

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

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PURPOSE. Neovascular age-related macular degeneration (AMD) is a major cause of legal blindness in the elderly. Diets with omega3-long-chain-polyunsaturated-fatty-acid (ω 3-LCPUFA) correlate with a decreased risk of AMD. Dietary ω 3-LCPUFA versus ω 6-LCPUFA inhibits mouse ocular neovascularization, but the underlying mechanism needs further exploration. The aim of this study was to investigate if adiponectin (APN) mediated ω 3-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% ω 3 or 2% ω 6-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-AMPK α /AMPK α and qPCR for *Apn*, *Mmps*, and *IL-10* were used to define mechanism.

RESULTS. ω 3-LCPUFA intake suppressed laser-induced CNV in WT mice; suppression was abolished with APN deficiency. ω 3-LCPUFA, mediated by APN, decreased mouse *Mmps* expression. APN deficiency decreased AMPK α phosphorylation in vivo and exacerbated choroid-sprouting ex vivo. APN pathway activation inhibited HRMEC proliferation and decreased *Mmps*. In *Vldlr*^{-/-} mice, ω 3-LCPUFA increased retinal *AdipoR1* and inhibited NV. ω 3-LCPUFA decreased *IL-10* but did not affect *Mmps* in *Vldlr*^{-/-} retinas.

CONCLUSIONS. APN in part mediated ω 3-LCPUFA inhibition of neovascularization in two mouse models of AMD. Modulating the APN pathway in conjunction with a ω 3-LCPUFA-enriched-diet may augment the beneficial effects of ω 3-LCPUFA in AMD patients.

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

Choroidal neovascularization (CNV) and retinal angioma-tous 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 (ω 3 LCPUFA),

docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA) is associated with a decreased risk of AMD.⁴⁻⁷ The Age-Related Eye Disease Study (AREDS) reported 30% less central geographic atrophy and neovascular AMD in participants with a diet containing high ω 3 LCPUFA, DHA, and EPA (0.106% of total energy intake) than those with low ω 3 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 ω 3 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 ω 3 LCPUFA level or were otherwise well-nourished.¹⁰⁻¹² The effects of ω 3 LCPUFA on modulating neovessel formation needs further exploration, particularly for patients on a Western diet with low ω 3 LCPUFA intake.^{13,14} Previous studies show that a ω 3



LPCPUFA-enriched diet reduces laser-induced CNV in mice¹⁵⁻¹⁷; however, the complete underlying mechanisms are still unknown.¹⁶⁻¹⁹

APN plays anti-angiogenic and anti-inflammatory roles in vascular diseases.^{20,21} In the mouse model of oxygen-induced proliferative retinopathy, APN is involved in ω -3 LPCPUFA's inhibition of retinal neovascularization.²² Therefore, the APN pathway may be involved in ω -3 LPCPUFA's protection in neovascular AMD. We hypothesized that the APN signaling pathway mediates ω -3 LPCPUFA 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 (*Vldlr*^{-/-}) with retinal angiomatic 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). C57BL/6J and *Apn*^{-/-} (backcrossed with 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 C57BL/6J and *Apn*^{-/-} mice. Mice were fed defined rodent diets with either 2% ω -3 (1% DHA and 1% EPA) or 2% ω -6 LPCPUFA (AA)²³ (Supplementary Table S1) 7 days before and after laser photocoagulation. ARASCO (42% AA), DHASCO (43% DHA), and MEG-3 (45% EPA and 24% DHA), respectively, were obtained from DSM Nutritional Products (TE Heerlen, Netherlands) and were integrated into the rodent feed at Research Diets (New Brunswick, NJ, USA). The eyes were enucleated and fixed in 4% paraformaldehyde (PFA) for 1 hour at room temperature. The choroid was penetrated with 1% Triton X-100 PBS for 1 hour at room temperature and stained overnight with fluorescent Griffonia Bandeiraea Simplicifolia Isolectin B4 (Alexa Fluor 594, I21413, Molecular Probes, Grand Island, NY, USA; 10 μ g/mL) in 1 mM CaCl₂ in 1% Triton X-100 PBS. The choroid was washed with PBS and whole mounted, and images were taken at 100 \times or 200 \times magnification on a Zeiss AxioObserver.Z1 microscope. Lesion area was quantified and exclusion criteria were followed per previous publications.^{15,24}

