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Optimal omegas

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Optimal omegas

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Editorial: Optimal omegas

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Editorial on the Research Topic
Optimal omegas

The Frontiers "*Optimal omegas*" Research Topic was assembled to provide a scientific update on field human health studies, the modulation of metabolic pathways, traditional and novel dietary sources of omega-3 fatty acids, sustainable production of foods providing omega-3 fatty acids and bioavailability studies.

Trends toward sustainable plant-based diets mean that modern dietary staples are shifting with momentum, leading to ramifications for intakes of omega-3 fatty acids and the essential fatty acid metabolic pathway. Subsequently, there are growing demands for sustainable food-derived sources of omega-3 (ω-3) fatty acids.

Two publications in the Research Topic used National Health and Nutrition Examination Survey (NHANES) data. Li Y. et al. identified that omega-3 and omega-6 polyunsaturated fatty acids were negatively associated with neutrophil-lymphocyte ratio, white blood cell counts, systemic immune-inflammation index and platelet-lymphocyte ratio. Interestingly, Yan et al. found that omega-3 intakes exceeding 2.05 g and ω-6 intakes ranging from more than 11.42 g to ≤to 19.16 g lowered frailty risk amongst middle-aged and elderly individuals.

An updated systematic review of randomized controlled trials found that eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) both lower triglyceride levels, with docosahexaenoic acid having a greater effect (Choi and Calder). Li K. et al. found that mussel oil was more potent than fish oil in preventing atherosclerosis, with the downregulation of p38MAPK/NF-κB signaling pathway being one plausible mechanism. Derbyshire et al. discussed potential ways forward focusing on innovative delivery methods to utilize omega-3 long-chain polyunsaturated fatty acid rich oils including the use of fortification strategies, bioengineered plants, microencapsulation, and microalgae.

A pilot study in patients with the homozygous ancestral (minor) FADS1 genotype demonstrated that FADS1 genotypes significantly decreased blood levels of ω-6 polyunsaturated fatty acids (PUFAs), particularly arachidonic acid (AA) without having a notable impact on the ω-3 PUFAs such as EPA and DHA, liver fat content and AA-derived lipid mediators (Rabehl et al.).

Macura et al. found the resilience of DHA homeostasis in the retina and retinal pigmented epithelium (RPE) of 2-month-old mouse offspring, even following high-dose fish oil supplementation during pregnancy and lactation. Another significant finding in this study was an upregulation of major facilitator superfamily domain-containing protein (Mfsd2a), a key DHA transporter and transcytosis regulator during development. The post-market cohort study showed that AlmegaPL®, an EPA-only polar lipid supplement derived from the microalga Nannochloropsis, effectively reduced triglycerides (TG) and remnant cholesterol (RC) in a real-world consumer setting (Ganuza et al.).

A clinical trial conducted by Rafieipoor et al. revealed that ω-3 supplementation (3 g/day for 2 months) did not have a significant effect on managing chronic kidney disease-associated pruritus (CKD-aP) in patients undergoing hemodialysis.

Overall, these articles have provided some valuable field insights within the omega scientific field. In particular, non-animal derived sources of omegas is an exciting area to monitor for the future and one for which ongoing research is highly valuable.

Author contributions

ED: Writing – original draft, Writing – review & editing. KL: Writing – original draft, Writing – review & editing. ID: Writing – original draft, Writing – review & editing.

Conflict of interest

ED was employed by Nutritional Insight Limited.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The supplementation of a high dose of fish oil during pregnancy and lactation led to an elevation in Mfsd2a expression without any changes in docosahexaenoic acid levels in the retina of healthy 2-month-old mouse offspring

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Introduction: During fetal development, the proper development of neural and visual systems relies on the maternal supplementation of omega-3 fatty acids through placental transfer. Pregnant women are strongly advised to augment their diet with additional sources of omega-3, such as fish oil (FO). This supplementation has been linked to a reduced risk of preterm birth, pre-eclampsia, and perinatal depression. Recently, higher doses of omega-3 supplementation have been recommended for pregnant women. Considering that omega-3 fatty acids, particularly docosahexaenoic acid (DHA), play a crucial role in maintaining the delicate homeostasis required for the proper functioning of the retina and photoreceptors the effects of high-dose fish oil (FO) supplementation during pregnancy and lactation on the retina and retinal pigmented epithelium (RPE) in healthy offspring warrant better understanding.

