Adiponectin Mediates Dietary Omega-3 Long-Chain Polyunsaturated Fatty Acid Protection Against Choroidal Neovascularization in Mice

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters

Citation
Fu, Z., R. Liegl, Z. Wang, Y. Gong, C. Liu, Y. Sun, B. Cakir, et al. 2017. “Adiponectin Mediates Dietary Omega-3 Long-Chain Polyunsaturated Fatty Acid Protection Against Choroidal Neovascularization in Mice.” Investigative Ophthalmology & Visual Science 58 (10): 3862-3870. doi:10.1167/iovs.17-21796. http://dx.doi.org/10.1167/iovs.17-21796.

Published Version
doi:10.1167/iovs.17-21796

Citable link
http://nrs.harvard.edu/urn-3:HUL.InstRepos:34375122

Terms of Use
This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA
Retinal Cell Biology

Adiponectin Mediates Dietary Omega-3 Long-Chain Polyunsaturated Fatty Acid Protection Against Choroidal Neovascularization in Mice

Zhongjie Fu,1 Raffael Liegl,1 Zhongxiao Wang,1 Yan Gong,1 Chi-Hsiu Liu,1 Ye Sun,1 Bertan Cakir,1 Samuel B. Burnim,1 Steven S. Meng,1 Chatarina Löfqvist,2 John Paul SanGiovanni,3 Ann Hellström,2 and Lois E. H. Smith1

1Department of Ophthalmology, Boston Children’s Hospital, Harvard Medical School, Boston, Massachusetts, United States
2Department of Ophthalmology, Sahlgrenska Academy at University of Gothenburg, Gothenburg, Sweden
3National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, Maryland, and Georgetown University School of Medicine, Washington, District of Columbia, United States

Correspondence: Lois E. H. Smith, Department of Ophthalmology, Boston Children’s Hospital, Harvard Medical School, Boston, MA 02115, USA; lois.smith@childrens.harvard.edu.

ZF and RL contributed equally to the work presented here and should therefore be regarded as equivalent authors.

Submitted: March 2, 2017
Accepted: June 20, 2017

Citation: Fu Z, Liegl R, Wang Z, et al. Adiponectin mediates dietary omega-3 long-chain polyunsaturated fatty acid protection against choroidal neovascularization in mice. Invest Ophthalmol Vis Sci. 2017;58:3862–3870. DOI: 10.1167/iovs.17-21796

PURPOSE. Neovascular age-related macular degeneration (AMD) is a major cause of legal blindness in the elderly. Diets with omega-3-long-chain-polyunsaturated-fatty-acid (ω3-LCPUFA) correlate with a decreased risk of AMD. Dietary ω3-LCPUFA versus ω6-LCPUFA inhibits mouse ocular neovascularization, but the underlying mechanism needs further exploration. The aim of this study was to investigate if adiponectin (APN) mediated ω3-LCPUFA suppression of neovessels in AMD.

METHODS. The mouse laser-induced choroidal neovascularization (CNV) model was used to mimic some of the inflammatory aspect of AMD. CNV was compared between wild-type (WT) and Apn−/− mice fed either otherwise matched diets with 2% ω3 or 2% ω6-LCPUFAs. Vldlr−/− mice were used to mimic some of the metabolic aspects of AMD. Choroid assay ex vivo and human retinal microvascular endothelial cell (HRMEC) proliferation assay in vitro was used to investigate the APN pathway in angiogenesis. Western blot for p-AMPKα/AMPKα and qPCR for Apn, Mmps, and IL-10 were used to define mechanism.

RESULTS. ω3-LCPUFA intake suppressed laser-induced CNV in WT mice; suppression was abolished with APN deficiency. ω3-LCPUFA, mediated by APN, decreased mouse Mmps expression. APN deficiency decreased AMPKα phosphorylation in vivo and exacerbated choroid-sprouting ex vivo. APN pathway activation inhibited HRMEC proliferation and decreased Mmps. In Vldlr−/− mice, ω3-LCPUFA increased retinal AdipoR1 and inhibited NV. ω3-LCPUFA decreased IL-10 but did not affect Mmps in Vldlr−/− retinas.

CONCLUSIONS. APN in part mediated ω3-LCPUFA inhibition of neovascularization in two mouse models of AMD. Modulating the APN pathway in conjunction with a ω3-LCPUFA-enriched-diet may augment the beneficial effects of ω3-LCPUFA in AMD patients.

Keywords: age-related macular degeneration, omega-3 long-chain polyunsaturated fatty acids, adiponectin, neovascularization

Choroidal neovascularization (CNV) and retinal angiomaticous proliferation (RAP) in age-related macular degeneration (AMD) is a major cause of vision loss in elderly people. CNV leads to exudation, hemorrhage, neural retinal dysfunction, and eventually loss of central vision. Therefore, prevention and suppression of CNV is of great interest to improve quality of life. The causes of CNV still remain incompletely defined, but genetic deficiencies, inflammation, extracellular matrix (ECM) integrity and remodeling, and dietary lipid and metabolites could all contribute to the development of CNV.1–3 Dietary lipids and their metabolites have been implicated to alter many of these processes.1,2 In this work we focus on the joint actions of fatty acids and adiponectin (APN) on neovascular AMD and their downstream mediators, inflammation and ECM integrity.

