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 (ω-3 LCPUFA) correlate with a decreased risk of AMD. Dietary ω-3 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. Vildir−/− 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, Mmp9, 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 Mmp9 expression. APN deficiency decreased AMPKα phosphorylation in vivo and exacerbated choroid-sprouting ex vivo. APN pathway activation inhibited HRMEC proliferation and decreased Mmp9. In Vildir−/− mice, ω3-LCPUFA increased retinal AdipoR1 and inhibited NV. ω3-LCPUFA decreased Il-10 but did not affect Mmp9 in Vildir−/− 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
The laser-induced CNV model and very low density lipoprotein retinal NV. We explored this question in two animal models: neovascular AMD. We hypothesized that the APN signaling APN Mediates x 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 Aplyr–/– mice (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 Aplyr–/– mice. Mice were fed defined rodent diets with either 2% ω-3 (1% DHA and 1% EPA) or 2% ω-6 LCPUFA (AA)13 (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 2% or 3% LCPUFA-enriched diet reduces laser-induced CNV in mice13–17; 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 LCPUFA’s inhibition of retinal neovascularization.22 Therefore, the APN pathway may be involved in ω-3 LCPUFA’s protection in neovascular AMD. We hypothesized that the APN signaling pathway mediates ω-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 (Vldlr–/–) with retinal angiomaticus proliferation (RAP) and CNV.

**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 LCPUFA (AA)25 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 simplicifolia Isolectin B4 (Alexa Fluor 594, 121413, Molecular Probes, Grand Island, NY, USA; 10 μg/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× or 200× magnification on a Zeiss AxioObserver.Z1 microscope. Lesion area was quantified and images were merged to form one image with AxiosVision 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 Aplyr–/– mice were killed. RPE/choroid/sclera complex (“choroid explants”) from the peripheral area was dissected and cut into approximately 1 × 1 mm pieces.26 The choroid explants were immediately embedded in 30 μL 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 CSM 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× 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 adiporRon (2.5, 5.0, 10, and 25 μM) and vehicle (0.1% DMSO) for 24 hours. Ten microliters MTT reagent (ATCC, #30-1010K) was added to 100 μL EGM2 in each well. After 6-hour incubation at 37°C, cells were washed with PBS and 100 μL DMSO was added per well. The absorbance was recorded at 570 nm.

**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 Apm (F: 5’-GAA GCC GCT TAT GTG TAT CGC-3’, R: 5’-GAA TGG GTA CAT TGG GAA CAG TAC-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’-GGGA GAT CCT TCG AGG AGC ACT ACT T-3’, R: 5’-GGG ATT CAC AGG CAT AAG AAG-3’); Mmp9 (F: 5’-TGG GAG CAT GGA GAT GAT GAG TAC-3’, R: 5’-AAG TGA GAA TCT CCC CCA ACA CAC C-3’); Mmp7 (F: 5’-ACT AGG AGC ATT GGC GGC TCT TGG C-3’, R: 5’-GCT AAC ACT GCC CCA GAC C-3’); Mmp2 (F: 5’-ATC GCT ATG TCA TGA GGA ACA ACT-3’, R: 5’-GGA TGG GTA CAT TGG GAA CAG TAC-3’); Mmp1 (F: 5’-ATG ACC TGG GTA CAT TGG GGA ACA ACT-3’, R: 5’-GGA TGG TAT TGT TGC TGC TGC-3’); and 18S ribosomal RNA (F: 5’-AGGAAAGGCGACCCACACGA-3’, R: 5’-CACCCACCACCCACGGAATCG-3’) for HRMECs using...
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 (1:1000, Sigma, P8340) and protease inhibitor (1:100, P0044, Sigma-Aldrich Corp., St. Louis, MO, USA) 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 LCPUFAs 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 LCPUFAsupplemented diets, as shown in our previous reports.15–17

Figure 1. Dietary ω-3 LCPUFAs versus ω-6 LCPUFAs 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 LCPUFAs-fed retinas. n = 12 to 14 mice per group. Unpaired t-test. Arrow (top): optic nerve head. Scale bar: 200 μm (top); 50 μm (bottom).

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 = 0.45, 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 AMPKα 29,30 which transcriptionally regulates the expression of MMPs.31,32 A decreased choroidal-retinal p-AMPKα/AMPKα 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 phosphorylate AMPKα and reduce MMP2 and MMP9 levels, which in turn inhibited neovessel formation.

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 = 0.0009) and total lesion area by 29% (P = 0.0095) (Figs. 5C–E).
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 choroidal-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.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 ω-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 LCPUFAs 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. 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 diet.

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 (200x magnification). (D) Representative images of isoclectin-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 = 3 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.
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-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 neovascular AMD and are genetically associated with Vldlr (VEGFA) in both models (Supplementary Fig. S1). Interestingly, these were independent of vascular endothelial growth factor A (VEGFA) in both models (Supplementary Fig. S1).

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.60–70 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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References

14. Simopoulos AP. Evolutionary aspects of diet, the omega-6/omega-3 ratio and genetic variation: nutritional implications
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