Methods: The fatty acid content and the changes in the expression of the genes regulating cholesterol homeostasis and DHA transport in the retina and RPE were evaluated following the high-dose FO supplementation.

Results: Our study demonstrated that despite the high-dose FO treatment during pregnancy and lactation, the rigorous DHA homeostasis in the retina and RPE of the two-month-old offspring remained balanced. Another significant finding of this study is the increase in the expression levels of major facilitator superfamily domain-containing protein (Mfsd2a), a primary DHA transporter. Mfsd2a also serves as a major regulator of transcytosis during development, and a reduction in Mfsd2a levels poses a major risk for the development of leaky blood vessels.

Conclusion: Impairment of the blood-retinal barrier (BRB) is associated with the development of numerous ocular diseases, and a better understanding of how to manipulate transcytosis in the BRB during development can enhance drug delivery through the BRB or contribute to the repair of central nervous system (CNS) barriers.

KEYWORDS

fish oil, retina, Mfsd2a, RPE, EPA, DHA, pregnancy, lactation

1 Introduction

The proper development and function of the retina depend on the adequate supply of omega-6 (n-6) and omega-3 (n-3) long-chain polyunsaturated fatty acids (LC-PUFAs) (1, 2). Docosahexaenoic acid (DHA, C22:6n-3), an omega-3 fatty acid, is one of the main retinal structural lipids, comprising up to 50% of the total photoreceptor rod outer segment lipid content (3). The change in the optimal amount of DHA levels can affect the fluidity of the membrane (4) and, consequently, the activity and regeneration of rhodopsin, ultimately affecting phototransduction (2, 4–6). In addition, the proper DHA supply prenatally and early in life is necessary for the proper visual and cognitive functions in the offspring (7–9). Furthermore, the optimal supply of n-3 LC-PUFAs during pregnancy and lactation is associated with a decreased rate of preterm birth (10), reduced risk of pre-eclampsia (11), post-partum depression (12), and the development of allergies (13). In addition, this perinatal and early postnatal period of life is considered particularly susceptible to the effects of environmental factors such as nutrition on beneficial epigenetic changes (14). It was shown that PUFA supplementation can alter epigenetic patterns related to allergic manifestation, and FO consumption was associated with altered histone acetylation in placentas (15). For example, the levels of cord blood T-cell PKCζ could be altered by FO supplementation in an epigenetic manner, affecting the development of allergic inflammation in children (16).

DHA deficiency during pregnancy and lactation affects human retinal development (17, 18), and supplementation with PUFAs throughout pregnancy is recommended as beneficial (19). Mammalian cells lack the ability to synthesize n-6 and n-3 PUFAs *de novo*. Consequently, the fetus relies on maternal transfer through the placenta and subsequent intake through milk and dietary supplements post-birth to accumulate these essential fatty acids. Therefore, it is crucial for the mother to maintain sufficient PUFAs in her diet or through dietary supplements (20). Research has validated a correlation between the concentration of 22:6n-3 in maternal plasma and its placental transfer (21).

Breast milk serves as the main source of PUFAs for newborns, and its PUFA composition is influenced by maternal dietary habits (22). However, several reports have highlighted insufficient n-3 LC-PUFA intake among a significant number of pregnant women in Europe (23–25). Additionally, pregnancy is linked to a notable decline in maternal DHA levels (26–28). Given these current dietary patterns, mothers may struggle to fulfill the heightened fetal DHA requirements. Consequently, prevailing guidelines suggest that pregnant women should aim for a daily intake of 250–500 mg of eicosapentaenoic acid (EPA) and DHA, with at least 200 mg specifically from DHA (29–31). The European Food Safety Authority recommends an extra 100–200 mg of DHA daily (32).