In clinical investigations, dietary intake of fish containing omega 3-long-chain-polyunsaturated-fatty-acid (ω3-LCPUFA), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA) is associated with a decreased risk of AMD.1–7 The Age-Related Eye Disease Study (AREDS) reported 30% less central geographic atrophy and neovascular AMD in participants with a diet containing high ω3-LCPUFA, DHA, and EPA (0.106% of total energy intake) than those with low ω3-LCPUFA intake (0.013% of total energy intake).8 In a large cohort of female health professionals in the United States, there was a 42% reduced incidence of AMD with higher (two servings of fish per week) versus lower fish (and ω3-LCPUFA) intake.9 Some studies show that there is no improvement in AMD progression associated with DHA and EPA capsule supplementation, although these patients had a high baseline serum ω3-LCPUFA level or were otherwise well-nourished.10–12 The effects of ω3-LCPUFA on modulating neovessel formation needs further exploration, particularly for patients on a Western diet with low ω3-LCPUFA intake.13,14 Previous studies show that a ω3-
LCPUFA-enriched diet reduces laser-induced CNV in mice\textsuperscript{15–17}; however, the complete underlying mechanisms are still unknown.\textsuperscript{16–19}

APN plays anti-angiogenic and anti-inflammatory roles in vascular diseases.\textsuperscript{20,21} In the mouse model of oxygen-induced proliferative retinopathy, APN is involved in \(\omega-3\) LCPUFA's inhibition of retinal neovascularization.\textsuperscript{22} Therefore, the APN pathway may be involved in \(\omega-3\) LCPUFA's protection in neovascular AMD. We hypothesized that the APN signaling pathway mediates \(\omega-3\) LCPUFA protection against choroidal/retinal NV. We explored this question in two animal models: the laser-induced CNV model and very low density lipoprotein receptor deficient mice (\textit{Vldlr}\textsuperscript{−/−}) with retinal angiomaticus proliferation (RAP) and CNV.

### Methods

**Ethics Statement**

All animal studies adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee at Boston Children’s Hospital (ARCH protocol number 16-06-3155R). \textit{C57BL/6j} and \textit{Apxr}\textsuperscript{−/−} (backcrossed with \textit{C57BL/6j} for 11 generations) from the Jackson Laboratory were used.

**Laser-Induced CNV in Mice**

Four laser burns were induced by a green Argon laser pulse (Micron IV, Phoenix Research Laboratories, Pleasanton, CA, USA) with duration of 70 ms and power of 240 mW in 6- to 8-week-old \textit{C57BL/6j} and \textit{Apxr}\textsuperscript{−/−} mice. Mice were fed defined rodent diets with either 2\% \(\omega-3\) (1\% DHA and 1\% EPA) or 2\% \(\omega-6\) LCPUFA (AA)\textsuperscript{23} (Supplementary Table S1) 7 days before and 6 LCPUFA (AA)\textsuperscript{23} (Supplementary Table S1) 7 days before and 1 week-old \textit{C57BL/6j} and \textit{Apxr}\textsuperscript{−/−} mice were killed. RPE/choroid/sclera complex ("choroid explants") from the peripheral area was dissected and cut into approximately 1 \(\times\) 1 mm pieces.\textsuperscript{26} The choroid explants were immediately embedded in 30 \(\mu\)L growth factor–reduced Matrigel (BD Biosciences, San Jose, CA, USA; Cat. 354230) in 24-well tissue culture plates (day 0, D0). The explants were grown in CSC complete medium (Cell Systems, Kirkland, WA, USA; Cat. 420-500) supplemented with growth factor Boost and 1\% Penicillin/Streptomycin (GIBCO, Grand Island, NY, USA; Cat. 15142) at 37°C with 5\% CO\textsubscript{2}. At D6, images were taken under 25× magnification. The sprouting area was quantified using ImageJ.

**Human Retinal Microvascular Endothelial Cell (HRMEC) Proliferation Assay**

HRMECs (passage 7 or 8) were grown in EGM2 and 1\% antibiotic-antimycotic (GIBCO, #12540-060) on 0.1\% gelatin-coated 96-well cell culture plate. HRMECs were treated with APN receptor agonist adipor2 (2.5, 5.0, 10, and 25 \(\mu\)M) or vehicle (0.1\% DMSO) for 24 hours. Ten microliters MIT reagent (ATCC, #30-1010K) was added to 100 \(\mu\)L EGM2 in each well. After 6-hour incubation at 37°C, cells were washed with PBS and 100 \(\mu\)L DMSO was added per well. The absorbance was recorded at 570 nm.

HRMECs (passage 7 or 8) were grown on 0.1\% gelatin-coated six-well cell culture plate and treated with APN receptor agonist adipor2 (25 \(\mu\)M) or vehicle for 2 hours. The cells were collected for RNA extraction.

