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The high-dose fish oil supplementation increased Mfsd2a expression without altering DHA levels in the retina of healthy mice

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ABSTRACT

The recommended fish oil (FO) supplementation doses often yield low omega-3 polyunsaturated fatty acids (PUFAs) tissue bioavailability, and higher doses (up to 10 g per day) have been increasingly recommended. However, the exact effects of such FO supplementation on the healthy retina and retinal pigmented epithelium (RPE) are unknown. Our study showed that the high dose FO treatment did not imbalance the rigorous docosahexaenoic acid (DHA, C22:6n3) homeostasis in the retina and RPE in the three-month-old female B6/SLJ mice. Instead, we have found the significant increase in the expression of Mfsd2a, the main DHA transporter. Mfsd2a is also an essential regulator of blood vessel transcytosis and the decrease in Mfsd2a expression can be a risk factor for developing leaky blood vessels. Therefore, the high-dose FO supplementation emerges as the prophylactic fortifier of the retinal blood vessels.

1. Introduction

The omega-3 and omega-6 long-chain polyunsaturated fatty acids (LC-PUFAs) are necessary for the proper development and function of the retina (SanGiovanni & Chew, 2005; Sinclair, 2019), and their unbalanced ratio plays an important role in the development of many chronic and inflammatory conditions. Docosahexaenoic acid DHA (C22:6n3), an omega-3 fatty acid, is one of the main structural lipids of the neuronal and vascular retina, whose homeostasis is crucial for the normal functioning of photoreceptors (PRs) (Bennett & Mitchell, 2008, Sinclair 2019). DHA is a bioactive molecule that can affect the photoreceptor membrane by altering its permeability, fluidity, thickness, and lipid phase properties. In addition, omega-3 LCPUFAs influence retinal cell gene expression, cellular differentiation, and cellular survival (SanGiovanni & Chew, 2005). However, the capacity of the retina to synthesize it's own DHA de novo is limited, and the maintenance of retinal DHA content relies on the uptake from extraretinal sources, such as blood-borne lipids (Scott & Bazan, 1989). Thus, the intake of LC-PUFAs through the intake of fish and other marine foods and through the supplementation, remains the primary source of these lipids. In addition, the dietary intake of alpha-linolenic acid (ALA) emerges as an alternative source of LC-PUFAs and is particularly important for the maintenance of the DHA content in the retina (Sinclair, Guo, & Abedin, L., 2022).

Fish oil (FO) is a rich source of n-3 LC-PUFAs and is widely used for omega-3 supplementation (Hooperton, Trepanier, James, Chouinard-Watkins & Bazinet, 2018). However, FO supplementation has, in most studies, given inconclusive results, and not all treated subjects are responsive to the treatment (Zhang et al., 2021). For example, in human preterm infants, FO administration was shown to reduce the frequency of ROP needing laser therapy (Beken et al., 2014). In contrast, some studies found no protective effects of FO on ROP development (Najm et al., 2017).

Recent findings emphasize that the low dose supplementation results in low DHA bioavailability in the brain, considerably reducing the beneficial effects of the FO (Arellanes et al., 2020). The usual

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recommended dose is of omega-3 supplementation is 450–500 mg of omega-3 fatty acids per day (Vannice & Rasmussen, 2014; Dietary Guidelines for Americans 2015–2020; Scientific Opinion on the Tolerable Upper Intake Level of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA) https://www.efsa.europa.eu/en/efsajournal/pub/2815). The daily prophylactic intake for non-demented humans recommended by the fish oil manufacturer used in the present study is 360–540 mg of EPA and 240–360 mg of DHA. However, supplementation with much higher doses (even up to 10 g per day) has been increasingly recommended (Arellanes et al., 2020). Moreover, high doses of n-3 fatty acids were recently suggested for clinical trials in patients suffering mild traumatic brain injury (mTBI) (Patch et al., 2021) in the early therapy period for major depressive disorder (MDD, Luo et al., 2020), and as anti-amyloidogenic in Alzheimer's disease (AD) animal models (Milanovic et al., 2018; Jovic et al., 2019). DHA and FO supplementation is also considered an important potential therapeutic for abnormal retinal neovascularization (Connor et al., 2007; Sapieha et al., 2012) and the retinopathy of prematurity (ROP, Hellstrom et al., 2021). Furthermore, the daily consummation of higher doses of LC-PUFAs for preventive purposes has been strongly recommended. In addition, the EFSA Panel on Dietetic Products, Nutrition, and Allergies (2012) concluded that it does not appear that high-dose EPA and DHA combined supplementation (up to 5 g/day for up to 16 weeks) can increase the risk of spontaneous bleeding episodes or bleeding complications or affect glucose homeostasis, immune function, or lipid peroxidation and is, therefore, considered safe for use.

However, it is not known whether the high dose FO supplementation is affecting the retinal or RPE lipid content or how it is affecting the expression of the main DHA transporter – major facilitator superfamily domain-containing protein2a (Mfsd2a). This is especially important considering that the steady DHA content in the membranes of the photoreceptors is crucial for the proper function of rhodopsin. The increase in DHA levels past an optimal amount (around 19 %) can change the fluidity of the membrane (Querques, Forte, & Souied, 2011) and the activity and regeneration of rhodopsin, thus affecting the phototransduction (Litman, Niu, Polozova & Mitchell, 2001; Querques, Forte, & Souied, 2011). In addition, omega-3 fatty acids have well-documented cholesterol-lowering properties (Micallef & Garg, 2008; Bahety et al., 2017). Cholesterol is another major retinal lipid, and the significant lowering of cholesterol solubility can also elicit changes in the activity of rhodopsin, enhancing light activation (Albert, Alezander, & Boesze-Battaglia, 2016).

Although the uptake of DHA by the retina involves lipoprotein receptors (SanGiovanni & Chew, 2005), adiponectin receptors (Bazan, 2018), and multiple fatty acid transporters (Tachikawa et al., 2018), the predominant pathway for DHA uptake by retina may be through the Mfsd2a pathway (Nguyen et al., 2014; Ben-Zvi et al., 2014; Wong et al., 2016). Importantly, Mfsd2a has been introduced as one of the major regulators of the blood-retinal barrier (BRB) permeability (Zlokovic, 2008). Dysfunction of BRB is implicated in several debilitating, blinding retinal diseases such as diabetic retinopathy (DR), retinopathy of prematurity (ROP), retinal vein occlusion, and retinitis pigmentosa (Choi et al., 2007; Chen et al., 2011; Luo et al., 2011; Strong, Liew & Michaelides, 2017). These eye diseases are the leading causes of vision impairment affecting adults and children worldwide. The importance of discovering novel therapies that restore function to compromised BRB cannot be overemphasized. Mfsd2a-related lipid transport was recently shown to be essential for the suppression of transcytosis (vesicular trafficking between the luminal and abluminal endothelial membrane), thus limiting transcellular passage (Andreone et al., 2017). The decreased Mfsd2a expression is associated with vascular permeability impairments without affecting endothelial junctions resulting in increased vesicle trafficking (increased transcytosis) and leaky barriers (Ben-Zvi et al., 2014; Yang et al., 2017). In contrast, the increased Mfsd2a overexpression was shown to decrease transcytosis, having anti-angiogenic properties in human intestinal microvascular endothelial cells (Ungaro et al., 2017). In addition, the increased Mfsd2a expression and decreased transcytosis were shown to alleviate vascular dysfunction in diabetic retinopathy (Zhang et al., 2021). Interestingly, it was shown that DHA supplementation has much stronger neuroprotective effects if the Mfsd2a expression in the retina is increased (Zhang et al., 2021). Considering that in a sizeable portion of patients, the treatment effectiveness of n-3 LC-PUFAs in the case of retinal neovascularization is low and DHA therapy is ineffective (Zhang et al., 2021), the manipulations of the Mfsd2a expression levels became an exciting target of investigations. However, how the high dose omega-3 supplementation affects the expression levels of Mfsd2a is unknown.

It is conceivable that doses higher than generally recommended may also be needed for prophylactic omega-3 supplementation and the maintenance of retinal health and good vision. The high dose omega-3 supplementation for the healthy subject is already greatly supported, particularly in the case of numerous American University sports programs, including the US National Collegiate Athletic Association (NCAA), that is recommending 9000 mg/day on n-3 PUFAs (Arellanes et al., 2020). The exact effects of high-dose FO supplementation on the specific molecular changes in the retina and RPE are unknown. In the present study, we used three months old female B6/SJL mice to assess the effects of short-term (3 weeks long) high-dose FO supplementation on the fatty acid content, Mfsd2a expression, and cholesterol metabolism-related gene expression.

