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Neurobiology

A High Omega-3 Fatty Acid Diet Reduces Retinal Lesions in a Murine Model of Macular Degeneration

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Age-related macular degeneration (AMD) is one of the leading cause of blindness among the elderly; however, current therapy options are limited. Epidemiological studies have shown that a diet that is high in ω-3 polyunsaturated (n-3) fatty acids can slow disease progression in patients with advanced AMD. In this study, we evaluated the effect of such a diet on the retinas of *Ccl2*$^{-/-}$/*Cx3cr1*$^{-/-}$ mice, a model that develops AMD-like retinal lesions that include focal deep retinal lesions, abnormal retinal pigment epithelium, photoreceptor degeneration, and A2E accumulation. *Ccl2*$^{-/-}$/*Cx3cr1*$^{-/-}$ mice that ingested a high n-3 fatty acid diet showed a slower progression of retinal lesions compared with the low n-3 fatty acids group. Some mice that were given high levels of n-3 fatty acids had lesion reversion. We found a shunted arachidonic acid metabolism that resulted in decreased pro-inflammatory derivatives (prostaglandin E_2 and leukotriene B_4) and an increased anti-inflammatory derivative (prostaglandin D_2). We also measured lower ocular *TNF-α* and *IL-6* transcript levels in the mice fed a diet of high n-3 fatty acids. Our findings in these mice are in line with human studies of AMD risk reduction by long-chain n-3 fatty acids. This murine model provides a useful tool to evaluate therapies that might delay the development of AMD. ***(Am J Pathol 2009, 175:799–807; DOI: 10.2353/ajpath.2009.090089)***

Age-related macular degeneration (AMD) is the most common cause of legal blindness of elderly people in the world.[1] The pathological features of AMD involve the destruction and deterioration of the photoreceptor and retinal pigment epithelium (RPE) specific to the macula. There are two types of AMD, the exudative or 'wet' form with choroidal neovascularization, and the atrophic or 'dry' form with atrophy of the photoreceptors and RPE. To date, except for the suppression of choroidal neovascularization of the late stage wet form, there is no definitive treatment for AMD. Care for earlier stage AMD and dry AMD is limited to risk factor management. Cessation of smoking, reduction in body mass, and taking specific vitamins and other nutrient supplements may help to slow disease progression.[2]

Omega-3 and ω-6 polyunsaturated fatty acids (n-3 PUFAs, or n-3 fatty acids; and n-6 PUFAs, or n-6 fatty acids) are two classes of PUFAs, which are metabolically and functionally distinct and often have opposing physiological effects. Mammals depend on dietary intake of n-3 fatty acids through sources such as fish oil, because mammalian cells lack enzymes necessary to synthesize the 18-C precursor of n-3 fatty acids and to convert n-6 to n-3 fatty acids.[3] n-3 fatty acids are highly concentrated in the brain and retina and are believed to be important in neuronal development and damage repair.[4] Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA, the precursor to DHA) are two major n-3 fatty acids and are concentrated in retina and retinal vascular endothelium.[5] Photoreceptors are abundantly enriched in DHA with amounts up to approximately 50% of photoreceptor rod outer segment lipids.[6] Vital retinal functions depend on the existence of an adequate proportion of DHA in retinal lipids.[7] DHA prevents the apoptosis of photore-

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ceptor cells that otherwise inevitably occurs during their early development *in vitro*.[8]

The n-3 fatty acids have been shown to exert many preventive and therapeutic actions for an array of diseases such as atherosclerosis and are recommended as a risk management option for AMD.[2] Epidemiological retrospective studies of n-3 fatty acids or fish intake on the prevalence of advanced AMD suggest a protective relationship.[5,9–12] However, no human clinical trials or intervention studies using an AMD animal model have evaluated the effect of n-3 fatty acids on AMD or AMD-like pathology in the literature.

We reported recently that *Ccl2*$^{-/-}$/*Cx3cr1*$^{-/-}$ mice developed a broad spectrum of AMD-like pathologies with early onset and high penetrance.[13,14] In this study, we hypothesized that dietary intake of n-3 fatty acids would alleviate the retinal lesions which develop spontaneously in *Ccl2*$^{-/-}$/*Cx3cr1*$^{-/-}$ mice. To test this hypothesis, we raised the mice on diets that were low or high in n-3 fatty acids and measured a clinical endpoint (funduscopy), histopathology, as well as the level of 2-[2,6-dimethyl-8-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1E,3E,5E,7E-octatetra-enyl]−1-(2-hydroxyethyl)−4-[4-methyl-6(2,6,6-trimethyl-1-cyclohexen-1-yl) 1E,3E,5E,7E-hexatrienyl]-pyridinium (A2E) to evaluate the effects of those diets.

Materials and Methods

Animals

Ccl2$^{-/-}$/*Cx3cr1*$^{-/-}$ mice and wild-type control (C57BL/6) were bred in-house. The study was conducted in compliance with the Association for Research in Vision and Ophthalmology statement for the use of animals, and all animal experiments were performed under protocols approved by the National Eye Institute's Institutional Animal Care and Use Committee.