**Real-Time PCR**

Freshly isolated sclera/choroid/RPE retina complex or retinas or HRMECs were lysed with QIAzol lysis reagent and incubated on ice for 15 minutes, and 20\% chloroform was added and incubated for 5 minutes at room temperature. The mixture was centrifuged at 12,000g for 15 minutes, and the supernatant was collected for RNA extraction according to the manufacturer's instructions using a PureLink RNA Mini Kit (#1218301A; Ambion, Grand Island, NY, USA). RNA was then reverse transcribed using iScript cDNA synthesis kit (#1708891; Bio-Rad, Hercules, CA, USA). The sequences of primers were \textit{Apxr} (F: 5′-GAA GCC GCT TAT GTG TAT CGC-3′; R: 5′-GAA TGG GTA CAT TGG GAA CAG TAC-3′); \textit{AdipoR1} (F: 5′-TCT TCG GGA TGT TCT TCC TGG-3′; R: 5′-TGG GTA AAA AGT CCG AGA GAC C-3′); \textit{Vegfa} (F: 5′-GGA GAT CCT TCT AGG AGC ACT T-3′; R: 5′-GCC ATT TAG CAG CAT A TA A GAA-3′); \textit{Mmp2} (F: 5′-TGG GAG CAT GGA GAT GAT GAA TAC-3′; R: 5′-AAG TGA GAA TCT CCC CCA ACA CAC-3′); \textit{Mmp9} (F: 5′-ACT AGC GGC GCT TCT GAG G-3′; R: 5′-ATG TCG AGG GCC GCT TCT GAG G-3′); \textit{Mmp2} (F: 5′-TAC AGG ATC ATT GGC TGC TAC ACA CC-3′; R: 5′-GCT CAC ATG GCC GCA GAC TAC-3′); \textit{Mmp9} (F: 5′-TGC ACC GCC GCT TCT GAG G-3′; R: 5′-ATG TCG AGG GCC GCT TCT GAG G-3′). Quantitative analysis of gene expression was generated using an Applied Biosystems (Grand Island, NY, USA) 7300 Sequence Detection System with the SYBR Green Master mix kit, and gene expression was calculated relative to internal control \textit{Cyclophilin A} (F: 5′-CAG ACC CCA CTG TCG CTT T-3′; R: 5′-TGG CTT TGG AAC TTT GTC TGC AA-3′) for mouse retinas and \(18S\) ribosomal RNA (F: 5′-ACGGAAGGGCCACCCAGA-3′; R: 5′-CACCCACCCACCCAGGAAATG-3′) for HRMECs using microscope and merged to form one image with AxioVision 4.6.3.0 software. Vascular lesions were analyzed using the SWIFT_MACTEL method, a plugin in ImageJ.\textsuperscript{25}

**Choroid Assay Ex Vivo**

Three-week-old \textit{C57BL/6j} and \textit{Apxr}\textsuperscript{−/−} mice were killed. RPE/choroid/sclera complex ("choroid explants") from the peripheral area was dissected and cut into approximately 1 \(\times\) 1 mm pieces.\textsuperscript{26} The choroid explants were immediately embedded in 30 \(\mu\)L growth factor–reduced Matrigel (BD Biosciences, San Jose, CA, USA; Cat. 554230) in 24-well tissue culture plates (day 0, D0). The explants were grown in CSC complete medium (Cell Systems, Kirkland, WA, USA; Cat. 420-500) supplemented with growth factor Boost and 1\% Penicillin/Streptomycin (GIBCO, Grand Island, NY, USA; Cat. 15142) at 37°C with 5\% CO\textsubscript{2}. At D6, images were taken under 25× magnification. The sprouting area was quantified using ImageJ.
the ΔΔCt method. The relative mRNA levels were presented as the ratio of change versus internal control.

Western Blot
Choroidal-retinal explants were homogenized and protein was extracted in radioimmunoprecipitation assay buffer (RIPA) (#89900; Pierce, Grand Island, NY, USA) supplemented with phosphatase inhibitor (1:100, Sigma, P8340) and protease inhibitor (1:1000, Sigma, P8340). Forty micrograms protein lysate were used to detect the levels of phospho-AMPKα² (p-AMPKα, 1:500, #2535; Cell Signaling, Beverly, MA, USA) and AMPKα² (1:500, #2532, Cell Signaling) overnight at 4°C. Signals were detected using 1:5000 corresponding horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (ECL, Pierce). GAPDH (1:3000, sc-32233; Santa Cruz, Dallas, TX, USA) was used as internal control.

Statistical Analysis
Animal data are presented as mean ± SEM. All data were used except for low quality images that were not sufficient for analysis. Both male and female mice were used. For drug treatments, the mice were randomly assigned to treatment and vehicle control in the same litter. For in vitro study, the experiment was repeated two or three independent times. Two-tailed unpaired t-test, ANOVA with Bonferroni’s multiple comparison test was used for comparison of results as specified in the figure legends (Prism v5.0; GraphPad Software, Inc., San Diego, CA, USA). Statistically significant difference was set at P ≤ 0.05.

RESULTS

Dietary Intervention of ω-3 Versus ω-6 LCPUFA Decreased Laser-Induced CNV in Mice

In the mouse model of laser-induced CNV, there was a 20% decreased lesion area (P = 0.0435; Fig. 1; Supplementary Table S2) in mice with ω-3 versus ω-6 LCPUFA-enriched diets, as shown in our previous reports.15–17

APN Pathway Mediated ω-3 LCPUFA Protection Against Laser-Induced CNV

In laser-induced CNV with dietary ω-3 LCPUFA supplementation, the lesion area was increased 60% in Apn⁻/⁻ versus WT mice (P = 0.0053, Figs. 2A-B). However, APN deficiency did not affect lesion area in mice fed a ω-6 LCPUFA-enriched diet (Figs. 2C-D), indicating that APN played a major role in ω-3 LCPUFA dependent inhibition in CNV. We also examined the lesion severity in mice fed animal chow (with undefined composition) provided by our animal facility. APN deficiency worsened CNV lesion formation (P = 0.0438; Supplementary Fig. S2A; Supplementary Table S3) and activation of the APN pathway with APN receptor agonist AdipoRon administration significantly reduced the lesion size (P = 0.0233; Supplementary Fig. S2B; Supplementary Table S3).