2. Material and methods

2.1. Animals

Female B6/SJL mice were used in this study. All animal procedures complied with the Directive (2010/63/EU) on the protection of experimental animals or animals used for other scientific purposes. The Ethical Committee for the Use of Laboratory Animals approved these procedures (resolution No. 01–06/13, Institute for Biological Research, University of Belgrade). Animal procedures used in this paper complied with the EEC Directive (86/609/EEC) on animal protection, including the efforts to minimize animal suffering. The animals were housed under standard conditions (12 h light/dark cycle, 23 ± 2 °C, relative humidity 60–70 %), and their health status was routinely checked. The pelleted commercial rodent chow (Milanovic et a., 2019) was available ad libitum (AL).

2.2. Treatment

For the FO treatment, we used three months old female B6/SJL mice fed on commercial rodent chow (Table 1). Animals were divided into

Table 1
Primer sequences for expression studies.

Gene orientation sequence		
HMGCR	F	TTG GTC CTT GTT CAC GCT CAT
	R	TTC GCC AGA CCC AAG GAA AC
SREBP1-C	F	ACG GAG CCA TGG ATT GCA
	R	AAG TCA CTG TCT TGG TTG TTGATGA
LXRBETA	F	AGC GTC CAT TCA GAG CAA GTG
	R	CAC TCG TGG ACA TCC CAG ATC T
ABCA	F	AGG CCG CAC CAT TAT TTT GTC
	R	GGC AAT TCT GTC CCC AAG GAT
APOE	F	GGC CCA GGA GGA GAA TCA ATGA G
	R	CCT GGC TGG ATA TGG ATG TTG
MFSD2A	F	AGA AGC AGC AAC TGT CCA TTT.
	R	CTC GGC CCA CAA AAA GGA TAA T
HPRT	F	CTC ATG GAC TGA TTA TGG ACA GGA C
	R	GCA GGT CAG CAA AGA ACT TAT AGC C

F-forward primer,
R – reverse primer.

two groups – the treated group (n = 14), which was supplemented with the commercial fish oil (DietPharm, FidaFarm Croatia) as a rich resource of omega-3 fatty acids (the fatty acid content is described in Milanovic et al., 2019), and the control group (n = 14), which received the same volume of the water as vehicle. The 100 μl of fish oil was administered to the treated group daily via oral gavage for 21 days. The dose in the current study was selected as a high-dose DHA or EPA treatment. The daily dose of 3000 mg of DHA for human use amounts to 50 mg/kg of body weight. The animal equivalent dose (AED) is calculated considering FDA guidelines for dose conversion between species. Briefly, AED (mg/kg) is calculated as human dose (mg/kg) multiplied by the correction factor for mice (Km): AED (mg/kg) = 50 mg/kg × 12.3 = 615 mg/kg. So, for the human dose of 50 mg/kg, the AED dose amounts to 615 mg/kg. Thus, by supplementation with 12 mg DHA and 18 mg EPA (amount of DHA and EPA per 100 μl), animals were treated with 545.5 mg/kg DHA and 818.2 mg/kg of EPA daily.

The same amount (100 μl) of water was given to the control group daily via oral gavage during the treatment period. Considering that dietary fish oil contains other fatty acids (in addition to omega-3 and omega-6 fatty acids), and iodine, furan fatty acids, as well as antioxidant vitamin E that potentially can have a particular biological effect per se (Hooperton, Trepanier, James, Chouinard-Watkins & Bazinet, 2018) we avoided using other oils as a control. Control oils may contain some active innate or added components that may hinder conclusions of the FO's direct effects. Thus, we avoided comparing the effects of two oils (oil of interest and control oil). One capsule (1 ml of FO) was used for 3–4 animals to avoid fatty acid degradation, and 100 μl per animal was administered using oral gavage.

2.3. Tissue collection

The animals were four months old at the moment of sacrifice. Mice were anesthetized (100 mg/kg, Ketamidor, Richter Pharma, Wells, Austria, 16 mg/kg Xulased, Bioveta, a.s. intraperitoneally), and each animal was perfused with 50 ml 0.1 M phosphate buffer (PBS) for 30 min and then decapitated. The eyes were enucleated, the optic nerve severed, and the cornea, lens, and vitreal body were removed. The retina was peeled off and removed for further analysis. The eyecup, including RPE, choroid, and sclera (RPE in further text), was separated and used for further analysis. All the tissue samples were stored at −80 °C prior to the RNA, protein, and fatty acid isolation. Retina and RPE were isolated from 7 eyes and processed separately for RNA isolation and qPCR. From another 7 eyes, retinas and RPE were isolated and processed separately for fatty acid isolation. The same animals were used for blood collection for the biochemical analyses. Western blot was performed on 4–6 retina and RPE samples. The eyes from control and FO-treated animals (n = 4) were enucleated and processed for immunohistochemistry.

2.4. Retina and RPE fatty acid methyl ester (FAME) preparation

Total lipids were extracted from the retina and RPE with chloroform/methanol, modified by (Kates, 1972). As previously reported, extracted lipids were converted into fatty acid methyl-esters with 3MHCl in methanol (Ichihara & Fukubayashi, 2010). Lipids were transferred into a glass cuvette, and 1.5 ml of 3 M HCl were added, mixed, heated in the water bath at 85 °C for 45 min, and cooled Hexane (Sigma Aldrich) was added for FAME extraction. Following the centrifugation for 15 min at 4000 rpm, the hexane (the upper layer) containing the fatty acid methyl-esters was transferred into vials using Pasteur pipettes and immediately analyzed.

2.4.1. Gas chromatographic condition

Fatty acid methyl esters (FAME) were analyzed using gas chromatography Agilent technologies AGILENT 6890/7890 GC ChemStationOpeartion with FID detector. The FAMEs were separated on a CP-Sil88 capillary column (a 100 m fused silica capillary column of 0.25 mm internal diameter, coated with 0.2 μm cyano-propyl-polysiloxane as a stationary phase), provided by SUPELCO (Bellefonte, PA, USA). Chromatographic conditions were: 1 μl injections of the FAME mixture were made at a split ratio of 20:1; the conditions for the split inlet were: the injector temperature of 250 °C with the injector split flow of 20 ml/min, the pressure of 31,623 psi and the total flow of 24 ml/min; the oven temperature program started at 80 °C, and increased by 4 °C/min up to 220 °C (hold time 5 min), then by 4 °C/min up to 240 °C, and then held at 240℃ for 10 min: the carrier gas was helium (the constant flow of 1.0 ml/min) and makeup gas-nitrogen, with the flow of 25 ml/min.; FID detector temperature was 270℃. The run time was 55 min. ChemStations were used for collecting and elaboration of results (identification and quantification of peaks). Chromatographic peaks were identified by comparing their retention times with appropriate FAMEs (Supelco FAME Mix, Bellefonte, PA). The quantification was based on the ratio between all peak areas and the corresponding peak. The results were expressed as % of individual fatty acids of total fatty acids. The efficiency of the column was expressed as the number of theoretical plateaus of 3 standard fatty acids, palmitic, stearic, and oleic, was in the range of 362870–510262, while the reproducibility of the response was determined as a percentage of the relative standard deviation (RSD%) of the successive measurements of the same reference solution and was in the range of 2,3–4,6 for the same standard fatty acids.

2.5. Real time quantitative polymerase chain reaction (qRT-PCR)

2.5.1. RNA isolation, and reverse transcription

Total RNA was extracted from the eyes isolated from control and FO-treated animals (N = 5–7 per group) according to the manufacturer's instructions for the TRIZOL isolation system (Invitrogen Life Technologies, USA). The RNA pellet was dissolved in 20 μl of DEPC water, RNA concentration was determined by spectrophotometry, and RNA integrity was verified by 1 % agarose gel electrophoresis. Six μg of total RNA was treated with RNase-free DNase I (Thermo Fisher Scientific, Waltham, MA, USA) and reverse transcribed in the same tube with a High-Capacity cDNA Archive Kit (Applied Biosystems, USA), following the manufacturer's protocol. The cDNA was stored at −20 °C until further use.

