Officer according to EU guidelines.

**Study Design**

The animals were allocated to three different groups ($n = 15$ group): wild type untreated, $ABCA4^{{</-}}$ untreated, and $ABCA4^{{</-}}$ treated with $o3$ PUFAs. The $ABCA4^{{</-}}$ mice were randomly allocated to the latter two groups. The $o3$ treatment was a fish oil formulation that contained 172 mg EPA and 34 mg DHA (EPA/DHA $= 5:1$) (KD Pharma, Bexbach, Saarland, Germany and Ophthalmos, Nicosia, Cyprus). The animals in the treatment group were given the supplement daily by gavage administration for 3 months. The total oral dosing volumes did not exceed 10 mL/kg. The general health and appearance of the animals were assessed daily, and body weight measurements were recorded weekly. At the end of the study, the mice were euthanized by cervical dislocation.
Fatty Acid Analysis
To determine the AA/EPA ratio before and after long-term treatment, we collected blood samples (n = 10) from each group on day (D) zero, D30, and D90. Samples were processed to separate the fatty acids and then analyzed using gas chromatography (GC). Additionally, after study completion (D90), both retinas from three animals were collected from each group for the same purpose. Blood samples were collected on Whatman (GE Healthcare, Life Sciences, Buckinghamshire, UK) filter paper and stored at −20°C. Samples were processed as previously described34,35 and once supernatants were collected and dried using an analytical evaporator, the samples were redissolved in n-hexane and analyzed using a GC flame-ionization detector (FID).

Gas Chromatographic Flame-Ionization Detection
An Agilent GC-6890 system (Hewlett Packard Agilent Technologies, Santa Clara, CA, USA) was equipped with an FID. We used an H2-flow rate of 35 mL/min, an air-flow rate of 350 mL/min, and a carrier-gas (He) flow rate of 2.5 mL/min. The temperatures of the injection port and detector were set as previously described.34 The injection volume was 1 mL in the splitless 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.

Quantification of A2E
For A2E quantification, the cornea and lens from four eye pairs per group were removed and immediately snap frozen and stored at −80°C until needed for further processing. Eyecups were homogenized and A2E was extracted from the eyecups in chloroform/methanol/PBS solution. The organic layer was separated, evaporated to dryness, and stored at −80°C. Sample analysis was performed on a Waters Xevo TQD MS instrument (Waters, Milford, MA, USA) equipped with the standard z-spray analysis was performed on a Waters Xevo TQD MS instrument using the ion transition of m/z 529.2 → 105.1 and quantified using an external calibration curve prepared from synthetic A2E (Gene and Cell Technologies, Vallejo, CA, USA), as described elsewhere.41

Transmission Electron Microscopy
Electron microscopy was used to quantify lipofuscin granules in the RPE cells in order to observe any difference among the groups. For this analysis, the cornea and lens from five animals per group were removed, and the dissected eyecups were fixed with 2.5% glutaraldehyde, postfixed with 1% osmium tetroxide, dehydrated in a graded ethanol series, cleared in propylene oxide, and embedded in an epon and araldite resin mixture (Agar Scientific, Essex, UK). Ultrathin (80 nm) sections were prepared using a Reichert Jung ultramicrotome (Leica, Wetzlar, Germany). Sections with a silver-gold interference color were mounted on 200-mesh copper grids (Agar Scientific) and contrasted with uranyl acetate and lead citrate. Images were obtained on a JEM 1010 transmission electron microscope (JEOL, Tokyo, Japan) equipped with a Mega View III digital camera (Olympus, Hamburg, Germany).