Activation of the APN Pathway by Dietary ω-3 LCPUFA Supplementation Inhibited Matrix Metalloproteinase (MMP) Expression in Laser-Induced CNV

To explore the underlying mechanism of APN pathway activation causing CNV suppression, we examined changes in potential APN downstream targets Mmp2 and Mmp9 in WT and Apn⁻/⁻ mice with either ω-3 or ω-6 LCPUFA-enriched diets. ω-3 LCPUFA supplementation increased Apn mRNA in WT choroid-retina complex (P = 0.0010; Fig. 3A). Dietary ω-3 versus ω-6 LCPUFA decreased Mmp2 and Mmp9 in WT choroid-retina complex (Mmp2: ω-3 to ω-6 = 0.4, P = 0.0006; Mmp9: ω-3 to ω-6 = 0.43, P = 0.0451; Fig. 3A). The decreases were abolished with APN deficiency (Mmp2: ω-3 to ω-6 = 1.1, P = 0.6607; Mmp9: ω-3 to ω-6 = 0.91, P = 0.6673; Fig. 3B). We then investigated the downstream target of the APN pathway AMPKα²,29,30 which transcriptionally regulates the expression of MMPs.31,32 A decreased choroidal-retinal p-AMPKα/AMPKα² ratio (Apn⁻/⁻ to WT = 0.4) was found in Apn⁻/⁻ versus WT CNV mice fed with ω-3 LCPUFA (Fig. 3C). Our findings...
suggested that dietary ω-3 LCPUFA supplementation activated the APN pathway to phosphorylate AMPKα and reduce MMP2 and MMP9 levels, which in turn inhibited neovessel formation.

**Activation of the APN Pathway Inhibited Endothelial Cell Activity Ex Vivo and In Vitro**

The inhibitory effects of the APN pathway on ocular vessel growth were further confirmed in the choroidal sprouting assay ex vivo and HRMECs proliferation assay in vitro. APN deficiency resulted in a larger choroid sprouting area (Apn−/− to WT = 1.67; P = 0.0445; Fig. 3D). Activation of the APN pathway with adipoRon (a dual agonist for APN receptor ADIPOR1 and ADIPOR2) dose-dependently inhibited HRMEC proliferation (Fig. 3E). AdipoRon treatment significantly decreased Mmp2 (AdipoRon to vehicle = 0.57; P = 0.0083; Fig. 3F) and Mmp9 expression in HRMECs in vitro (AdipoRon to vehicle = 0.75; P < 0.0001; Fig. 3F). The results were consistent with activation of the APN pathway mediating ω-3 LCPUFA inhibition of laser-induced CNV through activation of AMPKα and reduction in Mmp2 and Mmp9 mRNA expression (Fig. 4).

**Dietary ω-3 LCPUFA Supplementation Suppressed Lesion Formation in Vldlr−/− Mice**

In addition to the inflammatory contribution to CNV formation, metabolic alterations may also lead to disease progression of AMD. In Vldlr−/− mice, the absence of VLDLR is associated with RAP, similar to that seen with neovascular AMD. To assess if dietary intervention of ω-3 LCPUFA also attenuated vascular lesion formation caused by dysregulated metabolism, Vldlr−/− mice were fed either ω-3 or ω-6 LCPUFA-enriched diets from P1. The mother’s milk reflects the lipid content of their diet. In Vldlr−/− mice with ω-3 versus ω-6 LCPUFA diets, there was no difference in retinal Apn but an increase in AdipoR1 mRNA levels (ω-3 to ω-6 = 3.9; P = 0.008; Fig. 5A). Retinal AdipoR1 was increased during development from P3 to P17, and induced in Vldlr−/− versus Vldlr−/+ mice (Fig. 5B). At P16, dietary ω-3 versus ω-6 LCPUFA decreased the number of neovascular lesions by 18% (P = 0.0099) and total lesion area by 29% (P = 0.0095) (Figs. 5C–E).
Interestingly, \(\omega-3\) versus \(\omega-6\) LCPUFA diets did not impact retinal Mmp2 and Mmp9 expression in Vldlr\(^{-/-}\) mice (Fig. 5F). Instead, there were increased anti-inflammatory marker IL-10 mRNA levels in \(\omega-3\) versus \(\omega-6\) LCPUFA-fed Vldlr\(^{-/-}\) mice (\(\omega-3 \) to \(\omega-6 = 13.3; P = 0.0009;\) Fig. 5F). Activation of the APN pathway with adipoRon (50 mg/kg, oral gavage daily from P3–16) also increased IL-10 expression (Fig. 5G) and decreased Mmp2 and Mmp9 (Supplementary Fig. S3) in Vldlr\(^{-/-}\) mouse retinas. However, in the laser-induced CNV model, \(\omega-3\) versus \(\omega-6\) LCPUFA did not change IL-10 expression in WT choroidal-retinal explants. APN deficiency increased gene expression of Mmp2 and Mmp9 but not IL-10 in laser-induced CNV (Supplementary Fig. S4). These observations suggested that activation of the APN pathway might target different downstream pathways to modulate neovascularization in different models, such as laser-induced CNV and Vldlr\(^{-/-}\) mice. APN is one but not the only way mediating \(\omega-3\) protective effects in Vldlr\(^{-/-}\) mice.