2.5.2. Quantitative real-time RT-PCR (qRT-PCR)

20 ng of the resulting cDNA was used for PCR analysis in a final volume of 10 μl using RT^{2}SYBR Green qPCR Mastermix (Applied Biosystem). RT-PCR amplifications were performed in an ABI 7500 thermal cycler (Applied Biosystems) in the default cycling mode (50 °C for 30 min, 95 °C for 15 min, followed by 40 cycles of 94 °C for 60 s, 57 °C for 60 s, 72 °C for 60 s, and then incubation ay 70 °C for 10 min). The results obtained by qRT-PCR were analyzed in RQ Study add-on software for the 7000 v 1.1 SDS instrument, with a confidence level of 95 % ($P < 0.05$). Quantification was performed by the 2-DDCt method (Livak & Schmittgen, 2001) and the change of mRNA levels was expressed relative to the control value. Sequences of the used primers (by Vivogen, Serbia) are given in Table 1.

2.6. Western blot analysis

The extracted retinal and RPE tissues (N = 4–6) were homogenized in 10 w/v of RIPA buffer as previously described (Jovic et al., 2019). Following the centrifugation (21 000 rcf, 30 min, 4 °C), the supernatant was collected, and protein concentrations were determined using Micro BCA Protein Assay Kit (Pierce Biotechnology). Equal amounts of proteins (15 μg) were loaded per lane and, after the 10 % SDS acrylamide gel electrophoresis separation, blotted onto nitrocellulose membranes (Amersham Bioscience). Following the incubation in the blocking solution (5 % nonfat dry milk in Tris-buffered saline/0.1 % Tween 20, TBST) at room temperature (RT) for 1 h, the membranes were incubated with rabbit polyclonal Mfsd2a (1:8000, 10539, Abcam,) mouse monoclonal ABCA1 (1:500, #18180, Abcam), and rabbit monoclonal HMGCR

(1:1000, # 174830, Abcam) primary antibodies in TBST, overnight at + 4C. The membranes were then incubated with the HRP labeled secondary anti-mouse (1:3000; sc-2370, Santa Cruz) and anti-rabbit antibody (1:5000; sc-2370, Santa Cruz) for 1 h in TBSTat RT. The signal was detected (enhanced chemiluminescence, ECL, Amersham Bioscience) following the exposure to an X-ray film. The signals were analyzed by computerized densitometry (image analysis program ImageQuant ver. 5.2, Amersham) with the Ponceau S staining of the membranes serving as the loading control. The relative values of the signals were normalized to the corresponding Ponceau S staining and statistically analyzed.

2.7. Immunohistochemistry

The eyes were enucleated and fixed in 4 % paraformaldehyde at 4 °C (O/N), cryoprotected in 30 % sucrose, and embedded in 7.5 % gelatin:15 % sucrose. 18 μm cryo-sections were used in the analysis. Sections were degelatinized at 37 °C for 30 min in 1xPBS and permeabilized using Triton (0.5 %) for 15 min, followed by blocking (1 % bovine serum albumin, BSA in 1xPBST (0.1 % Triton in 1xPBS) for 1 h at RT, and incubated overnight at 4 °C with Mfsd2a (1:100, 10539, Abcam), and goat polyclonal anti-CD13 (1:100, R&D Systems, AF2335) primary antibodies. To label brain vessels, sections were incubated with 488-conjugated Lycopersicon esculentum lectin (Vector, 488 DL1174) together with primary antibodies (CD13 and Mfsd2a). Sections were subsequently washed in PBST and incubated with anti-rabbit secondary antibody conjugated to Alexa 568, Invitrogen) used at the 1:500 concentration in PBS for 2 h at room temperature (RT). After washing in PBS, slides were covered with DAPI mounting medium (Sigma, Serbia) and evaluated using fluorescent microscopy. Micrographs were captured on an Axio Observer Microscope (Z1 AxioVision 4.6 software system, Carl Zeiss, Germany) at a magnification of 20 ×. Findings reported by microscopic images were representative of observations performed in 3 separate staining for each group (n = 4). In all pictures, the apical is up.

2.8. Quantification of blood vessel coverage

Quantification was performed from representative images taken on a Zeiss microscope at 20 × magnification with conditions kept identical for all groups. CD13- and the lectin-positive area was measured using threshold processing (Otsu, ImageJ), and the pericyte coverage was expressed as % area occupied by Mfsd2a and CD13 in the lectin-positive area. In each animal (n = 4 per group), five fields from 2 to 4 nonadjacent retinal sections were analyzed.

2.9. Statistical analysis

Data were analyzed using the Prism program (GraphPad Software). The nonparametric Man-Whitney *U* test was used to compare two experimental groups, considering that data did not meet the normal distribution criteria. Statistical significance was set at $p < 0.05$.

3. Results

3.1. The high-dose FO treatment altered n-3 and n-6 PUFA content in retinas and RPE

The effects of the 3-week-long FO supplementation on the composition of lipids (n-3 and n-6 fatty acids) in the retina and RPE were analyzed immediately after the treatment (Fig. 1.A-E). We measured the effects of the FO supplementation on the n-3 fatty acids (EPA, DPA, and DHA) in the retina and RPE. A significant increase in the levels of EPA (3.52 folds) and DPA (1.67 folds) was observed in the retina (Fig. 1.A), while only EPA levels were increased in RPE (2.02 folds, Fig. 1.B). Interestingly, the levels of DHA were unaltered in both structures (Fig. 3. A, B). The main effects of FO supplementation on the composition of n-6 fatty acids in the retina (Fig. 1.C) were an increase in the levels of linoleic acid (1.42 folds) and adrenic acid (3.8 folds) and a decrease in the levels of arachidonic acid (20 %). In RPE, the significant decrease in the levels of dihomo-gamma-linoleic acid (DHGLA) in the RPE (49.4 %) was the main effect of the FO supplementation on the n-6 fatty acids (Fig. 1.D). The analyses of the n-6:n-3 ratio did not show any differences between the control and FO-treated animals, either in the retina or in RPE (Fig. 1.E). Similarly, both structures' AA/DHA ratio was unaltered (data not shown).

3.2. The effects of the high dose FO treatment on the total PUFA, mono-unsaturated fatty acids (MUFA), and saturated fatty acids (SFA) in the retina and RPE

The FO content showed the presence of other fatty acids besides essential PUFAs, especially palmitic, palmitoleic, and oleic (Table 2). Therefore, we analyzed whether FO intake modulates the content of mono-unsaturated fatty acids (MUFAs) and saturated fatty acids (SFAs) in both retina and RPE. Supplementation with FO rich in palmitic acid induced elevation in retinal palmitic acid (35.5 %, $p < 0.05$) (Table 2). Interestingly, the FO supplementation induced a decrease in the levels of palmitoleic acid in RPE (2.8 folds, $p < 0.01$) (Table 2). The other individual SFAs did not differ in either of the structures analyzed. Similarly, total SFA changed after the FO treatment in neither the retina nor RPE. Total PUFA and MUFA also did not change noticeably. In both structures, the FO treatment had no effect on either n-6 or n-3 fatty acids.

3.3. The high-dose FO treatment significantly increased the expression of DHA transporter Mfsd2a in the retina

The major facilitator superfamily domain-containing protein 2a (Mfsd2a) has been recognized as the major mediator of DHA transport in the retina (Wong et al., 2016). We sought to understand if the expression levels of Mfsd2a were affected following the high-dose FO supplementation (Fig. 2). The analysis of mRNA expression levels by qRT-PCR showed that the *Mfsd2a* mRNA levels were significantly increased in both retina (13.93 folds) and RPE (29.4 folds) (Fig. 2A). One of the suggested targets of Mfsd2a expression is sterol regulatory element-binding protein 1-c (*Srebp1-c*) (Zhang et al., 2021). In addition, Srebp1-c functions as an enhancer of the transcription of the genes required for fatty acid synthesis (Horton, 2002). The qRT-PCR analysis showed that the FO treatment significantly decreased the expression levels of *Srebp1-C* (39.5 %) in the retina (Fig. 2B). At the same time, the changes in *Srebp1-C* expression levels in RPE were insignificant (Fig. 2B). We then analyzed if the Mfsd2a protein levels were altered after the FO supplementation. The protein expression analyses showed a substantial increase in Mfsd2a expression (2.31 folds) in the FO-treated retina, while the change in its expression levels in RPE was negligible (Fig. 2C).