Experimental Protocol

Ccl2$^{-/-}$/*Cx3cr1*$^{-/-}$ breeding pairs were divided into two groups and fed diets through pregnancy and lactation that were either high or low in long chain n-3 fatty acid content. The pups were weaned onto the same diets as their dams. The two pelleted diets used (provided by Dyets Inc., Bethlehem PA) were based on AIN-93G formulations with several modifications to obtain the low and high n-3 fatty acid levels required in this study.[15] The two diets contained 10% fat by weight and had a similar content of linoleic acid and α-linolenic acid with EPA (C20:5n-3) and DHA (C22:6n-3), along with docosapentaenoic acid (C22:5n-3), as the nutritional variables. To confirm their fatty acid content, lipid profiles were performed for both diets before administration to the mice. The lipid composition for the major fatty acids present in each diet is indicated in Table 1. The efficiency of altering the n-3 fatty acids content in mice by those diets has been tested by measuring the n-3:n-6 ratio in the milk of the mother and the retina of pups in a similar study using identical diets as in this study.[16] In addition, the lipid composition of the retina from mice on the two diets was measured with transmethylation and gas chromatography at 12 weeks of age as described previously.[17]

Table 1. Nutrient and Fatty Acid Composition of Pelleted Diets Given to High n-3 Fatty Acid and Low n-3 Fatty Acid Groups

Ingredient	Amount (g/kg)
Casein, ALACID, Vitamin free*	200
Carbohydrate:	600
Corn starch	150
Sucrose	100
Dextrose	199
Maltose-dextrin	150
Cellulose	50
Mineral and salt mix†	35
Vitamin mix‡	10
L-Cystine	3
Choline bitatrate	2.5

Fatty acids	Low n-3 fatty acids (g/kg)	High n-3 fatty acids (g/kg)
Menhaden oil§	—	18
Olive oil	56	43.2
Flaxseed oil	0.5	—
Safflower oil	11.8	12.8
Hydrogenated coconut oil	31.7	26
TBHQ¶	0.00374	—
Mixed Tocopherol‖	0.0187	—

Fatty acid composition	wt % of fatty acids	wt % of fatty acids
Σ Saturated	38.5	37.2
Σ monounsaturated	45.5	39.3
18:2n-6 fatty acids (LA)	14.8	14.6
18:3n-3 fatty acids (α-LNA)	0.68	0.66
20:5n-3 fatty acids (EPA)	0.09	1.9
22:5n-3 fatty acids (DPA)	—	0.4
22:6n-3 fatty acids (DHA)	—	1.9
n-6/n-3 fatty acids	19.2	2.9

*ALACID casein (NZMP North America Inc, Santa Rosa, CA).
†Dyets Inc., Bethlehem, PA. catalogue #210025.
‡Dyets Inc., Bethlehem, PA. catalogue #310025.
§Omega Protein, Inc. (ingredient declaration: Menhaden oil, Tocopherol, TBHQ; fatty acid composition: wt % of Menhaden oil: 1.8% 18:3n3; 11% 20:5n-3; 2.2% 22:5n-3; 11.4% 22:6n-3).
¶TBHQ is tert-butylhydroquinone (Eastman Chemical Company, Kingsport, Tenneessee).
‖Cognis Nutrition, Cincinnati, OH.

Mice were fed until 8 months of age with either a high n-3 fatty acid or low n-3 fatty acid diet until being harvested for histology as well as other testing. Some were fed continuously until up to 12 months of age. Since we did not observe any retinal lesions in the wild-type (C57BL/6) control mice in our previous studies,[13,14] the wild-type mouse was not included in our feeding experiment, but age-matched tissue samples of wild-type mice fed with a regular diet were used as the reference for certain measurements.

Fundus Photography

After pupil dilation, sequential funduscopic examinations were performed every 5 weeks from 3 to 5 weeks of age until 8 months of age, using a Kowa fundus camera (Kowa Optimed, Torrance, CA) and Volk 90D lens (Volk

Optical, Mentor, OH) following intraperitoneal injection of ketamine (1.4 mg/mouse) and xylazine (0.12 mg/mouse) for systemic anesthesia and topical administration of 1% tropicamide ophthalmic solution (Alcon Inc, Fort Worth, Texas) for pupil dilation. All mice were identified with ear tags, each photograph was labeled, and the record was kept. We evaluated the lesion change by comparing the sequential photos taken in the same fundus area. Progression was defined as >10% increase of deep retinal and subretinal spot (lesion) number, >50% increase in spot diameter in at least 1/3 of the spots, >5 fused spots, or appearance of >2 chorioretinal scars in comparison with the previous observation. Regression was defined as >10% decrease of deep retinal lesions' number, >50% decrease in spot diameter in at least 1/3 of the spots. To avoid a subjective bias, evaluation of the pictures was conducted without knowledge of the treatment by a masked observer. Each individual lesion was identified and viewed in sequential fundus photographs of that eye.

Histopathology

Eyes were harvested following euthanasia of the mice after 8 months on the diet. The tissues were fixed in 10% formalin for at least 24 hours. All tissues were then embedded in methacrylate. The eyes were serially sectioned in the vertical pupillary-optic nerve plane. Each eye was cut into 6 sections. All sections were stained with hematoxylin and eosin. If an ocular lesion was observed, another 6 to 12 sections would be cut through the lesion. These slides were also stained with Periodic Acid Schiff to highlight Bruch's membrane and the basement membrane of small neovascular vessels.