### DISCUSSION

Many signaling pathways including lipids are associated with the development of CNV.\(^{38-40}\) Because many clinical studies suggest a strong protective effect of dietary \(\omega-3\) LCPUFA on neovascular AMD, it is particularly important to understand the underlying mechanisms. Laser-induced CNV is a widely used model of neovascularization driven by inflammation.\(^{54,55}\) Vldlr\(^{-/-}\) mice as a model of metabolically driven neovascular AMD have abnormal lipid metabolism and photoreceptor energy deficits that drive RAP (and CNV).\(^{25}\) Our data showed that \(\omega-3\) LCPUFA protects against AMD-like neovascularization in these two animal models through the activation of the APN pathway.
There are indications in other systems that ω-3 LCPUFA and APN are linked. APN is an important metabolic modulator mainly derived from white adipocytes. Dietary intake of ω-3 LCPUFA increases circulating APN levels in premature infants, diabetic patients, and the elderly. ω-3 LCPUFA activates peroxisome proliferator-activated receptor γ, a nu p s t r e a m transcriptional regulator of APN expression. Dietary supplementation of ω-3 LCPUFA reduces white-adipose endoplasmic reticulum stress and increases the production of APN to inhibit hypoxia-induced retinal neovascularization in vivo and white adipocytes in vitro. Interestingly, mutations in APN or ADIPOR1 are associated with severe AMD.

Exploring the role of APN and neovascularization, we examined retinal vascular development. AdipoR1 gradually increased during retinal vascular formation in both Vldlr−/− and WT mice from P3 to P17, demonstrating a key role of the APN pathway during the period of normal retinal neurovascular development. Vldlr−/− had consistently higher expression of AdipoR1 during this period, suggesting that the APN/AdipoR1 pathway might also be involved in the pathologic NV in Vldlr−/− retinas. ω-3 LCPUFA versus ω-6 LCPUFA increased APN receptor mRNA AdipoR1 in WT mice, suggesting that ω-3 LCPUFA could act through APN. In laser-induced CNV, APN deficiency abolished dietary ω-3 LCPUFA-induced inhibition of CNV formation but did not affect CNV in mice fed a ω-6

**FIGURE 5.** Dietary ω-3 LCPUFA decreased retinal neovascular lesions in Vldlr−/− mice. Vldlr−/− mice were fed either ω-3 or ω-6 LCPUFA-enriched diets from postnatal day (P) P1. (A) In Vldlr−/− retinas, ω-3 LCPUFA induced gene expression of APN receptor adipoR1 but not Apn (n = 3 to 4 mice per group). Unpaired t-test. (B) Increased AdipoR1 mRNA levels in Vldlr−/− versus Vldlr−/− retinas from postnatal day 3 to 17. Unpaired t-test. (C) At P16, retinal neovascularization was examined. 3D reconstruction demonstrated blood vessels invading from the OPL toward RPE, representative images of neovascular lesion (200× magnification). (D) Representative images of isolecin-stained vessels (red) in retinal whole mounts. (E) Dietary ω-3 versus ω-6 LCPUFA supplementation decreased the number and total lesion area of neurovascular lesions (n = 7 to 10 mice per group). Unpaired t-test. (F) ω-3 versus ω-6 LCPUFA did not affect retinal Mmp2 and Mmp9 expression in Vldlr−/− mice. Retinal IL-10 was increased in ω-3 LCPUFA-fed Vldlr−/− mice. n = 3 to 4 mice per group. Unpaired t-test. (G) Change in IL-10 in Vldlr−/− mice treated with either 50 mg/kg adipoRon or vehicle from P3 to 15. n = 3 to 4 retinas per group. (H) In the model of laser-induced CNV, there was no change in IL-10 expression in choroidal-retinal complex from WT mice fed ω-3 versus ω-6 LCPUFA-enriched diets. n = 5 mice per group. Unpaired t-test.
LCPUFA-enriched diet, confirming that APN mediated ω-3 and not ω-6 LCPUFA prevention of CNV.

APN receptor activation is known to promote antiangiogenic, and anti-inflammatory functions. However, the neovascular inhibitory effects of APN activation were independent of vascular endothelial growth factor A (VEGFA) in both models (Supplementary Fig. S1). Interestingly, although APN mediated the protective effect of dietary ω-3 LCPUFA on neovascularization in both the laser-induced CNV model and in Vldlr−/− mice, the downstream mechanisms were different. ω-3 LCPUFA inhibited laser-induced CNV through the suppression of MMP2 and MMP9 but inhibited NV in Vldlr−/− mice through the induction of IL-10. In laser-induced CNV mice, APN deficiency reduced AMPKα phosphorylation and completely reversed ω-3 LCPUFA-induced effects on MMP2 and MMP9, proteases which degrade ECM and basement membrane, to promote tumor growth and angiogenesis.52–55 MMP2 and MMP9 are expressed in vitreous56 and retina57,58 in human eyes with neovascular AMD and are genetically associated with AMD.59–60 Plasma MMP9 levels are higher in subjects with CNV versus control groups.61 Inhibition of AMPK activity partially abolished metformin inhibition of Mmp9 and Mmp9 mRNA levels.62 Phosphorylation of AMPKα leads to the reduction in MMP9 levels in mouse embryonic fibroblasts.32 AMPKα phosphorylation is increased with activation of the APN pathway.62,63 Therefore, ω-3 LCPUFA, mediated by APN, inhibits MMPs production. As previously reported, ω-3 LCPUFA is associated with decreased MMP2 and MMP9 activity.64–67