3.4. The high-dose FO supplementation increased Mfsd2a blood vessels expression without changing the pericyte coverage

The main expression site of Mfsd2a in the retina is in endothelial cells (EC) (Lobanova et al., 2019). We thus hypothesized that the increase in the Mfsd2a protein expression should be the result of the increased Mfsd2a expression in the retinal blood vessels (i.e., blood-retinal barrier, BRB). We performed immunohistochemical analyses of Mfsd2a expression (anti-Mfsd2a, red) on retinal blood vessels (labeled with anti-Lectin, green) in control and FO-treated retinas (Fig. 3A). ImageJ quantification revealed a 1.32. folds increase of the Mfsd2a expression on the retinal blood vessels following the FO supplementation (Fig. 3B).

It was shown that the changes in the levels of Mfsd2a expression on blood vessels could be in correlation with the changes in the degree of pericyte coverage (Ben-Zvi et al., 2014). We thus analyzed the pericyte coverage on the retinal blood vessels to establish if there is an association with the changes in Mfsd2a expression. Immunohistochemical

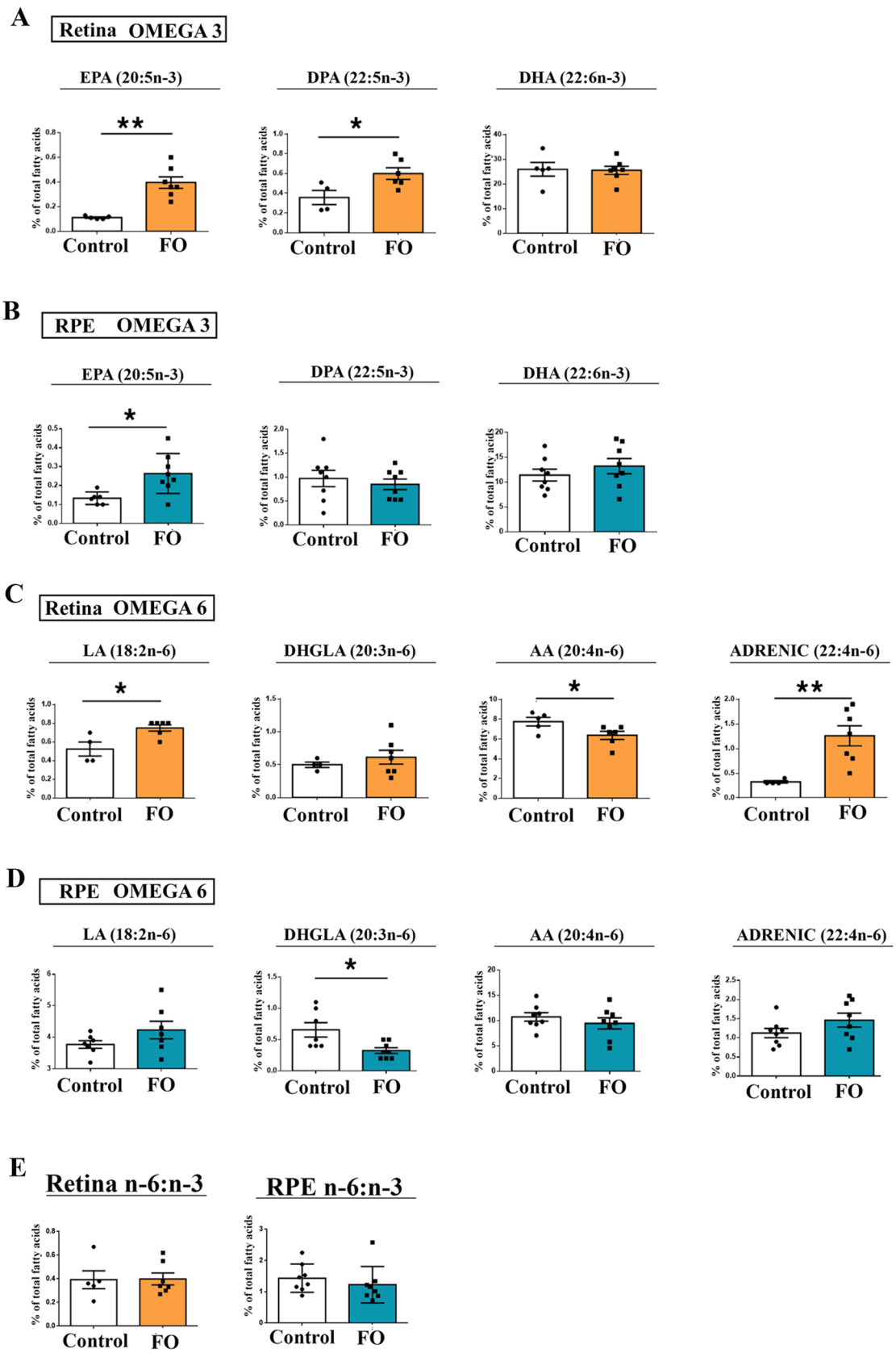

Fig. 1. The high-dose FO supplementation affects n-3 and n-6 PUFA levels in retina and RPE of the control and FO treated mice. The relative content of n-3 LC-PUFA in the retina (A) and RPE (B) of control and FO-treated mice (FO). The relative content of n-6 LC-PUFA in the retina (C) and RPE (D) of control and FO-treated mice (FO). (E) Omega-6/omega-3 LC-PUFA ratio in retina and RPE of control and FO-treated mice (FO). Linoleic acid (LA, 18:2n-6); dihomo-gamma-linoleic acid (DHGLA, 20:3n-6); arachidonic acid (AA, 20:4n-6); adrenic acid (22:4n-6), eicosapentanoic acid (EPA, 20:5n-3); docosapentaenoic acid, (DPA, 22:5n-3); docosahexaenoic acid (DHA, 22:6n-3). Data are presented as mean ± S.E.M. *$p < 0.05$, ** $p < 0.01$.

Table 2
SFA, MUFA, and PUFA in the retinas and RPE of control and FO supplemented mice.

Fatty Acid %	Retina	Retina FO	RPE	RPE FO
Palmitic acid (16:0)	16.46 ± 1.94	**22.31 ± 1.84[a]**	23.94 ± 2.50	24.23 ± 3.29
Stearic acid (18:0)	34.78 ± 2.19	31.81 ± 2.98	27.04 ± 3.9	27.03 ± 2.07
SFA	51.24 ± 1.94	54.13 ± 2.78	50.98 ± 4.06	51.25 ± 4.69
Palmitoleic acid (16:1n-7)	0.16 ± 0.09	0.22 ± 0.13	0.7625 ± 0.55	**0.28 ± 0.06[b]**
Oleic acid (18:1n-9)	6.42 ± 1.85	7.27 ± 1.68	14.84 ± 2.40	13.66 ± 0.66
Vaccenic acid (18:1n-7)	1.92 ± 0.12	1.77 ± 0.12	2.16 ± 0.07	2.23 ± 2.65
MUFA	8.50 ± 0.80	9.02 ± 2.65	17.76 ± 0.11	15.89 ± 0.75
n-6	9.70 ± 1.51	10.30 ± 1.78	17.08 ± 3.20	16.05 ± 2.24
n-3	26.52 ± 6.36	26.66 ± 4.41	12.58 ± 3.40	14.33 ± 4.44
PUFA	34.84 ± 4.80	33.97 ± 3.47	26.76 ± 4.78	27.48 ± 4.33

SFA, saturated fatty acids; MUFA, mono-unsaturated fatty acids; *n*-6, omega-6 polyunsaturated fatty acids (PUFA); *n*-3, omega-3 PUFA. Values are presented as mean ± SEM. Significantly different from the control retina: [a] $p < 0.05$. Significantly different from the control RPE: [b] $p < 0.05$.

staining using a pericyte marker (CD13, red) and an endothelial cell marker (Lectin, green) was performed on the retinas from the control and FO-treated animals (Fig. 3C). Image J analyses did not reveal any differences between the pericyte coverage in the FO-treated retinas when compared to the controls (Fig. 3D), indicating that the increase in Mfsd2a blood vessel expression is not the result of the increased pericyte coverage.