As the appearance of lipofuscin is somewhat variable in ABCA4−/− mice, a previous morphologic definition43 was used in this study for recognizing lipofuscin granules as follows: Lipofuscin in ABCA4−/− mice is a type of intracellular granule that appears under the electron microscope as a membrane-bound body with heterogeneous staining and very variable shape, generally darker than the cytosol. Melanin granules are easily and reliably distinguished from lipofuscin by electron microscopy, in that melanosomes are uniformly electron dense (black), are not fused with other organelles, and appear spindle-shaped, ovoid, or round. Melanosomes that were fused with lipofuscin were considered melanolipofuscin and were included in the combined category of lipofuscin and melanolipofuscin. We used ImageJ software (http://imagej.nih.gov/ij, provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) for lipofuscin and melanolipofuscin quantification. For each image, we determined the total area of RPE cytoplasm. This measure did not include nuclei, apical microvilli, or the extracellular space in the region of the basal infoldings. The extent of the lipofuscin/melanolipofuscin area was expressed as the number of square micrometers occupied by lipofuscin per 1000 µm2 RPE cytoplasm.

Retinal Nuclear Staining
To assess how the treatment affected retinal photoreceptors (i.e., thickness of the ONL), we used eyes from each group (n = 4 or 5). Eyes were enucleated on D90 and fixed with 2% paraformaldehyde, cryoprotected, and embedded in optimum cutting temperature media (OCT; Sakura Finetek, Torrance, CA, USA) as previously described.34 We cross-sectioned whole eyes and obtained 20 sections (28 µm apart), including those containing the inferior pole, optic nerve, and superior pole. Briefly, 1-µm-thick cryosections were stained using TO-PRO-3 iodide (T3605, 1:10,000; Thermo Fisher Scientific, Waltham, MA, USA). Samples were examined by Leica TCS SP5 confocal microscopy (Leica). ONL measurements were made at different regions around the entire retinal section (center, middle, and periphery). ImageJ software was used to calculate the ONL thickness at each location, and the mean ONL thickness of the entire retina was compared among the different groups.

Mass Spectrometry–Based Proteomics
To further assess the effect of treatment on inflammatory mediators, proteomic analysis of proteins extracted from the eyecups was performed. The cornea and lens from four eyes per group were removed, and the dissected eyecups were immediately snap frozen and stored at −80°C until needed for further processing. Frozen eyecups from each group were incubated in lysis buffer for 30 minutes on ice, followed by sonication for 30 seconds using a model 150VT ultrasonic homogenizer (Biologics, Inc., Manassas, VA, USA). Lysates were clarified by centrifuge, the supernatant was collected, and the proteins were precipitated in a 10-fold excess volume of ice-cold acetone overnight at −20°C and subsequently resuspended in urea buffer. Protein concentration was determined using the bicinchoninic acid protein assay, and the samples were further processed using a modified filter-aided sample preparation (FASP) protocol.42 Peptides were analyzed on a Waters Synapt G2Si HDMS instrument (Waters) operated in ion-mobility mode using the UDMA approach.43 Each sample was analyzed in triplicate. Raw mass spectrometry data were analyzed using Progenesis QI for proteomics software (version 3.0, Waters) and were subjected to protein identification against the UniProt mouse reference proteome database.
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Once proteins were identified, Panther Classification System (version 12.0) was used to determine their functional classification (available in the public domain; http://pantherdb.org/). Predicted functional partners of an individual protein were determined by the STRING database (version 10.5; available in the public domain, https://string-db.org). Proteins with fold change > 1.5 or < -0.666 and P < 0.05 were further analyzed using DAVID Bioinformatics Resources 6.8 (available in the public domain, https://david.ncifcrf.gov/).

**Immunofluorescence Staining**

To inspect how the treatment affected complement component 3 (C3), eyes (n = 4 or 5) from each group were enucleated on D90 (same eyes used in the nuclear staining) and fixed, cryoprotected, and embedded in OCT media (Sakura Finetek). Whole eyes were cross-sectioned as previously described and incubated with a mouse monoclonal IgG1 C3 antibody (1:500, Santa Cruz Biotechnology, Dallas, TX, USA) followed by Alexa Fluor 488 donkey anti-mouse IgG (1:200; Invitrogen, Carlsbad, CA, USA). The slides were examined by Leica TCS SP5 confocal microscopy (Leica).