Surprisingly, unlike in laser-induced CNV, MMP2 and MMP9 were not decreased in Vldlr−/− mice on a ω-3 LCPUFA diet. However, the anti-inflammatory factor IL-10 was increased 13-fold in ω-3 LCPUFA versus ω-6 LCPUFA-fed Vldlr−/− mice. A dual agonist of AdipoR1/R2, AdipoRon, increased IL-10 in Vldlr−/− mice, suggesting that the inhibitory regulation of ω-3 LCPUFA on CNV was through APN/AdipoR1 and IL-10. There was no measurable change in IL-10 expression in ω-3 LCPUFA versus ω-6 LCPUFA-fed laser-induced CNV mice, suggesting that ω-3 LCPUFA may exert protective effects through APN but with different downstream mechanisms in the two neovascular AMD models with respect to inflammation and metabolic alterations.

Further work is needed to explore how to manipulate the APN pathway to facilitate ω-3-LCPUFA’s effect in CNV prevention. Our observations suggest that APN could potentially be a biomarker for the effects of dietary ω-3 LCPUFA in AMD progression, and indicate a plausible target to treat AMD and other choroidal/retinal neovascular diseases. The current treatment for CNV, anti-VEGF therapy, does not treat all patients effectively and requires repeated intravitreal injections, associated with complications.68–70 The inhibitory effects of ω-3 LCPUFA in AMD seen in the present study were independent of VEGFA. The potential use of ω-3 LCPUFA and pharmaceutical modifiers of its protective effects on neovascular AMD is of great interest. Increased dietary ω-3 LCPUFA consumption in conjunction with the modulation of the APN pathway may prevent disease progression in neovascular AMD through inflammation and metabolic alterations.

Acknowledgments

Supported by Grants NIH EY024864, EY017017, EY022275, P01 HD18655, BCH IDDRc, 1U5HD090255; Lowry Medical Research Institute, European Commission FP7 project 305485 PREVENTROP (LEHS); The Swedish Research Council (DNR# 2016-01131), and Gothenburg County Council (ALFGBG-507741) long-term support by De Blånds Vänner och Kronprinsessan Margareta Arbetssnämmnd för synskadade, European Commission FP7 project 305485 PREVENTROP (AH); European Commission FP7 Project 305485 PREVENTROP (CL); Knights Templar Eye Foundation and Blind Children’s Center (ZF); Knights Templar Eye Foundation (CHL); Boston Children’s Hospital OED/BRREC/CTREC Faculty Career Development Grant (YS); The German Research Foundation (DFG), and Li2650/1-1 (RL).

Disclosure: Z. Fu, None; R. Liegl, None; Z. Wang, None; Y. Gong, None; C.-H. Liu, None; Y. Sun, None; B. Cakir, None; S.B. Burnim, None; S.S. Meng, None; C. Löfqvist, None; J.P. SanGiovanni, None; A. Hellström, None; L.E.H. Smith, None

References

1. Ding X, Patel M, Chan CC. Molecular pathology of age-related macular degeneration. Progress Retinal Eye Res. 2009;28:1–18.

2. Stahl A, Krohne TU, Sapieha P, et al. Lipid metabolites in the pathogenesis and treatment of neovascular eye disease. British J Ophthalmol. 2011;95:1496–1501.

3. Shaw PX, Stiles T, Douglas C, et al. Oxidative stress, innate immunity, and age-related macular degeneration. AIMS Mol Sci. 2016;3:196–221.

4. Merle BM, Delyfer MN, Korobelnik JE, et al. High concentrations of plasma n3 fatty acids are associated with decreased risk for late age-related macular degeneration. J Nutr. 2013;143:505–511.

5. Tan JS, Wang JJ, Flood V, Mitchell P. Dietary fatty acids and the 10-year incidence of age-related macular degeneration: the Blue Mountains Eye Study. Arch Ophthalmol. 2009;127:656–665.

6. Ho L, van Leeuwen R, Witteman JC, et al. Reducing the genetic risk of age-related macular degeneration with dietary antioxidants, zinc, and omega-3 fatty acids: the Rotterdam study. Arch Ophthalmol. 2011;129:758–766.

7. Seddon JM, George S, Rosner B. Cigarette smoking, fish consumption, omega-3 fatty acid intake, and associations with age-related macular degeneration: the US Twin Study of Age-Related Macular Degeneration. Arch Ophthalmol. 2006;124:995–1001.

8. Sangiovanni JP, Agron E, Meleth AD, et al. ω-3 Long-chain polyunsaturated fatty acid intake and 12-y incidence of neovascular age-related macular degeneration and central geographic atrophy: AREDS report 30, a prospective cohort study from the Age-Related Eye Disease Study. Am J Clin Nutr. 2009;90:1601–1607.

9. Christen WG, Schaumberg DA, Glynn RJ, Buring JE. Dietary omega-3 fatty acid and fish intake and incident age-related macular degeneration in women. Arch Ophthalmol. 2011;129:921–929.

10. Patel CK, Fung TH, Muqit MM, et al. Non-contact ultra-widefield imaging of retinopathy of prematurity using the Optos dual wavelength scanning laser ophthalmoscope. Eye (Lond). 2013;27:589–596.