3.5. The high-dose FO treatment decreased the cholesterol, HDL, and triglycerides levels in the plasma and the expression of the genes regulating cholesterol synthesis in the retina

Genes regulating cholesterol and fatty acid synthesis were altered in the retina of Mfsd2a-/- animals (Lobanova et al., 2019; Zhang et al., 2021). In addition, the FO supplementation has cholesterol-lowering effects and the biochemical analyses of the serum from the FO-treated mice showed a significant lowering of the cholesterol, HDL, and triglyceride plasma levels (Fig. 4A). Therefore, we analyzed if the FO treatment altered the expression levels of the genes regulating cholesterol synthesis: liver X receptor beta (Lxrb) responsible for the integration of pathways of cholesterol input and output (Kalaany & Mangelsdorf, 2006), and endoplasmic reticulum-bound 3 hydroxy-3-methylglutaryl-coenzyme-A reductase (Hmgcr), the rate-limiting enzyme in cholesterol synthesis (Siperstein & Fagan, 1966). The qRT-PCR analysis showed that the FO treatment significantly decreased the expression levels of Lxrb in the retina (33 %) while its expression was unaltered in RPE (Fig. 4B). The expression levels of Hmgcr were decreased in the retina (52.73 %) but increased in RPE (1.78 folds) following the FO treatment (Fig. 4.C).

We then analyzed if the FO supplementation affected the expression levels of the rate-limiting HMGCR protein by Western blot analysis and showed that HMGCR levels were not altered in the retina following the treatment (Fig. 4.D). However, HMGCR protein levels were significantly increased in the RPE after the FO supplementation (11.66 folds, Fig. 4.D).

3.6. The high-dose FO supplementation affected the expression levels of ABCA1 cholesterol transporter in the retina and RPE while the levels of APOE remained unchanged in both structures

As FO supplementation altered the expression of genes regulating cholesterol synthesis, we analyzed if the cholesterol turnover was also affected. The cells recycle cholesterol by a very efficient apolipoprotein-dependent process. The most abundant is the apolipoprotein E (ApoE, (Mauch et al., 2001). This process of lipidation is mediated by the ATP-binding cassette transporter A1 (ABCA1), which is located in plasma membranes and effluxes cholesterol and phospholipids out of many cells (Mauch et al., 2001). The RT-PCR analyses showed that FO treatment did not have any effects on the levels of *ApoE* mRNA expression in either the retina or RPE (Fig. 5A). However, in the retina of the FO-treated animals, the expression levels of *ABCA1* transporter were significantly lower than in controls (37 % decrease, Fig. 5B). In the same time, the FO treatment significantly increased abca1 mRNA expression levels in the RPE (5.2 folds, Fig. 3.B). Western analyses showed that ABCA1 expression levels were downregulated in the FO- supplemented retina (60 %, Fig. 5.C) while its levels in the RPE remained unaltered (Fig. 5.C).

4. Discussion

The supplementation with FO, rich in DHA and EPA, is recommended by scientists and clinicians as a daily routine for prophylactic preservation and maintenance of our vision (Vannice & Rasmunsen, 2014). Notably, the high dose omega-3 supplementation for the healthy subject is already exceedingly supported. In the present study, the high-dose FO supplementation of healthy 3-month-old WT mice significantly increased the expression levels of the major DHA transporter Mfsd2a in the retina and RPE with the concurrent increase in the Mfsd2a expression on retinal blood vessels. At the same time, the FO supplementation significantly altered the lipid profile and the expression levels of the cholesterol metabolism-related genes in both the retina and RPE.

The main effect of the high dose FO treatment on the fatty acid content was a significant increase in the levels of EPA and DPA, among n-3 LC-PUFA, in the retina (3.52 and 1.67 folds, respectively) and EPA in RPE (2.02 folds). At the same time, the levels of DHA were unaltered in both the retina and RPE, maintaining DHA homeostasis. These results, although in contrast with other studies, can be explained either by considering the difference in the duration and the dose of the supplementation (Nishizawa, Wang, Sekine & Saito, 2003). In addition, they can be the result of the retroconversion of DHA and DPA to EPA that occurs in humans and rodents (Brossard et al., 1996; Conquer & Holub, 1997; Nakamura & Nara, 2004). Most importantly, the increase in the expression levels of the specific DHA transporter, Mfsd2a, in both retina and RPE can be responsible for maintaining DHA homeostasis through the increased regulation of DHA transport between retina and RPE and between RPE and blood flow. These changes may reflect a compensatory mechanism aiming to maintain a relatively constant overall level of lipid saturation in the retina, required for maintaining the accurate fluidity of cell membranes. In our previous study, we analyzed the effects of the high-dose FO supplementation on the mouse brain and found an increase in the levels of DHA without the changes in the levels of Mfsd2a (Milanovic et al., 2018). Since the retina contains a higher proportion of phospholipid fatty acids compared with brain, with the highest concentration found in the light-sensitive outer segment membranes of rod and cone photoreceptor cells (Fliesler & Anderson, 1983), DHA homeostasis in the retina may be under more stringent control than in the brain.

On the other hand, the significant increase in EPA and DPA levels in the retina and EPA in RPE may have an atheroprotective role by reducing the accumulation of cholesterol-rich domains in atherosclerotic membranes, thus reducing the inflammation (Mason, Jacob, Shrivastava, Sheratt & Chattopadhyay 2016; Ghasemi Fard et al., 2021). In addition, DPA has also been shown to have distinct effects on lipid

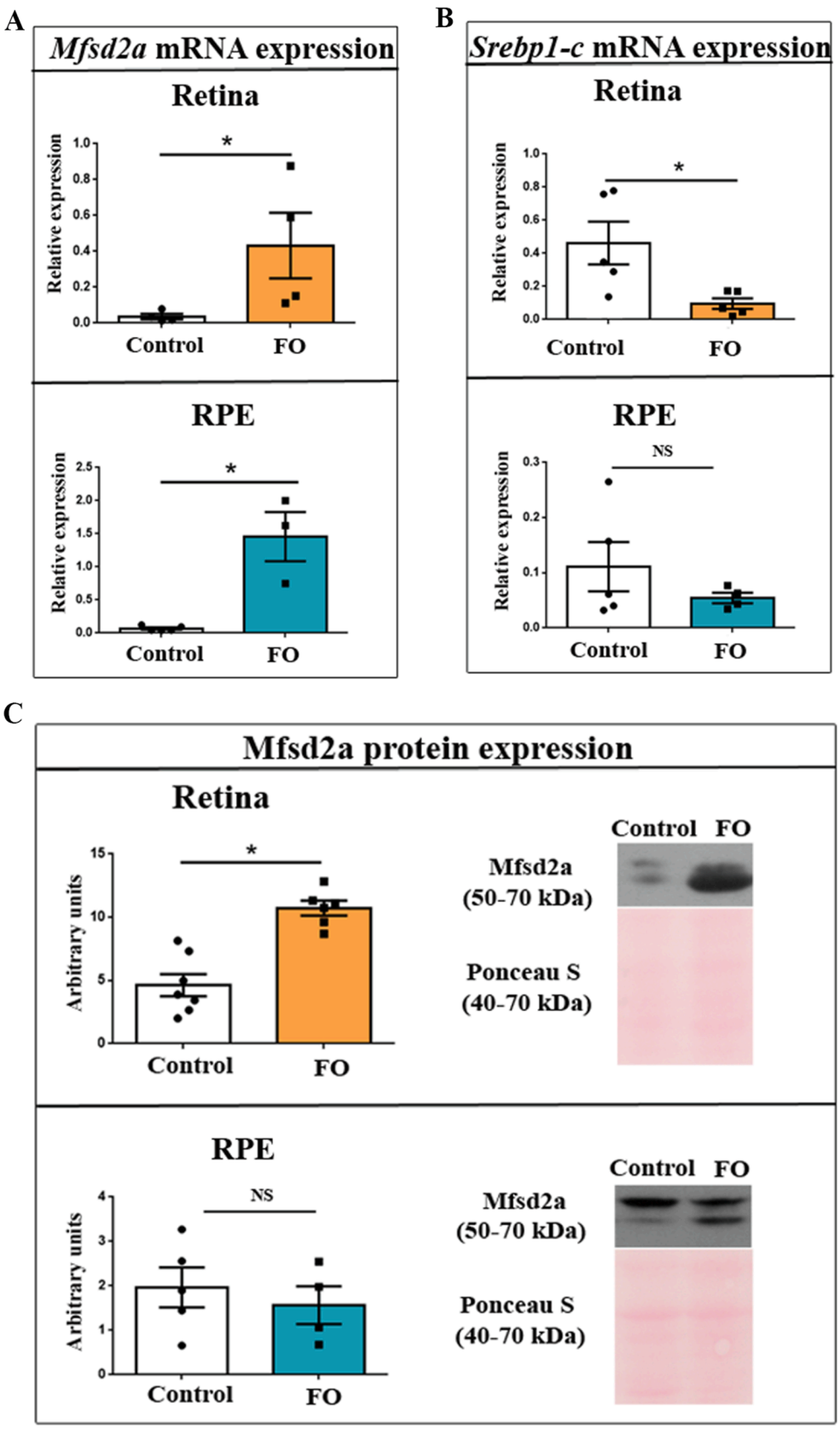

Fig. 2. The high-dose FO supplementation altered the expression levels of Mfsd2a in the retina and RPE. (A) The changes in the expression levels of *Mfsd2a* mRNA in the retina (yellow) and RPE (blue) were analyzed using the real-time polymerase chain reaction (RT-PCR). (B) The changes in the expression levels of *Srebp1-c* in the retina (yellow) and RPE (blue) were analyzed using RT-PCR. (C) Changes in the protein expression levels of Mfsd2a in the retina (yellow) and RPE (blue) as revealed by Western blot analysis. Representative immunoblots of Mfsd2a from the control and FO-treated (FO) retinas and RPEs. The data represent mean ± SEM value. *$p < 0.05$. NS = non-significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