Transmission Electron Microscopy

Electron microscopy was performed on 4% glutaraldehyde-formalin fixed whole eyes. The fixed neuroretina-RPE-choroid tissue was embedded in Ladd LX-112 epoxy resin. Six 1 μm-thick sections stained with toluidine blue were examined under light microscopy. Based on the lesions shown on the thick sections, ultrathin sections of these lesions were taken and were stained with uranyl acetate and lead citrate for examination under a JEOL/JEM-100B microscope. Two eyes in each diet group were used for transmission electron microscopic study.

A2E Extraction and Quantification

A2E is the major component of lipofuscin fluorophores generated from visual cycle flux of all-*trans*-retinal. The molecule is particularly relevant to aging and AMD pathogenesis.[18] The mice were kept in the dark for >12 hours before being sacrificed. Whole eyes were removed in a dark room under dim red light and homogenized. At least three eyes were pooled for each measurement. A2E was extracted with chloroform/methanol as previously described.[19] Detection and quantification was performed by liquid-chromatography mass spectrometry (LC/MS/MS) using a QTRAP 2000 linear ion trap tandem mass spectrometer (Applied Biosystems/MDS SCIEX, Concord, Ontario, Canada) with an Agilent 1100 LC system (Agilent, Wilmington, DE). A gradient of 80% to 98% methanol was used to separate A2E on a C18 column (Zorbax; Agilent) at a flow-rate of 0.3 ml/min. A2E was quantified using external A2E standards.

Enzyme-Linked Immunosorbent Assay

Because dietary n-3 can influence the metabolism of arachidonic acids, we measured several biological active metabolites of arachidonic acids. Serum was collected for different diet groups. Levels of serum prostaglandin E_2 (PGE_2), leukotriene B_4 (LTB_4), and prostaglandin D_2 (PGD_2) were determined by monoclonal antibody-based enzyme-linked immunosorbent assay. Assays were performed by following the manufacturer's instructions (Cayman Chemical Company, Ann Arbor, MI).

Quantification of Gene Expression by Reverse Transcription PCR

Approximately 100 retinal cells (RPE and neuronal cells) were microdissected from a frozen section of an ocular slide. The total RNA from the cells and universal mouse RNA (BD Bioscience, Palo Alto, CA) for assay normalization were subject to cDNA synthesis (Superscript II RNase H^- Reverse Transcriptase, Invitrogen, Grand Island, NY). Real-time PCR was performed using a Stratagene Mx3000 Real-Time PCR System and Brilliant SYBR Green QPCR Master Mix (Stratagene, CA). The primers for *TNF-α, IL-6, VEGFA,* and *VEGFR* were synthesized by SuperArray and supplied as the RT^2 Real-Time Gene Expression Assay kit. For the internal control, *β-actin* was amplified using primers 5′-CCCAGCACAATGAAGATCAA-3′ and 5′-ACATCTGCTGGAAGGTGGAC-3′. Following PCR, a thermal melt profile was performed for amplicon identification. To determine the Ct, the threshold level of fluorescence was set manually in the early phase of PCR amplification. ABI SDS 1.3.1 software and the $2^{-}\Delta\Delta$Ct analysis method were used to determine relative amounts of product using *β-actin* as an endogenous control. The fold change was normalized first by the level of *β-actin* from same cDNA sample. The average fold change due to gene manipulation was again normalized to the transcript level of the universal mouse RNA and presented graphically. Each sample was analyzed at least twice.

Statistical Analysis

The rates of progression and regression between groups were compared by χ^2 test. Multiple means were compared by one-way analysis of variance (analysis of variance), followed by Duncan's multiple range test for post hoc comparison of means. Differences were considered significant when $P < 0.05$.

Table 2. The Constituents of n-3 Fatty Acid in Eye Tissue after 12 Weeks of Low or High n-3 Diet Treatment

	Low n-3 diet	High n-3 diet
	Mean ± SD (wt % of total fatty acids)	Mean ± SD (wt % of total fatty acids)
12:0	0.47 ± 0.15	0.55 ± 0.44
14:0	1.49 ± 0.08	2.18 ± 1.24
16:0	18.00 ± 1.13	20.29 ± 2.00
18:0	13.21 ± 0.58	9.55 ± 4.45
20:0	0.44 ± 0.06	0.31 ± 0.15
22:0	0.56 ± 0.11	0.40 ± 0.21
14:1	0.15 ± 0.04	0.31 ± 0.21
16:1	4.61 ± 0.52	9.26 ± 5.94
18:1n9	25.31 ± 1.69	25.83 ± 6.92
18:1n7	2.88 ± 0.06	2.40 ± 0.25
20:1n9	0.78 ± 0.13	0.58 ± 0.21
18:2n6	3.50 ± 0.81	7.27 ± 3.76
18:3n6	0.03 ± 0.01	0.08 ± 0.03
20:2n6	0.32 ± 0.04	0.20 ± 0.23
20:3n6	0.38 ± 0.02	0.32 ± 0.09
20:4n6	6.74 ± 0.30	3.10 ± 1.84
22:4n6	1.35 ± 0.18	0.49 ± 0.39
22:5n6	1.45 ± 0.52	0.25 ± 0.12
18:3n3	0.08 ± 0.01	0.23 ± 0.20
20:5n3 (EPA)	0.07 ± 0.00	0.21 ± 0.07
22:5n3	0.29 ± 0.01	0.62 ± 0.05
22:6n3 (DHA)	10.31 ± 1.35	18.63 ± 3.32
Total n-3	10.94 ± 1.02	20.60 ± 6.85

Results

Two independent experiments were performed on *Ccl2*$^{-/-}$/*Cx3cr1*$^{-/-}$ mice fed diets either high or low in long chain n-3 fatty acids (Table 1) and yielded similar and reproducible results. The experimental end-point data (fundoscopy, histopathology, and A2E) were pooled and presented as below. The levels of the primary n-3 (EPA, DHA) or n-6 in the retina after 12 weeks on the low or high n-3 diet are shown in Table 2. The n-3 fatty acids accounted for 10.9% ± 1.02% and 20.6% ± 6.9% of the total lipid in the low and high n-3 fatty acid groups, respectively.