Very Low Density Lipoprotein Receptor Deficient (*Vldlr*^{-/-}) Mice

Vldlr^{-/-} mice develop pathological RAP similar to AMD.²⁵ Neovessels extend from the deep retinal vascular layer of the outer plexiform layer (OPL) toward the retinal pigment epithelium (RPE). Mice were fed defined rodent diets with either 2% ω -3 (DHA and EPA) or 2% ω -6 LPCPUFA (AA)²³ from postnatal day (P) 1. At P16, the eyes were enucleated and fixed in 4% PFA for 1 hour at room temperature. The retinas were dissected and stained overnight with fluorescent Griffonia Bandeiraea Simplicifolia Isolectin B4 (Alexa Fluor 594, I21413, Molecular Probes, 10 μ g/mL) in 1 mM CaCl₂ in PBS, then whole mounted with photoreceptors facing up. Images were taken at 50 \times magnification on a Zeiss AxioObserver.Z1

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.²⁵

Choroid Assay Ex Vivo

Three-week-old C57BL/6J and *Apn*^{-/-} mice were killed. RPE/choroid/sclera complex ("choroid explants") from the peripheral area was dissected and cut into approximately 1 \times 1 mm pieces.²⁶ The choroid explants were immediately embedded in 30 μ 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% CO₂. At D6, images were taken under 25 \times 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, #15240) on 0.1% gelatin-coated 96-well cell culture plate. HRMECs were treated with APN receptor agonist adipoRon (2.5, 5.0, 10, and 25 μ M) and vehicle (0.1% DMSO) for 24 hours. Ten microliters MTT reagent (ATCC, #30-1010K) was added to 100 μ L EGM2 in each well. After 6-hour incubation at 37°C, cells were washed with PBS and 100 μ 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 adipoRon (25 μ 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 (#12183018A; 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 *Apn* (F: 5'-GAA GCC GCT TAT GTG TAT CGC-3', R: 5'-GAA TGG GTA CAT TGG GAA CAG T-3'); *AdipoR1* (F: 5'-TCT TCG GGA TGT TCT TCC TGG-3', R: 5'-TTT GGA AAA AGT CCG AGA GAC C-3'); *Vegfa* (F: 5'-GGA GAT CCT TCG AGG AGC ACT T-3', R: 5'-GCG ATT TAG CAG CAG ATA TAA GAA-3'); *Mmp2* (F: 5'-TGG GAG CAT GGA GAT GGA TAC-3', R: 5'-AAG TGA GAA TCT CCC CCA ACA C-3'); *Mmp9* (F: 5'-ACT GCG GGC TCT TCT GAG G-3', R: 5'-CCC TGG ATC TCA GCA ATA GCA-3'); *bMmp2* (F: 5'-TAC AGG ATC ATT GGC TAC ACA CC-3', R: 5'-GGT CAC ATC GCT CCA GAC T-3'); *bMmp9* (F: 5'-TGT ACC GCT ATG GTT ACA CTC G-3', R: 5'-GCA GGG ACA GTT GCT TCT-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 *Cyclophilin A* (F: 5'-CAG ACG CCA CTG TCG CTT T-3'; R: 5'-TGT CTT TGG AAC TTT GTC TGC AA-3') for mouse retinas and 18S ribosomal RNA (F: 5'-ACGGAAGGGCACCACCAGGA-3', R: 5'-CACCACCACCCACGGAATCG-3') for HRMECs using