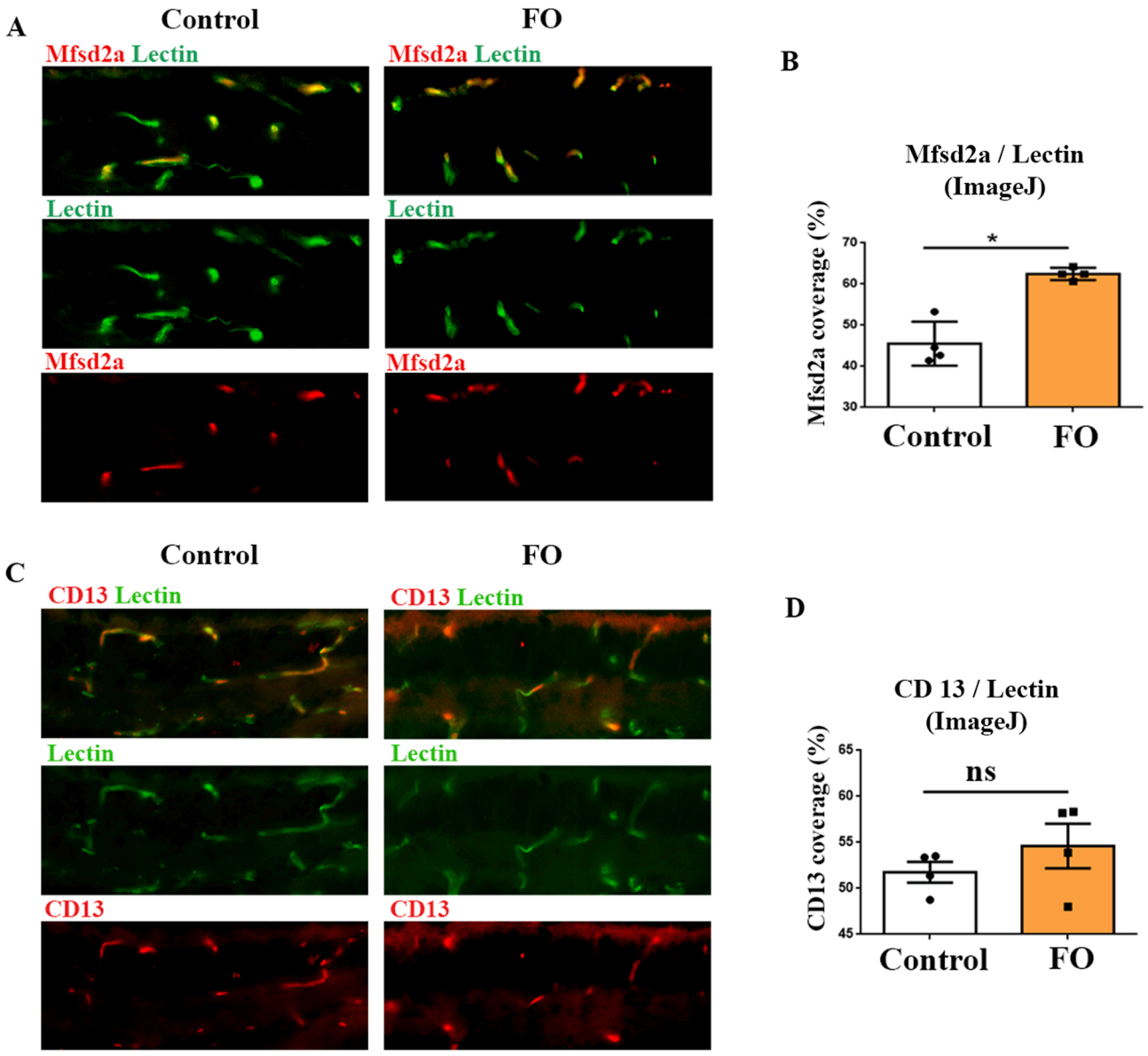

Fig. 3. The high-dose FO supplementation alters Mfsd2a BRB coverage without altering the pericyte coverage. (A) The Mfsd2a retinal blood vessel coverage in the control and FO-treated retinas was analyzed using immunohistochemistry, (Mfsd2a- red, Lectin, green, DAPI – blue). (B) The quantification of the Mfsd2a retinal blood vessel coverage was performed using ImageJ and the results were presented as the ratio of Mfsd2a/Lectin staining (graph). (C) Representative images of the degree of pericyte coverage (CD13, red) of retinal blood vessels (Lectin, green) in the control (Control) and the fish oil treated (FO) retinas. (D) The quantification of the CD13 retinal blood vessel coverage was performed using ImageJ and the results were presented as the ratios of CD13/Lectin staining (graph). The data represent mean ± SEM value. *$p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

dynamics and membrane lipid oxidation (Mason, 2019). At the same time, the effects of the FO treatment on n-6 PUFA in the retina were more prominent than in the RPE and consisted of an increase in the levels of linoleic and adrenic acid, possibly due to the presence of LA in the FO used for the supplementation LA (1.67 %, w/w). The increase in LA levels may account for the increase in the levels of adrenic acid. The metabolic products of adrenic acid belong to the group of bioactive lipids which were shown to have various beneficial effects including exertion of analgesia and reduction of endoplasmic reticulum (ER) stress (Singh, Barnych, Wagner, Wan, Morisseau, Hammock, 2021). On the other hand, the moderate increase of the AA levels in phospholipids of eyes is commonly observed in models of dietary DHA deficiency (Simopoulos, 2018). In addition, such increase was observed in the phospholipids from the eyes of Mfsd2a KO (Wong et al., 2016). Accordingly, in our study, with the observed increase in the expression of Mfsd2a, the levels of AA are slightly decreased.

The only significant changes that were observed in the levels of other fatty acids analyzed (Table 2) were the increase in the levels of palmitic acid in the retina (16:0. 1.73 folds), and the decrease in the levels of palmitoleic acid (16:1n-7; 2.72 folds). These alterations are probably the result of the higher content of palmitic acid in the FO (22.9 % w/w). Only a modest decrease in the levels of DHGLA was observed in the FO-treated RPE.

Most importantly, our results revealed that high-dose FO supplementation was able to induce the expression of Mfsd2a, a major DHA transporter in the retina (Wong et al., 2016) on both transcriptional and translational levels (Fig. 2), simultaneously increasing the Mfsd2a expression on the blood vessels (Fig. 3) after only three weeks of treatment. It was suggested that the low expression of Mfsd2a in the retina might be one reason why DHA therapy fails to alleviate the symptoms of diabetic retinopathy (DR) and that the combined use of Mfsd2a overexpression and DHA therapy may have synergistic effects (Zhang et al.,