**Statistical Analysis**

All statistical analyses were performed using GraphPad Prism statistical software (GraphPad Software, La Jolla, CA, USA, version 5). Data are expressed as the mean ± SEM. The 2-tailed Student’s t-test or 1-way ANOVA with a post hoc Tukey test was used to evaluate the differences among treated groups. Statistical significance was set at P < 0.05.

**Results**

**General Observations**

General appearance of the mice was monitored daily, and no abnormalities were noted. Body weight measurements were recorded weekly, and no significant changes were found over time in any of the groups.

**ω3 PUFAs Increase Blood EPA and Decrease Blood AA**

Blood fatty acids were examined prior to (D0), during (D30), and at the end of the study (D90) and are presented in terms of percentage of total fatty acids, as shown in Figure 1. The percentages of the ω3 PUFAs (EPA and DHA) and the percentages of the ω6 PUFAs (AA and dihomogamma-linolenic acid, DGLA) in the blood were determined. Overall, we found that the percent of EPA in the blood was approximately three times higher in the treatment group than in the other two groups on D30 and D90 (P < 0.0001) and that of AA was approximately 1.5 times lower (P < 0.0001). Specifically, on D30, the blood AA/EPA ratio was lower in the treatment group (1.13 ± 0.04) than in the ABCA4+/− untreated (1.53 ± 0.18) or wild-type (1.23 ± 0.27) group (P < 0.0001). No significant changes were noted in the percentage of DGLA or DHA in blood. At the end of the study on D90, fatty acid levels in all groups remained unchanged from D30 (ω3-treated group, EPA: 4.87 ± 0.04 [P < 0.0001]; AA: 5.56 ± 0.12 [P < 0.0001]), as did the AA/EPA ratio (ω3-treated group, 1.10 ± 0.03 [P < 0.0001]).

**ω3 PUFAs Increase Retinal EPA and Decrease Retinal AA**

On D90, retinas from each group were collected, and the percentages of the constituent fatty acids (EPA, DHA, DGLA, and AA) were determined using GC (Fig. 2). We found that retinal EPA levels in the ω3-treated group (1.56 ± 0.03) were significantly higher (approximately double) than those in the ABCA4+/− untreated (0.93 ± 0.06, P < 0.001) and wild-type (0.74 ± 0.06 P < 0.001) groups. In contrast, AA levels in the treatment group (5.87 ± 0.24) were significantly lower than those in the ABCA4+/− untreated (7.03 ± 0.32, P < 0.05) and wild-type (7.06 ± 0.14, P < 0.05) groups. Surprisingly, retinal DGLA was lower in the ABCA4+/− untreated (0.58 ± 0.02, P < 0.05) than in the wild-type (0.68 ± 0.03) or ω3-treated mice (0.64 ± 0.03). As expected from previous reports, DHA was the most abundant fatty acid in the retina, with levels being much greater than those for the other fatty acids. No significant differences in DHA were found between groups (Fig. 2B; wild type, 28.00 ± 0.74; ABCA4+/− untreated, 28.80 ± 0.52; ω3 treated, 29.50 ± 0.21).

**ω3 PUFAs Reduce A2E Bisretinoid Levels**

A2E bisretinoid levels were evaluated in both eyecups of four animals per group. Figure 3 shows A2E levels expressed as the fold change compared with the measured levels of A2E in wild-type mice. Analysis revealed that A2E levels were significantly higher in the ABCA4+/− untreated group (6.07 ± 1.89, P < 0.05) than in the wild-type (0.78 ± 0.22) or the ABCA4+/− ω3-treated (1.47 ± 0.39, P < 0.05) groups. Indeed, levels in the mice treated with ω3 were approximately four times lower than in the untreated ABCA4+/− mice. Interestingly, the A2E levels of the treated animals were comparable to those in the wild-type group and there was no statistical difference between them.