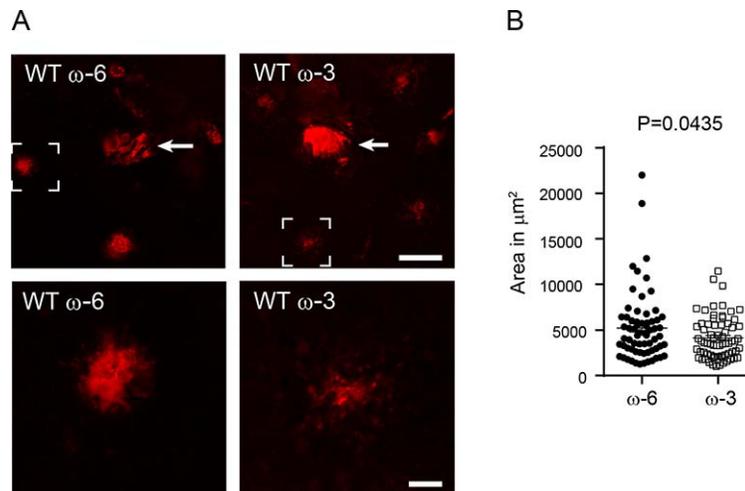


FIGURE 1. Dietary ω -3 LCPUFA versus ω -6 LCPUFA suppressed laser-induced CNV. Eight-week-old C57BL/6J (WT) mice were fed ω -3 or ω -6 LCPUFA-enriched diets starting 1 week before and continuing until 1 week after laser-induced CNV (day 0). At day 7, choroidal neovessels were examined. (A) Representative neovessel lectin-stained (red) choroidal whole-mounts. The brackets in the top panel are shown at the bottom. (B) Significantly reduced lesion areas were found in ω -3 versus ω -6 LCPUFA-fed retinas. $n = 12$ to 14 mice per group. Unpaired t -test. Arrow (top): optic nerve head. Scale bar: 200 μ m (top); 50 μ m (bottom).

the $\Delta\Delta$ Ct 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, P0044, Sigma-Aldrich Corp., St. Louis, MO, USA) and protease inhibitor (1:1000, Sigma, P8340). Forty micrograms protein lysate were used to detect the levels of phosphor-AMPK α^{27} (p-AMPK α , 1:500, #2535; Cell Signaling, Beverly, MA, USA) and AMPK α^{28} (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 \pm 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 $P \leq 0.05$.

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 ($P = 0.0033$, 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, $P = 0.0431$; 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 AMPK α ,^{29,30} which transcriptionally regulates the expression of MMPs.^{31,32} A decreased choroidal-retinal p-AMPK α /AMPK α ratio ($Apn^{-/-}$ to WT = 0.4) was found in $Apn^{-/-}$ versus WT CNV mice fed with ω -3 LCPUFA (Fig. 3C). Our findings