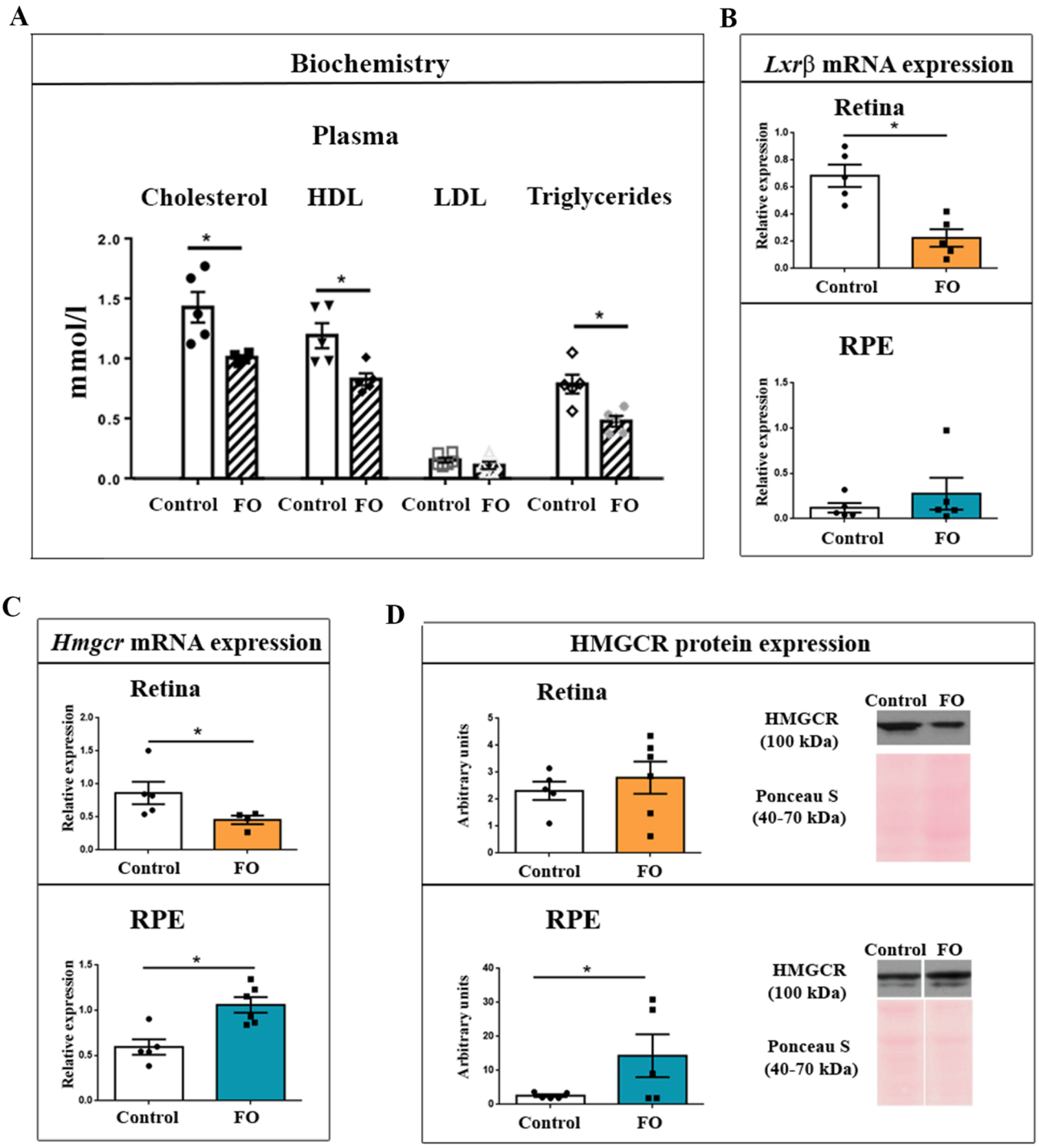

Fig. 4. The analyses of the cholesterol levels in the serum and the expression of the genes regulating cholesterol synthesis in the retina and RPE in the control and FO supplemented mice. (A) The biochemical analyses of cholesterol, HDL, LDL, and triglycerides levels (mmol/l) in the plasma of the control and FO treated mice. The expression levels of *Lxrβ*mRNA(B), and *Hmgcr* mRNA (C), were analyzed in the retina (yellow) and RPE (blue) of the control and FO supplemented mice by real time polymerase chain reaction (RT-PCR). (D) Densitometric quantification of HMGCR protein expression and representative immunoblots of HMGCR in control and FO-treated retina and RPE. Data are presented as mean ± SEM. * $p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2021). It is thus conceivable that the high dose FO supplementation can serve as a potential adjuvant in the therapies or as a prophylactic in the early stages of disease for the impaired BRB through the up-regulation of Mfsd2a expression. Recent findings provided insights into the molecular structure of the putative DHA binding site on the Mfsd2a receptor, opening the venue for developing new neurotherapeutic agents that can bind to that site and successfully penetrate the BRB (Cater et al., 2021). If the adjuvant supplementation with the FO can increase the number of binding sites for the potential candidate drug, the delivery of that drug through the otherwise difficult-to-cross BRB will be significantly improved.

Mfsd2a is mainly expressed in endothelial cells (Lobanova et al., 2019). However, a decreased Mfsd2a expression correlates directly with the reduced degree of pericyte coverage (Ben-Zvi et al., 2014).

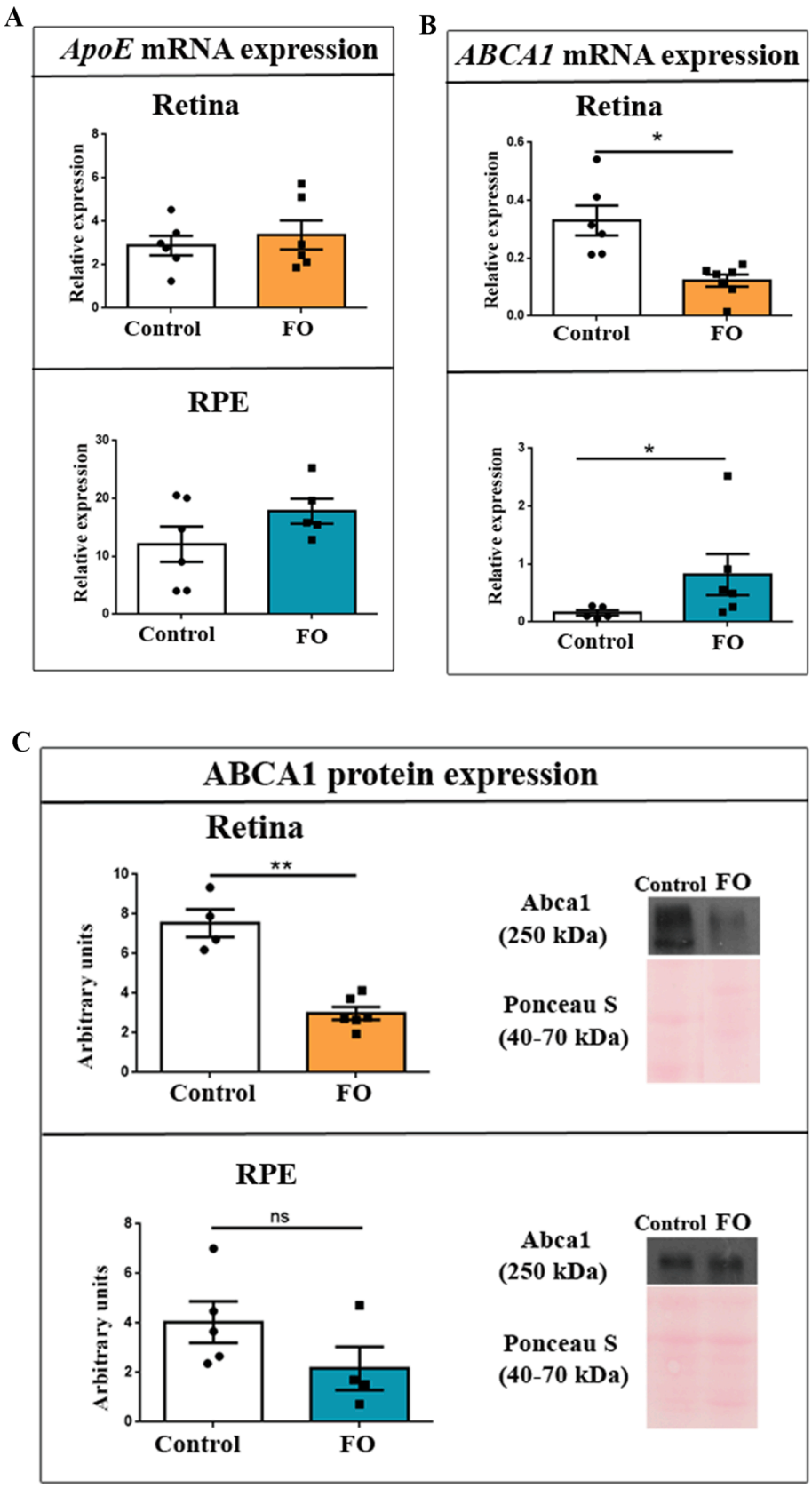

Fig. 5. The expression of genes regulating cholesterol transport and elimination in the retina and RPE of the control and FO supplemented mice. The expression levels of *ApoE* (A), and *Abca1* (B) were analyzed in retina (yellow) and RPE (blue) of the control and FO supplemented mice by real time polymerase chain reaction (RT-PCR). (C) Densitometric quantification of ABCA1 protein expression and representative immunoblots of ABCA1 in control and FO-treated retina and RPE. Data are presented as mean ± SEM. * $p < 0.05$; ** $p < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Therefore, endothelial–pericyte interactions may control the expression of Mfsd2a, which controls BRB integrity. However, the pericyte coverage of retinal blood vessels remained unaltered under the high-dose FO supplementation (Fig. 3), eliminating the role of pericytes in the regulation of Mfsd2a expression. The increased Mfsd2a expression in the RPE was not translated to the changes in the Mfsd2a protein levels possibly because of its more conservative response in general, due to its suggested physiological role as a "gate-keeper" (Zheng et al., 2012).