**ω3 PUFAs Reduce the Number of Lipofuscin Granules**

To assess the ultrastructural morphologic differences between the ABCA4+/− and wild-type animals, we examined the retina, RPE, and choroid tissue. In addition to normal-looking lipofuscin granules (Fig. 4A, red arrows), we found an accumulation of unusual, irregularly shaped, electron-dense material in the cytoplasm of RPE cells in the ABCA4+/− untreated animals (Fig. 4A, yellow arrows). This type of organelle was not as evident in the wild-type group and there was no statistical difference between them.

**ω3 PUFAs and the Photoreceptor Layer**

The ONL thickness represents the health of photoreceptor nuclei. To examine the photoreceptor layer, we therefore stained eyes from each group using a nucleic acid dye, and
compared ONL thickness among the three groups (Fig. 5). Although ONL thickness was slightly less in the \textit{ABCA4} \textsuperscript{+/+} untreated group (56.50 ± 3.24 \textmu m) than in the age-matched wild-type group (61.40 ± 1.86 \textmu m), the difference was not statistically significant. The \textit{ABCA4} \textsuperscript{+/+} model previously demonstrated that until 18 months, photoreceptor degeneration is similar to that of wild-type mice, being both slow and age-related.\textsuperscript{18} Therefore, no changes were expected to be seen at this point (11 months). Interestingly, the treatment group (75.66 ± 4.80 \textmu m) exhibited significantly larger ONL thickness.

![Figure 1](http://www.arvojournals.org/)

**Figure 1.** Fatty acid analysis includes percentages of total (A) AA, (B) DGLA, (C) EPA, and (D) DHA from blood samples collected on D0, D30, and D90 from the three groups. (E) The AA/EPA ratio for each group at D0, D30, and D90. Data represent mean ± SEM (\(n = 10\)).

![Figure 2](http://www.arvojournals.org/)

**Figure 2.** Fatty acid analysis from retina samples collected on D90 from the three groups. The percentage of (A) AA, DGLA, EPA, and (B) DHA is expressed as the mean ± SEM (\(n = 3\) per group).
C3 Immunofluorescence Staining Is Localized in the Retinal RPE Layer

The proteomic analysis revealed significantly lower levels of C3 in the treatment group. Therefore, to localize this effect, we stained cryosections with an anti-C3 antibody (Fig. 9). Although we did not quantify C3 staining, visual inspection showed that the eyes from ABCA4+/− untreated mice contained more C3 than either of the other two groups. C3 staining was more evident in the RPE layer and much less so in the photoreceptor layer.

Discussion

Stargardt disease is one of the most common forms of macular dystrophy and to date no treatment has provided evidence of disease regression. Therefore, this condition has a significantly negative impact on quality of life, and developing promising therapeutics is an urgent priority.

Our findings showed that treatment with ω3 PUFAs, when AA/EPA is maintained at levels between 1 and 1.5, reduced the accumulation of lipofuscin granules and A2E and decreased C3 level, limiting a complement-induced inflammatory response. Demonstration of a protective effect against photoreceptor damage would require a significantly longer experiment. If this could be shown, then supplementation with ω3 PUFAs, particularly EPA, could be considered as a treatment for Stargardt disease.

The way through which ω3 PUFAs act is complex; it primarily involves the generation of anti-inflammatory mediators, namely resolvins, which are generated from EPA and DHA and which promote the resolution of inflammation, in addition to the reduction in proinflammatory eicosanoids generated from AA. However, ω3 PUFAs might act through additional mechanisms that are unrelated to inflammation, perhaps involving a regenerative potential.

Following ω3 supplementation for 30 days, we observed a reduced AA/EPA ratio whose average was less than 1.5. This lower ratio resulted not only from increased anti-inflammatory EPA but also from reduced proinflammatory AA in both blood and retinal tissue. DHA is abundantly expressed in photoreceptors and vital retinal functions depend on its existence; this fact was confirmed by its high levels in the retina compared with the other FAs. Incorporation of EPA and DHA into cell membrane phospholipids displaces AA substrate for cyclooxygenase leading to the production of the corresponding 3-series prostanoylin (5, 14) which promote the resolution of inflammation, in addition to the reduction in proinflammatory eicosanoids generated from AA.