Fish oil (FO) serves as a natural source of n-3 fatty acids (33). Studies have demonstrated the safety and pivotal role of high-dose FO supplementation (up to 5 g/day for 16 weeks) in preventing and managing various diseases in the human population (32). Clinical trials in Denmark, Norway, and Australia involving pregnant women who consumed 2.2–2.7 g of omega-3 fatty acids through fish oil supplements (containing 920 mg, 1,183 mg, and 2.2 g DHA, respectively) revealed positive effects of high-dose FO supplementation (20, 34–36). The extended gestational period observed in the supplemented group was correlated with the DHA concentration ratio in neonatal cord blood. Notably, at 2.5 years of age, children in the fish oil-supplemented group exhibited superior eye and hand coordination scores compared to the control group, which were also in correlation with EPA and DHA in cord blood (20). Similarly, an assessment of the Norwegian infant cohort found that children born to mothers supplemented with FO demonstrated higher mental processing scores at the age of four (37). Additionally, the Australian government has revised its recommendations and, currently, advises pregnant women with low n-3 status to supplement with the augmented dose of n-3 LC-PUFA—specifically, 800 mg DHA and 100 mg EPA per day—primarily to mitigate the risk of preterm birth (38).

However, the impact of high-dose fish oil (FO) supplementation during pregnancy and lactation on the fatty acid composition in the retina and retinal pigmented epithelium (RPE) of offspring in early adulthood remains unclear. Despite the close association between the retinas and RPE (39), they diverge in their requirements for cholesterol and n-3 PUFA homeostasis, potentially responding disparately to FO supplementation during pregnancy and lactation. Moreover, fish oil supplementation has been reported to possess cholesterol-lowering properties in various systems (40–42). Cholesterol is yet another retinal lipid whose changes can affect the activity of rhodopsin, enhancing light activation (43). Given that the retina acquires cholesterol both from blood-borne uptake and local biosynthesis, there is a pressing need to gain a deeper understanding of how dietary treatments impact the transcriptional network of cholesterol-related gene expression.

The retina's absorption of DHA involves receptors for lipoproteins and adiponectin (1, 44), along with multiple transporters for fatty acids (45). Nevertheless, the primary route for DHA uptake into the retina is likely through the pathway mediated by the major facilitator superfamily domain-containing protein 2a (Mfsd2a) (46–48). Mfsd2a has been identified as a key regulator of blood–organ barrier permeability, encompassing both the blood–brain barrier (BBB) and the blood–retinal barrier (BRB) (49, 50). Mfsd2a-mediated lipid transport is crucial for inhibiting transcytosis (51), and a decrease in Mfsd2a expression is linked to impairments in vascular permeability. However, this reduction in Mfsd2a expression leads to a subsequent increase in vesicle trafficking (enhanced transcytosis) and compromised barriers, all occurring without alterations in endothelial junctions (47, 52). Conversely, studies have demonstrated that heightened Mfsd2a expression can reduce transcytosis (53).

In a previous study, we demonstrated that high-dose fish oil (FO) supplementation during adulthood led to significant alterations in lipid content and the increased expression of genes governing DHA transport (Mfsd2a) and cholesterol homeostasis in the retina and retinal pigmented epithelium (RPE) (54). However, the enduring effects on the retinas and RPE in the offspring resulting from high-dose FO supplementation during pregnancy and lactation remain unknown. To address this, we investigated the phospholipid content in the retinas and RPE, as well as changes in the expression levels of genes involved in DHA transport and cholesterol homeostasis in the retinas and RPE of 2-month-old B6/SJL female mice born to mothers who received high-dose FO supplementation during pregnancy and lactation.

2 Materials and methods

2.1 Animals

For this study, female B6/SJL mice were utilized. All procedures involving animals adhered to the EU Directive (2010/63/EU) concerning the protection of animals used for experimental and scientific purposes. Approval was obtained from the Ethical Committee for the Use of Laboratory Animals (resolution No. 01–06/13) at the Institute for Biological Research, University of Belgrade. The animal procedures also conformed to the EEC Directive (86/609/EEC) on animal protection, with diligent efforts made to minimize any potential suffering. The mice were housed in standard conditions (23 ± 2°C, relative humidity 60–70%, 12-h light/dark cycle), with regular health check-ups. They had unrestricted access to pelleted commercial rodent chow (see Table 1), available *ad libitum* (AL).