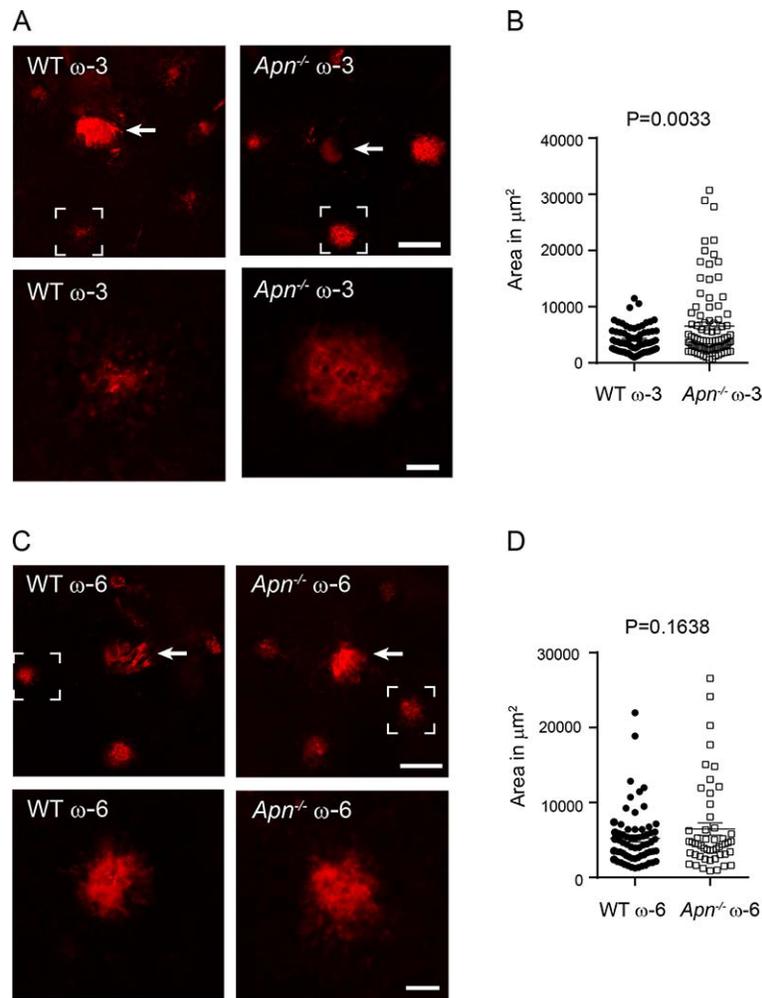


FIGURE 2. APN deficiency abolished dietary ω -3 LCPUFA protection against laser-induced CNV. Eight-week-old *Apn*^{-/-} and WT mice were fed ω -3 or ω -6 LCPUFA-enriched diets from 1 week before until 1 week after laser-induced CNV (day 0). At day 7, choroidal neovessels were examined. Representative lectin-stained (red) vessels in choroidal whole-mounts (A, C) and quantification of choroidal lesion area (B, D). The brackets in the top panel are shown at the bottom (A, C). Significantly larger lesions were observed in ω -3 LCPUFA-fed *Apn*^{-/-} versus WT mice but not in ω -6 LCPUFA-fed *Apn*^{-/-} versus WT mice (B, D). $n = 9$ to 14 mice per group (ω -3). $n = 8$ to 12 mice per group (ω -6). Unpaired *t*-test. Arrow (top): optic nerve head. Scale bar: 200 μm (top, panel A, C); 50 μm (bottom, panel A, C).

suggested that dietary ω -3 LCPUFA supplementation activated the APN pathway to phosphorylate AMPK α 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 vivo²⁶ 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)³³ 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

AMPK α 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,³⁴ metabolic alterations may also lead to disease progression of AMD.^{35,36} In *Vldlr*^{-/-} mice, the absence of VLDLR is associated with RAP,²⁵ similar to that seen with neovascular AMD.³⁷ 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.²³ 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).