11. Kabasawa S, Mori K, Horie-Inoue K, et al. Associations of cigarette smoking but not serum fatty acids with age-related macular degeneration and geographic atrophy: AREDS report 30, a prospective cohort study from the Age-Related Eye Disease Study. Arch Ophthalmol. 2011;129:921–929.

12. Souied EH, Aslam T, Garcia-Layana A, et al. Omega-3 fatty acids and age-related macular degeneration. Ophthalmic Res. 2015;55:62–69.

13. Blashal TG, Hibbeln JR, Ramsden CE, Majchrzak SF, Rawlings RR. Changes in consumption of omega-3 and omega-6 fatty acids in the United States during the 20th century. Am J Clin Nutr. 2011;93:950–962.

14. Simopoulos AP. Evolutionary aspects of diet, the omega-6/omega-3 ratio and genetic variation: nutritional implications...
for chronic diseases. *Biomed Pharmacother.* 2006;60:502–507.
15. Gong Y, Li J, Sun Y, et al. Optimization of an image-guided laser-induced choroidal neovascularization model in mice. *PloS One.* 2015;10:e0132643.
16. Gong Y, Fu Z, Edlin ML, et al. Cytochrome P450 oxidase 2C inhibition adds to omega-3 long-chain polyunsaturated fatty acids protection against retinal and choroidal neovascularization. *Arterioscl Thromb Vascular Biol.* 2016;36:1919–1927.
17. Gong Y, Shao Z, Fu Z, et al. Fenofibrate inhibits cytochrome P450 epoxide 2C activity to suppress pathological ocular angiogenesis. *EbiBioMedicine.* 2016;13:201–211.
18. Stahl A, Sapieha P, Connor KM, et al. Short communication: PPAR gamma mediates a direct antiangiogenic effect of omega 3-PUFAs in proliferative retinopathy. *Circul Res.* 2010;107:495–500.
19. Sapieha P, Stahl A, Chen J, et al. 5-Lipoxygenase metabolite 4-HDHA is a mediator of the antiangiogenic effect of omega-3 polyunsaturated fatty acids. *Sci Transl Med.* 2011;3:69ra12.
20. Higuchi A, Ohashi K, Kihara S, Walsh K, Ouchi N. Adiponectin suppresses pathological microvessel formation in retina through modulation of tumor necrosis factor-alpha expression. *Circul Res.* 2009;104:1058–1065.
21. Ohashi K, Ouchi N, Matsuzawa Y. Anti-inflammatory and anti-atherogenic properties of adiponectin. *Biochimie.* 2012;94:2137–2142.
22. Fu Z, Lofqvist CA, Shao Z, et al. Dietary omega-3 polyunsaturated fatty acids decrease retinal neovascularization by adipose-endoplasmic reticulum stress reduction to increase adiponectin. *Am J Clin Nutr.* 2015;101:879–888.
23. Connor KM, Santovigliano JP, Lofqvist C, et al. Increased dietary intake of omega-3-polyunsaturated fatty acids reduces pathological retinal angiogenesis. *Nature Med.* 2007;13:886–873.
24. Lambert V, Lecomte J, Hansen S, et al. Laser-induced choroidal neovascularization model to study age-related macular degeneration in mice. *Nature Protocols.* 2013;8:2197–2211.
25. Joyal JS, Sun Y, Gantner ML, et al. Retinal lipid and glucose metabolism dictates angiogenesis through the lipid sensor Ffar1. *Nature Med.* 2016;22:439–445.
26. Shao Z, Friedlander M, Hurst CG, et al. Choroid sprouting assay: an ex vivo model of microvascular angiogenesis. *PloS One.* 2013;8:e69952.
27. Ma ZG, Dai J, Wei WY, et al. Asiatic acid protects against cardiac hypertrophy through activating AMPKalpha signalling pathway. *Int J Biol Sci.* 2016;12:861–871.
28. Almudena GH, Nuria B, Oscar E, et al. Severely brown fat lipoprotein aggravates atherosclerotic process in male mice. *Endocrinology.* 2016;157:3517–3528.
29. Iwabu M, Yamauchi T, Okada-Iwabu M, et al. Adiponectin and AdipoR1 regulate PGC-1alpha and mitochondria by Ca(2+)
30. Kadotani IT, Yamashita IT. Adiponectin and adiponectin receptors. *Endocrine Rev.* 2005;26:439–451.
31. Esfahanian N, Shahiba Y, Nikbin K, et al. Effect of metformin on the proliferation, migration, and MMP-2 and -9 expression of human umbilical vein endothelial cells. *Molecular Med Rep.* 2012;5:1068–1074.
32. Morizane Y, Thanos A, Takeuchi K, et al. AMP-activated protein kinase suppresses matrix metalloproteinase-9 expression in mouse embryonic fibroblasts. *J Biol Chem.* 2011;286:16030–16038.
33. Okada-Iwabu M, Yamauchi T, Iwabu M, et al. A small-molecule AdipoR agonist for type 2 diabetes and short life in obesity. *Nature.* 2015;503:493–499.
34. Parmeggiani F, Romano MR, Costagliola C, et al. Mechanism of inflammation in age-related macular degeneration. *Mediators Inflamm.* 2012;2012:546786.
35. Barron MJ, Johnson MA, Andrews RM, et al. Mitochondrial abnormalities in ageing macular photoreceptors. *Invest Opthalmol Vis Sci.* 2001;42:3016–3022.
36. Ghaem Maralani H, Tai BC, Wong TY, et al. Metabolic syndrome and risk of age-related macular degeneration. *Retina.* 2015;35:459–466.
37. Yannuzzi LA, Negrao S, lida T, et al. Retinal angiomatous proliferation in age-related macular degeneration. *Retina.* 2001;21:416–444.
38. Wang Z, Cheng R, Lee K, et al. Nanoparticle-mediated expression of a Wnt pathway inhibitor ameliorates ocular neovascularization. *Arteriosclerosis Thromb Vascular Biol.* 2015;35:855–864.
39. Wang Z, Liu CH, Sun Y, et al. Pharmacologic activation of Wnt signaling by lithium normalizes retinal vasculature in a murine model of familial exudative vitreoretinopathy. *Am J Pathol.* 2016;186:2588–2600.
40. Gong Y, Shao Z, Fu Z, et al. Fenofibrate inhibits cytochrome P450 epoxide 2C activity to suppress pathological ocular angiogenesis. *EbiBioMedicine.* 2016;13:201–211.
41. Karbowska J, Kochan Z. Role of adiponectin in the regulation of carbohydrate and lipid metabolism. *J Physiol Pharmacol.* 2006;57(suppl 6):103–113.
42. Nakano Y, Itabashi K, Sakurai M, Aizawa M, Dobashi K, Mizuno K. Accumulation of subcutaneous fat, but not visceral fat, is a predictor of adiponectin levels in preterm infants at term-equivalent age. *Early Hum Develop.* 2014;90:213–217.
43. Ito R, Satoh-Asahara N, Yamakage H, et al. An increase in the EPA/AA ratio is associated with improved arterial stiffness in obese patients with dyslipidemia. *J Atherosclerosis Thromb.* 2014;21:248–260.
44. Zhang J, Wang C, Li L, et al. Dietary inclusion of salmon, herring and pompano as oily fish reduces CVD risk markers in dyslipidaemic middle-aged and elderly Chinese women. *Br J Nutr.* 2012;108:1455–1465.
45. Olza J, Mesa MD, Aguillera CM, et al. Influence of an eicosapentaenoic and docosahexaenoic acid-enriched enteral nutrition formula on plasma fatty acid composition and biomarkers of insulin resistance in the elderly. *Clin Nutr.* 2010;29:31–37.
46. Liu M, Liu F. Transcriptional and post-translational regulation of adiponectin. *Biochim J.* 2009:425:41–52.
47. Cao G, Chen Y, Zhang J, et al. Effects of adiponectin polymorphisms on the risk of advanced age-related macular degeneration. *Biomarkers.* 2015;20:266–270.
48. Kaarniranta K, Paananen T, Nevalainen T, et al. Adiponectin receptor 1 gene (ADIPOR1) variant is associated with advanced age-related macular degeneration in Finnish population. *Neurosci Lett.* 2012;513:233–237.
49. Dorrell MI, Friedlander M. Mechanisms of endothelial cell guidance and vascular patterning in the developing mouse retina. *Progress Retinal Eye Res.* 2006;25:277–295.
50. Higuchi A, Ohashi K, Shibata R, Sono-Romanelli S, Walsh K, Ouchi N. Thiazolidinediones reduce pathological neovascularization in ischemic retina via an adiponectin-dependent mechanism. *Arteriosclerosis Thromb Vascular Biol.* 2010;30:46–53.
51. Fu Z, Gong Y, Lofqvist C, Hellstrom A, Smith LE. Review: adiponectin in retinopathy. *Biochimica Biophys Acta.* 2016;1862:1392–1400.
52. Egeland M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. *Nature Rev Cancer.* 2002;2:161–174.
53. Mira E, Lacalle RA, Buesa JM, et al. Secreted MMP9 promotes angiogenesis more efficiently than constitutive active MMP9 bound to the tumor cell surface. *J Cell Sci*. 2004;117:1847–1857.