Clinical Ocular Features

Mouse fundus photographs were taken to record the lesion location, number, size and character. At 9 weeks of age, we took baseline pictures of the fundus of all mice and found no obvious difference in lesions between the low-n-3 fatty acids and high n-3 fatty acids group of *Ccl2*$^{-/-}$/*Cx3cr1*$^{-/-}$ mice. The sequential photographs taken at 12 weeks of age and 5-week intervals were compared as the lesions progressed or regressed in each follow-up examination. Less progression and greater regression of the retinal lesions were observed in the high n-3 fatty acids fed mice compared with the low n-3 fatty acids fed mice (Figure 1). Figure 1A demonstrates representative funduscopic images of a mouse on the low n-3 fatty acid diet with progression of the deep retinal lesions, and a mouse on the high n-3 fatty acid diet with regressed retinal lesions 15 weeks later after baseline measurements.

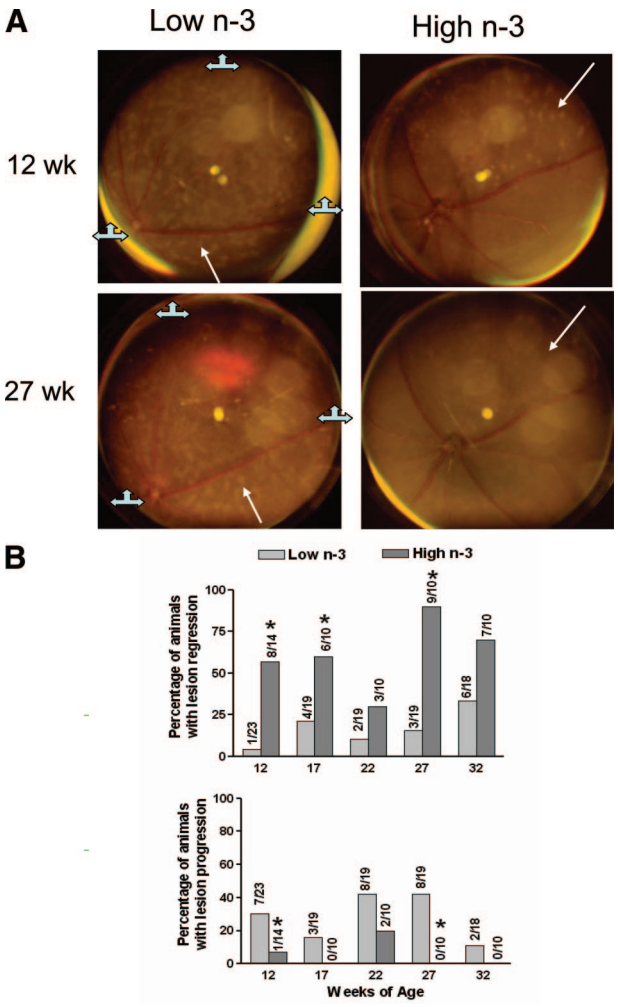

Figure 1. Periodic monitoring of *Ccl2*$^{-/-}$/*Cx3cr1*$^{-/-}$ mice fundus lesions. **A:** Representative funduscopic photographs from a low n-3-treated mouse show progressing deep retinal lesions characterized by increases in number and size, as well as scar formation within 15 weeks, and from a high n-3-treated mouse showing improving deep retinal lesions characterized by decreases in number and size within 15 weeks. **Arrows** indicate the same deep retinal local lesion in the sequential photographs: the size of the retinal lesion is enlarged in the mouse fed with low n-3 diet and the retinal lesion disappears in the mouse fed with high n-3 diet. The three markers in the low n-3 photo sets show the two images are slightly tiled. **B:** Summary of the observations. The regression and progression evaluation at 12th week was based on the comparison with the images taken at ninth week. *χ^2 test $P < 0.05$ in comparison with the low n-3 fatty acid diet group.