The Srebp pathway is one of the major signaling pathways that are highly increased in the eyes of Mfsd2a knockout mice (Wong & Silver, 2020). Especially important is the role of Srebp1-c in regulating endothelial cell function, migration, and proliferation (Zhou et al., 2004; Yao et al., 2006) and regulating sterol and fatty acid production (Horton et al., 2002). The Mfsd2a downregulation in DR was associated with the upregulation of Srebps (Zhang et al., 2021). Accordingly, we found that the expression of srebp-1c was downregulated simultaneously with the increase in Mfsd2a expression following the FO supplementation (Fig. 2). On the other hand, *srebp-1c* expression is regulated via LXR, a transcription factor responsible for the integration of pathways of cholesterol input and output. There are two LXR-responsive elements (LXREs) in the *Srebp-1c* promoter, and EPA and DHA were shown to inhibit the binding of the LXR/RXR heterodimer to the LXREs in the -*Srebp-1c* promoter and elicit a marked decrease in the level of *Srebp-1c* mRNA (Le Jossic-Corcos et al., 2005). However, EPA can induce suppression of Srebp1-c independently of LXR (Davidson, 2006). Thus, the observed increase in EPA levels in the retinas of FO-treated mice can be partially responsible for the decreased Srebp-1c levels and indirectly for the increase in the Mfsd2a levels. However, whether the FO supplementation downregulates *Srebp1-c* directly or through the up-regulation of Mfsd2a expression is unclear.

The other potential explanation for the mechanism with which omega-3 can regulate Mfsd2a expression is through the activation of the Wnt signaling pathway. Mice lacking LRP5 or Norrin exhibit increased retinal vascular leakage and, more importantly, exhibit excessive transcytosis across RMECs (Wang et al., 2020). Wnt signaling pathway directly regulates the transcription of an EC-specific inhibitor of transcytosis, Mfsd2a, in a β-catenin-dependent manner, and the overexpression of Mfsd2a rescues Wnt-deficient transcytosis in ECs and retinas (Wang et al., 2020). DHA was recently shown to enhance Wnt-signaling in Wnt3a dependent manner in the human iPSC-derived neural progenitor cells (NPCs). Importantly, this activation was dose-dependent, yielding a 3-fold increase in the pathway activation (Zhao et al., 2019). Wnt signaling is crucial for maintaining BRB and BBB under physiological conditions, and the aberrations in Wnt signaling are observed in several blinding retinal diseases. A more detailed understanding of the potential interaction of Wnt signaling with omega-3 fatty acids is necessary to understand better the mechanism with which FO regulates Mfsd2a expression.

The FO supplementation has lowered cholesterol, HDL, and triglyceride levels in the plasma of treated mice. Considering that almost 70 % of the lipids needed for normal retinal function are synthesized locally, we analyzed the essential genes responsible for regulating cholesterol metabolism (Figs. 4 and 5). A significant decrease in the transcription of the *lxrb* and *hmgcr* (regulators of cholesterol synthesis) and *abca1* (cholesterol transporter) was observed in retinas after the FO supplementation. The FO-induced decrease in the expression levels of *hmgcr*, a rate-limiting enzyme in cholesterol synthesis, is in accordance with the previously shown ability of EPA to inhibit *hmgcr* expression levels (Karanth, Tran, Kuberan & Schlegel, 2013; Cho et al., 2014). However, these transcriptional changes were insufficient to reduce the Hmgcr protein levels in the retina. *abca* and *apoE* genes regulate lipoprotein-mediated cholesterol transport and elimination. As *Abca1* is regulated primarily by Lxrb, the decrease in the *Lxrb* expression in the retina found in our study can be responsible for the down-regulation of Abca expression, both on transcriptional and translational levels. Considering that the increased Abca1 and Hmgcr expression are associated with cholesterol accumulation (Johnson, Yabu, Hanson, Shah & Zager, 2003), which is linked with several retinal neurodegenerative diseases, including age-related macular degeneration (AMD) (Pikuleva & Curcio, 2014) the FO induced decrease of Abca and *hmgcr* expression suggest a neuroprotective effect.

The ingested fatty acids are transported from the blood via RPE's apical microvilli enveloping the ROS of PRs. RPE can eliminate cholesterol through basolateral secretion of lipoprotein particles, which is mediated through *abca1* and *apoe* expression. Although retina and RPE are intimately connected structurally and functionally (Strauss, 2005), they differ in their requirements for cholesterol and n-3 PUFAs homeostasis and thus may respond differently to FO supplementation. We found the robust elevation of Hmgcr (mRNA and protein) and Abca1 (mRNA) levels in RPE after the FO treatment. The observed changes in hmgcr and abca1 expression on the transcriptional and translational level in RPE are in accordance with the protective role of RPE as a "gate keeper" in order to regulate cholesterol and nutrient flux from systemic circulation to the retina and back (Zheng et al., 2012).

5. The weaknesses and strengths of the study

This study was designed to evaluate effects of a high-dose, short-term (3 weeks) FO treatment on retinal fatty acid content, Mfsd2a expression, and cholesterol metabolism. As this kind of analyses has not been performed so far, these results can be used for the better assessment of the of LC-PUFA high-dose prophylactic supplementation for the healthy individuals. The weaknesses of the study include that the effects of the FO supplementation were only assessed immediately after the treatment. Future studies should asses the long-term effects of the short-term treatment. In addition, similar studies should be conducted using older healthy animals.

6. Conclusions

We have shown that FO/n-3 PUFA supplementation has robust effects on lipid composition, transcriptional networks, and protein expression in the retina and RPE. In general, the effects of the FO treatment were more prominent in the retina than in the RPE, supporting the protective role of this structure. Importantly, these effects of the FO supplementation were elicited without affecting DHA homeostasis, ensuring the proper function of photoreceptors. Instead, our results showed that the significant increase in EPA levels in both retina and RPE might be the alternative mechanism affecting cholesterol-related gene expression.

The principal finding of this research is that the expression levels of Mfsd2a, a DHA transporter, were increased in the retinas of the FO-treated mice with the simultaneous increase in Mfsd2a expression on retinal blood vessels (1.32 folds). Mfsd2a was recently described as a regulator of vesicular transcytosis, an essential route for drug delivery across the BRB. Thus, the findings from this work may provide a feasible direction for regulating vesicular transcytosis and developing new drug delivery systems. In such a case, the FO supplementation can serve either as prophylaxis in the healthy eye or as an adjuvant in developing targeted manipulations of the barrier during diseases. In the latter case, Mfsd2a may be an effective candidate for such manipulations, and the FO supplementation an effective adjuvant.

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All animal procedures complied with the Directive (2010/63/EU) on the protection of experimental animals or animals used for other scientific purposes. The Ethical Committee for the Use of Laboratory Animals approved these procedures (resolution No. 01-06/13, Institute for

Biological Research, University of Belgrade). Animal procedures used in this paper complied with the EEC Directive (86/609/EEC) on animal protection, including the efforts to minimize animal suffering.

CRediT authorship contribution statement

Irena Jovanovic Macura: Formal analysis, Validation. **Ivana Djuricic:** Formal analysis, Writing - original draft. **Tamara Major:** Formal analysis, Validation, Writing – review & editing. **Desanka Milanovic:** Formal analysis. **Marjana Brkic:** Formal analysis. **Sladjana Sobajic:** Conceptualization. **Selma Kanazir:** Conceptualization, Validation. **Sanja Ivkovic:** Conceptualization, Validation, Supervision, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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