54. Kvanta A, Shen WY, Sarman S, Seregard S, Steen B, Rakoczy E. Matrix metalloproteinase (MMP) expression in experimental choroidal neovascularization. *Curr Eye Res*. 2000;21:684–690.

55. Lambert V, Wielockx B, Munaut C, et al. MMP-2 and MMP-9 synergize in promoting choroidal neovascularization. *FASEB J*. 2003;17:2290–2292.

56. De La Paz MA, Itoh Y, Toth CA, Nagase H. Matrix metalloproteinases and their inhibitors in human vitreous. *Invest Ophthalmol Vis Sci*. 1998;39:3256–3260.

57. Steen B, Sejersen S, Berglin L, Seregard S, Kvanta A. Matrix metalloproteinases and metalloproteinase inhibitors in choroidal neovascular membranes. *Invest Ophthalmol Vis Sci*. 1998;39:2194–2200.

58. Zeng J, Jiang D, Liu X, Zhu X, Tang L. Matrix metalloproteinase expression in choroidal neovascular membranes. *Yan Ke Xue Bao*. 2004;20:191–193.

59. Fritsche LG, Igl W, Bailey JN, et al. A large genome-wide association study of age-related macular degeneration. *Genetics Med*. 2005;7:272–277.

60. Fritsche LG, Igl W, Bailey JN, et al. A large genome-wide association study of age-related macular degeneration highlights contributions of rare and common variants. *Nature Genetics*. 2016;48:134–145.

61. Cheung N, Lam DS, Wong TY. Anti-vascular endothelial growth factor treatment for eye diseases. *BMJ*. 2012;344:e2970.