More mice demonstrated a regression of retinal lesions in the high n-3 fatty acids group in comparison with the 9-week baseline and with the preceding observation (Figure 1B). At 12 weeks of age, 57% of the mice fed the high n-3 fatty acids exhibited lesion regression, in contrast, only 4% of the low n-3 fatty acids fed mice showed regressed lesions ($P < 0.05$). At 17 weeks, 60% of the high n-3 fatty acids fed but 21% of the low n-3 fatty acids fed mice had lesion regression ($P < 0.05$). At 22 weeks, 30% of the high n-3 fatty acids treated and 10% of the low n-3 fatty acids treated mice showed lesion regression. At 27 weeks, 90% of the high n-3 fatty acids treated and 16% of the low n-3 fatty acids treated mice showed lesion

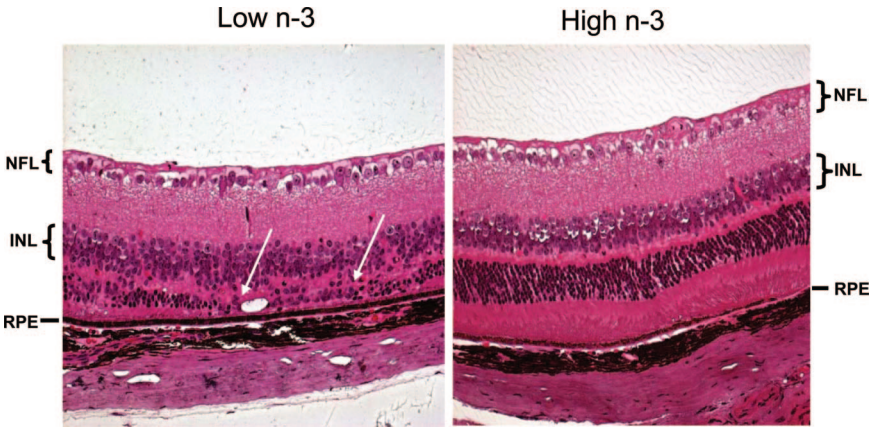

Figure 2. Representative ocular photomicrographs of *Ccl2*$^{-/-}$/*Cx3cr1*$^{-/-}$ mice. Photomicrographs show focal loss of photoreceptors and retinal degeneration (between two **arrows**) in a mouse on a low n-3 fatty acid diet for 27 weeks (**left**). In contrast, the retina is relatively normal with minimal RPE hypotrophy (**arrow**) in a mouse on a high n-3 fatty acids diet for 27 weeks (**right**). Staining is H&E (original magnification, ×200) NFL, nerve fiber layer; INL, inner nuclear layer; RPE, retinal pigment epithelium.

regression ($P < 0.05$). At 32 weeks, 70% of the high n-3 fatty acids treated and 33% of the low n-3 fatty acids treated mice showed lesion regression.

A higher percentage of mice demonstrated lesion progression in the low n-3 fatty acids group, in most examinations (Figure 1B). At 12 weeks, only 7% of high n-3 fatty acids fed but 30% of low n-3 fatty acids fed mice showed lesion progression ($P < 0.05$). At 17 weeks, high n-3 fatty acids treated mice were stable, but 16% of low n-3 fatty acids treated mice continued lesion progression. At 22 weeks, 20% of high n-3 fatty acids treated and 42% of low n-3 fatty acids treated mice showed lesion progression. At 27 weeks, no high n-3 fatty acids treated and 42% of low n-3 fatty acids treated mice showed lesion progression ($P < 0.05$). At 32 weeks, no high n-3 fatty acids treated and 11% of low n-3 fatty acids treated mice showed lesion progression.

Overall, a significantly smaller percentage of mice showed progression and a higher percentage showed regression in the high n-3 fatty acid diet group in comparison with low n-3 fatty acid diet group ($P < 0.05$).

Histological and Ultrastructural Features

Histopathological examination was conducted on 14 eyes from 14 mice fed with the high n-3 fatty acids diet and 18 eyes from 18 mice fed with the low n-3 fatty acids diet for 8 months. Loss of photoreceptors and mild focal hypopigmentation of the RPE cells were found in 4 out of 14 eyes of the high n-3 fatty acids group. In contrast, moderate to extensive retinal photoreceptor dystrophy, atrophy and RPE alterations were identified in 12 out of 18 eyes of the low n-3 fatty acids group (χ^2 test $P < 0.05$; representative images: Figure 2). These findings were confirmed by transmission electron microscopy (Figure 3). Loss of melanosomes and accumulation of lipofuscins were found to be more severe in the RPE cells of the low n-3 fatty acids mice, as compared with the high n-3 fatty acids group. No retinal lesions were seen in the normal control wild-type mice fed with regular diet.

Accumulation of A2E

Two-fold higher A2E was detected in the eyes of low n-3 fatty acids fed mice relative to the high n-3 fatty acids fed mice after 8 months of feeding ($P < 0.05$) (Figure 4A). However, the differences were no longer significant after 12 months of feeding ($P > 0.05$) (Figure 4A), which might result from loss of RPE cells and photoreceptors, the source of A2E, in the late stage of the disease. Figure 4B demonstrates the high signal-to-noise ratio of the A2E detection by the LC/MS/MS system.

Profile of Arachidonic Acid Derivatives

The concentrations of PGE_2 and LTB_4, two pro-inflammatory metabolites of arachidonic acid, were significantly lower in the serum of the high n-3 fatty acids fed mice than in the low n-3 fatty acids fed mice (Figure 5). In contrast, the serum concentration of PGD_2, an anti-inflammatory metabolite, was significantly higher in the mice fed with high n-3 fatty acids as compared with the low n-3 fatty acids group (Figure 5). The levels of these metabolites from the high n-3 fatty acids group were similar to those of the wild-type age-matched control mice that ingested regular chow. The levels of those metabolites from the low n-3 fatty acids group were similar to those of the age-matched *Ccl2*$^{-/-}$/*Cx3cr1*$^{-/-}$ mice that ingested regular chow. No wild-type mice showed any retinal lesions.