Our data also showed that levels of A2E were more than 10 times greater in ABCA4+/− untreated than in wild-type mice. However, in the disease-model mice that were treated with ω3, A2E levels were almost at wild-type levels. We also examined the morphologic effects of ω3 treatment on lipofuscin/melanopslipofuscin granules. Following ω3 supplementation, we found significantly fewer granules than in the ABCA4+/− untreated group. This result directly correlates with the lower A2E accumulation. These data establish that treatment with ω3 inhibits accumulation of A2E and lipofuscin in ABCA4+/− animals. However, the mechanism behind the inhibitory effect of ω3 against A2E is unknown and further experimentation is needed in order to unravel this effect.

To check the integrity of photoreceptors following treatment, we examined the ONL thickness and found significant preservation compared with the untreated controls. Therefore,
FIGURE 4. (A) Representative transmission electron microscopy micrographs of RPE cells from the three groups of mice. Homogeneous, electron-opaque granules are labeled with red arrows, and melanosomes with white arrows. Granules labeled with red arrows represent the more classical type of lipofuscin. Unusual granules of irregular shape and electron density (yellow arrows) accumulated in the RPE cytoplasm of ABCA4−/− untreated mice. These granules appear to fuse with melanosomes (blue arrows). The electron density of these confluent granules was occasionally as high as that of melanosomes (original magnification ×10,000, scale bar: 1 μm). (B) Quantification of lipofuscin and melanolipofuscin granules by electron microscopy. Considering the ABCA4−/− mice, the total area (μm²) occupied by lipofuscin and melanolipofuscin per 1000 μm² sectioned RPE cytoplasm was significantly less in those treated with ω3. Data represent the mean ± SEM (n = 5 per group).

* p < 0.05, ** p < 0.01, *** p < 0.001, one-way ANOVA with post-hoc correction.
we assume that reduced levels of lipofuscin and A2E are correlated with the protection that the treatment provided to the photoreceptor layer. In this experiment, we also confirmed that at 11 months, the loss of photoreceptors in the ABCA4+/C0/C0 mouse model is similar to that experienced by age-matched wild-type controls.18 This suggests that up to 18 months, where retinal degeneration is observed in the RPE cells of ABCA4+/C0/C0 mice and colocalize with endogenous autofluorescence.24 Our findings confirmed that C3 immunofluorescence staining is mainly located in the RPE layer, which correlates with A2E and lipofuscin accumulation in these cells.

The initial scope of this study was to show the therapeutic efficacy of ω3 in ABCA4+/C0/C0 animals, but not to identify the cellular or molecular pathways in which these fatty acids are involved. However, the identification of the two pathways from the proteomic analysis leads to new opportunities for exploring this topic. The most relevant finding was the reduced C3 level in the treatment group compared to that in the control groups. Dysfunction of complement regulators, including C3, has already been implicated in the pathophysiology of the ABCA4+/C0/C0 model,24 as well as in macular degeneration.25 Components of drusen and lipofuscin are thought to induce inflammation via multiple pathways, such as the complement cascade and the NLRP3 inflamasome. It was previously shown that C3 fragments become internalized in the RPE cells of ABCA4+/C0/C0 mice and colocalize with endogenous autofluorescence.24 Our findings confirmed that C3 immunofluorescence staining is mainly located in the RPE layer, which correlates with A2E and lipofuscin accumulation in these cells.