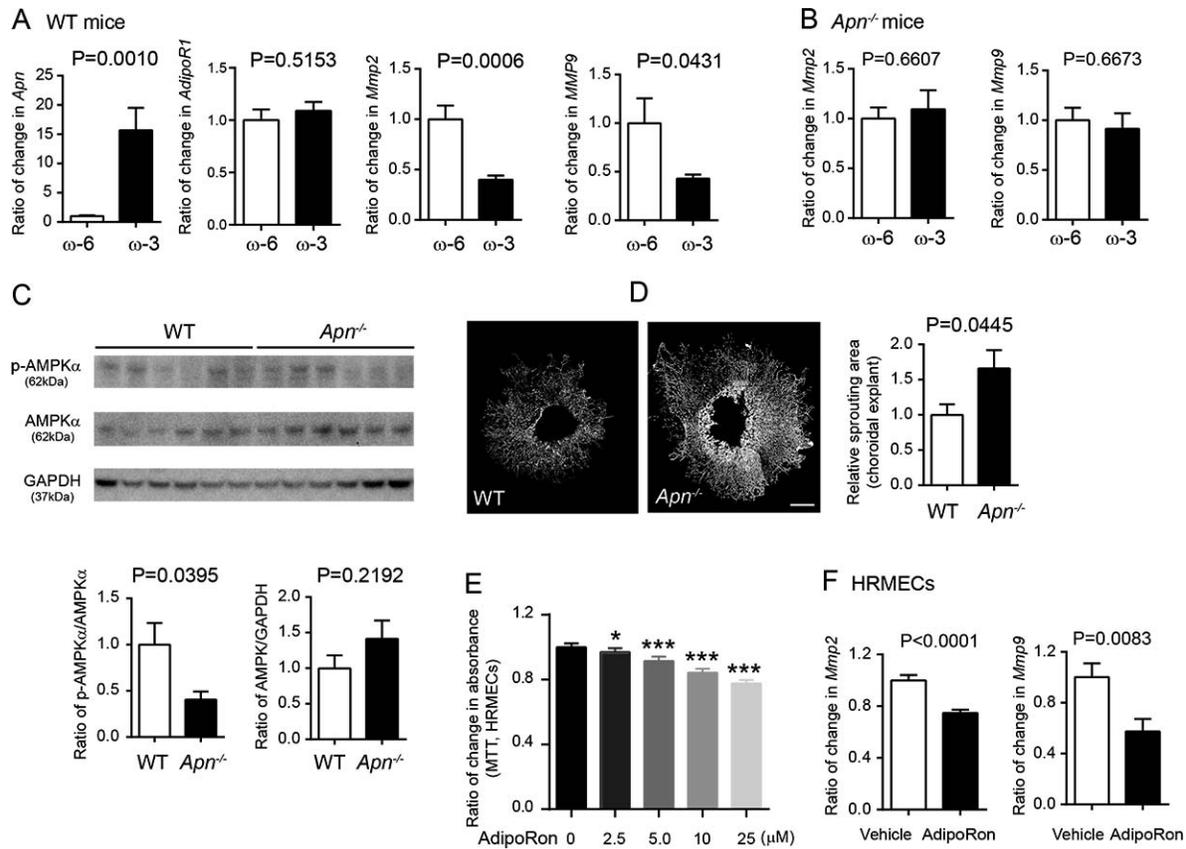


FIGURE 3. The APN pathway inhibited neovessel formation through the suppression of MMP expression in laser-induced CNV. (A) WT mice fed ω -3 versus ω -6 LCPUFA-enriched diets had increased *Apn*, and decreased *Mmp2* and *Mmp9*, as well as unchanged *AdipoR1* expression in choroidal-retinal explants. $n = 3$ mice per group. Unpaired *t*-test. (B) APN deficiency abolished ω -3 LCPUFA inhibition of choroidal-retinal *Mmp2* and *Mmp9* levels. $n = 3$ to 4 mice per group. Unpaired *t*-test. (C) APN deficiency decreased the levels of phosphorylated AMPK α in choroidal-retinal CNV explants. $n = 6$ mice per group. Unpaired *t*-test. (D) In the choroidal sprouting assay ex vivo, choroidal explants from *Apn*^{-/-} mice showed larger sprouting areas than WT mice. $n = 4$ to 6 explants per group. Unpaired *t*-test. Scale bar: 500 μ m. (E) In HRMEC MTT assay in vitro, activation of the APN pathway with APN receptor agonist adipoRon inhibited endothelial cell proliferation. $n = 10$ replicates per group. Unpaired *t*-test. (F) AdipoRon treatment (25 μ M) decreased *Mmp2* and *Mmp9* expression in HRMECs. Results were repeated in two independent experiments. Unpaired *t*-test.

Interestingly, ω -3 versus ω -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 ω -3 versus ω -6 LCPUFA-fed *Vldlr*^{-/-} mice (ω -3 to ω -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, ω -3 versus ω -6 LCPUFA did not change *IL-10* expression in WT choroid-

retina complex (ω -3 to ω -6 = 0.67; $P = 0.1554$; Fig. 5H). In mice fed with animal chow provided by animal facility with undefined composition, 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 ω -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.^{34,35} *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 ω -3 LCPUFA protects against AMD-like neovascularization in these two animal models through the activation of the APN pathway.

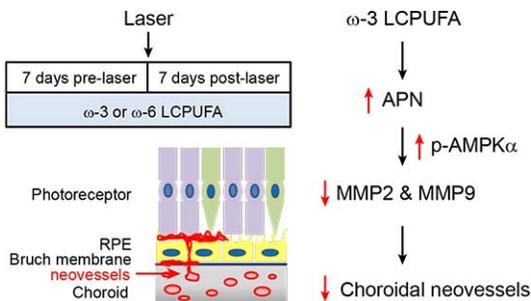


FIGURE 4. Schematic of the APN pathway in mediating ω -3 LCPUFA inhibition on laser-induced CNV.