Transcripts Profile in the Retina

RT-PCR data demonstrated a significant reduction of ocular *TNF-α* and *IL-6* mRNA detected by quantitative RT-PCR in the microdissected retinal cells of *Ccl2*$^{-/-}$/*Cx3cr1*$^{-/-}$ mice fed with the high n-3 fatty acids diet, as compared with those fed with the low n-3 fatty acids diet (Figure 6). The levels of *TNF-α* transcript expression in the high n-3 fatty acids group were close to that of the wild-type control mice fed with regular chow, which depicted normal retina. In the retinal tissue for the mice that ingested regular chow, *TNF-α* and *IL-6* transcripts were higher in *Ccl2*$^{-/-}$/*Cx3cr1*$^{-/-}$ mice than the wild-type mice (Figure 6). We did not observe significant changes of *VEGF* and *VEGFR* expression between low or high n-3 diets (data not shown).

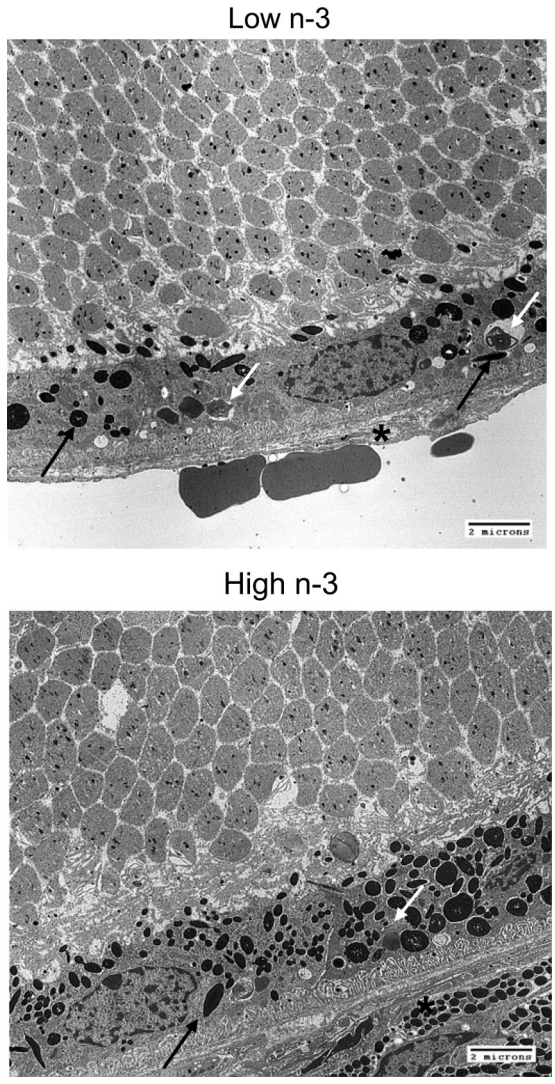

Figure 3. Representative retinal transmission electron micrographs of *Ccl2*$^{-/-}$/*Cx3cr1*$^{-/-}$ mice. **Upper** panel: Transmission electron micrograph of a retina from a mouse on a low n-3 fatty acids diet for 27 weeks shows a decrease of melanosomes (black **arrows**) and an increase of lipofuscin-containing lysosomes (white **arrows**) in the RPE cells. Bruch's membrane (**asterisk**) is thickened. **Lower** panel: Transmission electron micrograph of a mouse on a high n-3 fatty acids diet for 27 weeks shows many melanosomes (black **arrow**) and few lipofuscin-containing lysosomes (white **arrow**) in the RPE cells. Bruch's membrane (**asterisk**) appears normal. Scale bar = 2 μm.

Discussion

In this study, the *Ccl2/Cx3cr1*-deficient mice were given a defined isocaloric diet either high or low in n-3 fatty acids (DHA and EPA). The results demonstrated an obvious benefit of long chain n-3 fatty acid intake in a mouse strain generated by knocking out a chemokine (*Ccl2*) and a chemokine receptor (*Cx3cr1*), which exhibits certain pathological features of human AMD-like lesions.[13] Since aged mouse Bruch's membrane structures are not considered similar to human, the characteristic age changes of drusen formation, often seen in humans, are rarely seen in aged mouse Bruch's membrane.[20] However, an increase in size and number of lipofuscin granules are reported in the RPE.[21] Therefore, A2E measurement rather than identification of drusen should be the biomarker of mouse "AMD." The diet started from conception and the clinical outcome can be observed as early as 9 weeks of age. Although there is a significant variation among individual animals, the overall outcome of lesion reversion or progression is striking between the two groups fed with high or low n-3 fatty acid diets.