Lenis et al.50 reported that a reduction in complement negative regulatory proteins (e.g., complement receptor 1-like protein γ; CRRY), possibly due to A2E accumulation, might be responsible for the increased complement activation seen in the RPE of the ABCA4+/C0/C0 model. As their study demonstrated, increasing the expression of such complement negative regulatory proteins using targeted gene therapy could be a potential treatment strategy for Stargardt disease and other retinopathies associated with complement dysregulation. Interestingly, in our study, C3 protein level was lower in the ω3-treated group, which suggests that ω3 treatment acted to protect against the A2E-induced activation of the complement pathway, thus preventing inflammation and phagocytosis. The anti-inflammatory effect of ω3 PUFAs indicates a more straightforward therapeutic approach with fewer expected adverse effects and more cost-effectiveness than other types of approaches, therefore potentially leading to higher treatment compliance in the clinical setting.

In addition to the involvement of the complement cascade, the discovery of EphA3 implication in axon guidance following ω3 treatment was demonstrated following proteomic analysis. EphA3 belongs to the ephrin receptor subfamily of the tyrosine kinase family, which has been associated with mediating developmental events, particularly in the nervous system and in the retinotectal mapping of neurons. EphA3 activation and signaling are important for growth cone collapse, axon repulsion, and synaptic plasticity.51 Due to the complexity of these pathways, further bioinformatic analysis would provide a greater understanding on how EphA3 is involved in the mechanistic insight regarding ω3 treatment.

Dornstauder et al.31 used the ELOVL4 transgenic model, which displays extensive age-related retina dysfunction and A2E accumulation, to study the effect of dietary DHA

**Figure 5.** (A) Representative ocular photomicrographs from the three groups. TO-PRO-3 iodide (blue) was used for nuclear staining. Samples were examined with a Leica TCS SP5 confocal microscope (original magnification ×200, scale bar: 150 μm). Retinal outer nuclear layer (ONL) and inner nuclear layer (INL). (B) Measurements of the ONL were performed at different fields around the entire retinal section. Data represent the mean ± SEM (n = 4–5).

**Figure 6.** Protein classification of the significantly (P < 0.05) altered proteins following treatment with ω3, according to their functional activity, as determined by Panther Classification System (version 12.0).
supplementation. Results indicated that following DHA sup-
plementation for longer than 12 months, the mouse models
showed preserved retina function at mid-degenerative stages
and reduced A2E levels.\textsuperscript{31} This effect was not evident before 12
months, which indicates that although DHA used alone might
have a minor protective effect following chronic administra-
tion, it is not as evident or immediate as when combined with
EPA. Our treatment regimen included mainly EPA, and some
DHA, because it is hypothesized that administration of EPA
results in the best possible outcome, due to its strong
competition with AA, the generation of anti-inflammatory
mediators, and its incorporation into the retinal tissue.

Furthermore, based on our preclinical data, a phase II,
multicentered, placebo-controlled, double-blind clinical trial is
commencing to evaluate the potential of \( \omega_3 \) PUFAs (mainly
EPA) in patients with different stages of Stargardt disease and
dry AMD (clinicaltrials.gov; ID: NCT03297515).

In conclusion, our findings suggest that 3 months of \( \omega_3 \)
PUFAs (EPA and DHA, 5:1) supplementation (when AA/EPA
\( \sim 1-1.5 \)) reduces A2E levels, lipofuscin granule formation,
and C3 levels in the \( ABCA4^{+/+} \) mouse model of Stargardt disease,
consistent with slowing of the disease. Evidence of a more
pronounced retinal protective effect would require longer
treatment, and further studies are indicated. Further work is
needed to establish a better understanding of this effect, which
may include electoretinography to assess the function of the
retinal cells, examining the composition of additional phos-
pholipids (i.e., phosphatidylethanolamine, which may have a
direct effect on A2E accumulation)\textsuperscript{52} and a more detailed
analysis from the proteomic data collected. Moreover, addi-
tional studies are required in order to determine the optimal
AA/EPA blood ratio for the greatest beneficial effect. We believe
that \( \omega_3 \) supplementation can be considered a potential
therapeutic regimen for patients with Stargardt disease and
perhaps other types of maculopathies.
Therapeutic Potential of ω3 PUFAs in Stargardt Disease

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