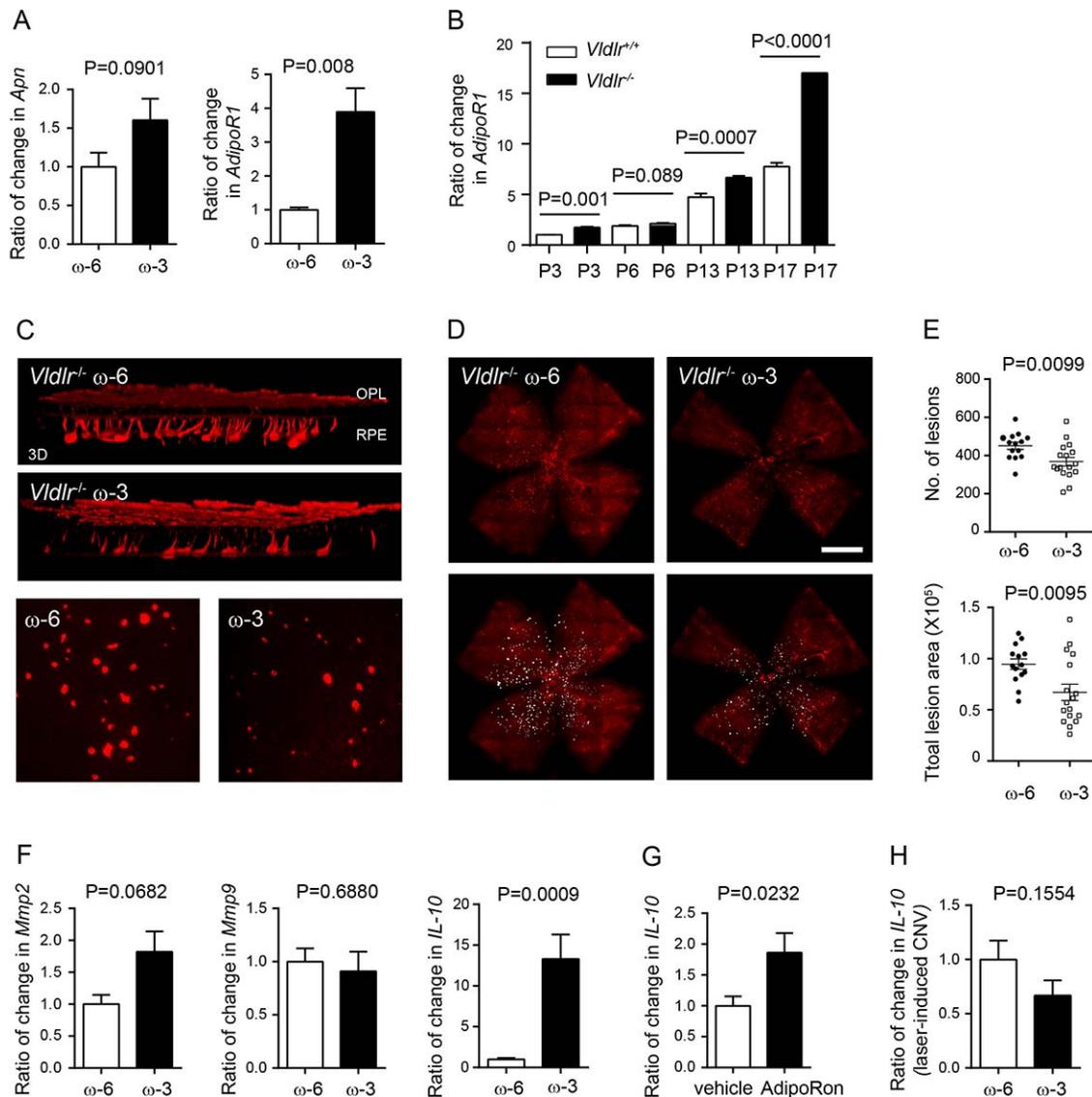


FIGURE 5. Dietary ω -3 LCPUFA decreased retinal neovascular lesions in *Vldlr*^{-/-} mice. *Vldlr*^{-/-} mice were fed either ω -3 or ω -6 LCPUFA-enriched diets from postnatal day (P) P1. (A) In *Vldlr*^{-/-} retinas, ω -3 LCPUFA induced gene expression of APN receptor *adipoR1* but not *Apn* ($n = 3$ 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 \times magnification). (D) Representative images of isoelectin-stained vessels (red) in retinal whole mounts. (E) Dietary ω -3 versus ω -6 LCPUFA supplementation decreased the number and total lesion area of neovascular 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. $n = 3$ mice per group. Unpaired *t*-test.

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.⁴¹ Dietary intake of ω -3 LCPUFA increases circulating APN levels in premature infants, diabetic patients, and the elderly.⁴²⁻⁴⁵ ω -3 LCPUFA activates peroxisome proliferator-activated receptor γ , an upstream transcriptional regulator of APN expression.^{18,46} 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.²² Interestingly, mutations in APN or ADIPOR1 are associated with severe AMD.^{47,48}

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.⁴⁹ *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

LCPUFA-enriched diet, confirming that APN mediated ω -3 and not ω -6 LCPUFA prevention of CNV.

APN receptor activation is known to promote anti-angiogenic, and anti-inflammatory functions.^{20,21,50,51} 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 AMPK α 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 retina^{57,58} 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 *Mmp2* and *Mmp9* mRNA levels.³¹ Phosphorylation of AMPK α leads to the reduction in MMP9 levels in mouse embryonic fibroblasts.³² AMPK α 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.

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