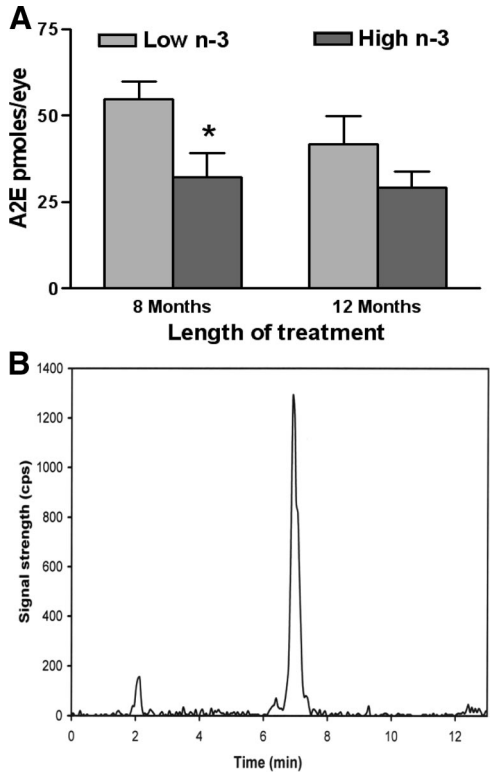

Figure 4. Quantification of A2E in eyes of *Ccl2*$^{-/-}$/*Cx3cr1*$^{-/-}$ mice. **A:** A2E, a major lipofuscin fluorophore that accumulates during AMD progression, was lower in the *Ccl2*$^{-/-}$/*Cx3cr1*$^{-/-}$ retina in the high n-3 fatty acids (n = 4) compared with the low n-3 fatty acids group (n = 4) at 8 months of age (*$P < 0.05$), and 12 months of age (n = 4 in the high n-3 fatty acid group and n = 3 in the low n-3 fatty acid, $P > 0.05$). **B:** LC/MS/MS chromatography represents the A2E peak eluting at 6.9 minutes.

The diet used in this study is designed to generate a differential ratio of n-3 fatty acids to n-6 fatty acids. The

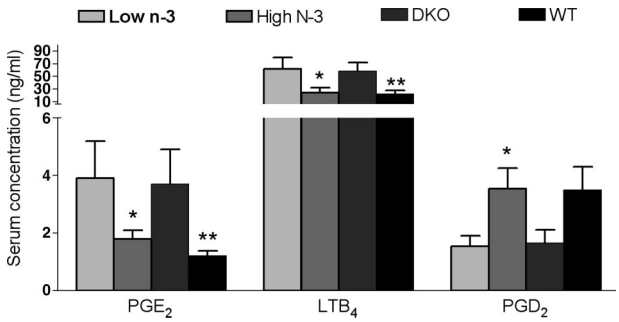

Figure 5. The serum concentration of arachidonic acid metabolites in *Ccl2*$^{-/-}$/*Cx3cr1*$^{-/-}$ mice treated with low n-3 or high n-3 chow; wild-type mice or *Ccl2*$^{-/-}$/*Cx3cr1*$^{-/-}$ mice with regular chow. The graph is plotted as mean ± SD. The concentrations of PGE_2 and LTB_4, pro-inflammatory metabolites of arachidonic acid, were lower, whereas PGD_2, an anti-inflammatory metabolite of arachidonic acid was higher in the serum of the high n-3 fatty acids *Ccl2*$^{-/-}$/*Cx3cr1*$^{-/-}$ group (n = 5) compared with that of low n-3 fatty acids diet group (n = 5). *$P < 0.05$ in comparison with the low n-3 fatty acids diet group. **$P < 0.05$ in comparison with the *Ccl2/Cx3cr1* deficient mice with regular chow.

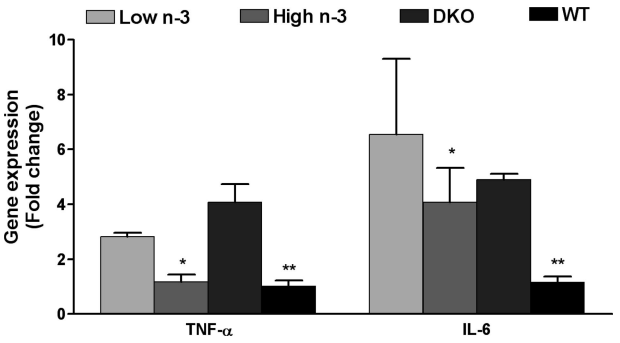

Figure 6. Gene transcripts in the retina of *Ccl2*$^{-/-}$*/Cx3cr1*$^{-/-}$ mice treated with low n-3 or high n-3 chow; wild-type mice or *Ccl2*$^{-/-}$*/Cx3cr1*$^{-/-}$ mice with regular chow. The graph is plotted as mean ± SD. Lower *TNF-α* and *IL-6* mRNA were detected by quantitative RT-PCR in the ocular tissue of *Ccl2*$^{-/-}$*/Cx3cr1*$^{-/-}$ mice fed with a high n-3 fatty acid diet ($n = 6$) as compared with those fed with a low n-3 fatty acid diet ($n = 6$). *$P < 0.05$ in comparison with *Ccl2*$^{-/-}$*/Cx3cr1*$^{-/-}$ low n-3 fatty acids group. **$P < 0.05$ in comparison with the *Ccl2/Cx3cr1* deficient mice with regular chow.

beneficial effects of n-3 fatty acids in various physiological and pathological situations are well-documented.[22] The n-3 fatty acids act as an energy source as well as an important unique cell component, especially in cellular membranes. DHA is particularly rich in retinal photoreceptor outer segments.[22] The highest concentrations in the body for DHA per unit area are found in the photoreceptor disk membranes and the overall percentage of DHA can reach 50% of total retinal fatty acids.[23,24] The unique fatty acid composition in retinal photoreceptor outer segment disk membranes is essential for maintaining a healthy retina. DHA affects membrane function by altering permeability, membrane order, thickness, lipid phase properties, and the activation of membrane-bound proteins.[5] The special structure of DHA, the position of the first unsaturated bond at the n-3 fatty acids (between Δ-20 and Δ-19) carbon, provides an advantage in efficiency of membrane dynamics over that observed in an otherwise structurally identical fatty acid with the first double bond at the n-6 carbon.[24] The n-3 fatty acids may have a neuroprotective effect in our model, which results in decelerated retinal lesion development in *Ccl2*$^{-/-}$*/Cx3cr1*$^{-/-}$ mice ingesting a high n-3 fatty acid diet.

The n-3 fatty acids and their derivatives play an extensive role in numerous biological processes, such as inflammatory cascades, apoptosis, and neuroprotection.[5] These are related to direct actions on plasma membranes, altered inflammatory response and control of gene expression. We focus on the role of n-3 fatty acids in inflammation because the role of inflammation in AMD pathogenesis is evident.[25,26] During AMD development, cellular debris derived from the aging RPE cells becomes sequestered below the RPE basal lamina into Bruch's membrane, which could constitute a chronic inflammatory stimulus. The entrapped cellular debris then becomes the target of encapsulation by a variety of inflammatory mediators and elicits a local chronic inflammatory response that exacerbates the effects of primary pathogenic stimuli.[26,27] During the late stage of the disease, more evidence can be found for inflammatory involvement.[25,28] The immunological protein expression profile in *Ccl2*$^{-/-}$*/Cx3cr1*$^{-/-}$ mice, the model used in this study, reveals increased complement C3 in Bruch's membrane, RPE, choroidal capillaries, and particularly in the deep retinal lesions, relative to the wild-type controls.[29] Complement component C3 and chemokine Ccl5 are elevated in the retina of these mice. Moreover, infiltration of mononuclear phagocytic cells, both macrophages and microglia are also found in the lesion site.[29]

We focused on the arachidonic acid metabolism pathway because it interacts with n-3 fatty acids metabolism in various ways such as plasma membranes.[5] Neuronal membranes are composed of high levels of PUFA, especially DHA and arachidonic acids.[22] Arachidonic acid (C20:4n-6) is the substrate for the synthesis of a range of biologically active compounds (eicosanoids) including prostaglandins, thromboxanes, and leukotrienes. These compounds can act as mediators in leukocyte chemotaxis and inflammatory cytokine production. When fish oil (high n-3 fatty acids) is provided, EPA is incorporated into cell membrane phospholipids at the expense of arachidonic acid, leading to less substrate available for eicosanoid synthesis.[30] In our study, high n-3 fatty acids decreased the production of inflammatory eicosanoids (PGE_2 and LTB_4) as compared with the mice ingesting low n-3 fatty acids, which was correlated with amelioration of retinal lesions. An similar result was reported by Ira mete al.[31] The reduced PGE_2 and LTB_4 could also be attributed to differential gene expression regulated by reactive mediators of n-3 fatty acids.[32,33]

We also observed an increased PGD_2 in serum of the high n-3 fatty acids group. PGD_2 is considered to be an anti-inflammatory mediator.[34,35] The dehydration end-product of PGD_2 is PGJ_2, which represses several pro-inflammatory genes including *tumor necrosis factor (TNF)-α, interleukin-1β*, and *inducible nitric oxide synthase*.[36,37] Even though the replacement of arachidonic acids with EPA reduces the generation of arachidonic acid metabolites in general; an increased PGI_2 after fish oil supplementation has been reported.[38] The newly identified EPA/DHA-derived inflammation-resolving mediator classes and their role on pathological retinal angiogenesis in mice provided the evidence to explain this pleiotropic effect of n-3 fatty acids.[16,39] This phenomenon might be attributed to an altered profile of lipid mediators using arachidonic acid and/or EPA as the substrates during inflammation,[39] or to modified expression of genes responsible for arachidonic acid metabolism.[32,33]

The protective effect of high n-3 fatty acids was correlated with the suppression of *TNF-α* and *IL-6* in the eye in this study. TNF-α is an inflammatory cytokine and has been found in a subset of microglia that is closely associated with retinal vessels.[16,40] Anti-TNF-α treatment has been reported to reduce both the size of, and leakage of, laser-induced choroidal neovasculature in mice.[41] Interestingly, TNF-α increases PGE_2 but decreases PGD_2 synthesis by zymosan-stimulated murine macrophages.[42] The role of n-3 on the interleukin (IL)-6 has been reported previously.[43] Aqueous IL-6 levels are reported to significantly correlated with the sizes of AMD associated choriodal neovascular membranes.[44] Even though vascular endothelial growth factor (VEGF) has been reported to be related to AMD,[44] particularly neo-

vascular AMD,[45] we did not find an altered expression due to different diets. This may be because most *Ccl2/Cx3cr1* deficient mice lack choroidal neovascularization.

In summary, a diet enriched in EPA and DHA can ameliorate the progression of retinal lesions in the *Ccl2/Cx3cr1* deficient mice. We suggest that this mouse strain is useful for the screening of therapeutic agents for AMD. One of the mechanisms underlying lower disease progression by long chain n-3 fatty acids may be via a shunted arachidonic acid pathway, leading to an increase of anti-inflammatory derivatives such as PGD_2 and decreases of pro-inflammatory derivatives such as PGE_2, LTB_4, TNF-α, and IL-6. The results in these mice are in line with the epidemiological studies of AMD risk reduction by long chain n-3 fatty acids.

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