2.2 Treatment

For the fish oil (FO) treatment, pregnant B6/SJL mice were separated into two groups: the treated group ($n = 7$) received supplementation with commercial fish oil (DietPharm, FidaFarm Croatia), a rich source of omega-3 fatty acids, whereas the control group ($n = 7$) was given the same volume of water as a vehicle. The treated group received 100 µL of fish oil (fatty acid composition in Table 2) daily through oral gavage. The FO treatment spanned 6 weeks, covering both gestation and the lactating period (Figure 1).

The chosen dose for this study constituted a high-dose treatment of either DHA or EPA. A daily human dose of 3,000 mg of DHA equates to 50 mg/kg of body weight. The animal equivalent dose (AED) was calculated according to FDA guidelines for species conversion, where AED (mg/kg) = human dose (mg/kg) multiplied by the correction factor for mice (Km). For the human dose of 50 mg/kg, the AED dose amounted to 615 mg/kg. Consequently, with 12 mg DHA and 18 mg EPA per 100 µL, animals were treated with 545.5 mg/kg of DHA and 818.2 mg/kg of EPA daily.

The control group received an equivalent amount of water (100 µL) administered daily through oral gavage during the same period. To maintain experimental purity, we refrained from using other oils as a control due to the potential biological effects of additional components in dietary fish oil, such as omega-3 and omega-6 fatty acids, iodine, furan fatty acids, and antioxidant vitamin E. In order to prevent DHA degradation, one capsule containing 1 mL of FO was used for every 3–4 animals (100 µL per animal) and was administered through oral gavage in under 45 s. Any remaining FO was discarded, and a new capsule was utilized for the subsequent set of animals. After the lactation period, the pups were weaned and provided with regular chow.

TABLE 1 Pelletized commercial diet content.

Nutrient	% of the total amount
Protein	17.2
Carbohydrate	60.9
Fat	3.7
PUFA/SFA	1.3
n-3/n-6 PUFA	0.05
Fiber	5.6
Ash	7.6

Adequate amount of vitamins and minerals SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids; n-3, omega-3; n-6, omega-6.

Five weeks post-termination of FO supplementation, mice were euthanized at the age of 2 months (Figure 1). Anesthesia was induced (100 mg/kg Ketamidor, Richter Pharma, Wells, Austria, and 16 mg/kg Xulased, Bioveta, a.s. intraperitoneally), followed by perfusion with 50 mL 0.1 M phosphate buffer (PBS) over 30 min before decapitation. Eyes were promptly enucleated, and from the seven eyes, retinas and RPE were isolated and processed for RNA isolation and qPCR. Another set of seven eyes had their retinas and RPE isolated and processed for fatty acid isolation.

2.3 Tissue collection

At the time of euthanization, the animals were 2 months old (Figure 1). Mice underwent anesthesia (100 mg/kg Ketamidor, Richter Pharma, Wels, Austria, and 16 mg/kg Xulased, Bioveta, a.s. intraperitoneally). Each animal underwent perfusion with 50 mL 0.1 M phosphate buffer (PBS) for 30 min and was subsequently decapitated. The eyes were enucleated, the optic nerve was severed, and the cornea, lens, and vitreal body were extracted. The retina was carefully peeled off for further analysis. The eyecup, encompassing the RPE, choroid, and sclera (referred to as RPE henceforth), was isolated and utilized for subsequent analysis. All tissue samples were stored at −80°C until RNA and fatty acid isolation. In seven eyes, retinas and RPE were individually isolated and processed for RNA isolation and qPCR. Another set of seven eyes had their retinas and RPE individually isolated and processed for fatty acid isolation. The same animals were used for blood collection to facilitate biochemical analyses.

2.4 Retina and RPE fatty acid methyl ester preparation

Retina and RPE total lipids were extracted using chloroform/methanol, following the method outlined by Folch and modified by Kates et al. (55). The conversion of the extracted lipids into fatty acid methyl esters (FAME) was accomplished using 3 M HCl in methanol, as detailed in previous reports (56). The lipids were placed in a glass cuvette, and 1.5 mL of 3 M HCl was added. After mixing, the mixture was heated in a water bath at 85°C for 45 min and then cooled. Hexane (Sigma Aldrich) was introduced for FAME extraction. Following centrifugation for 15 min at 4,000 rpm, the hexane (upper layer) containing the fatty acid methyl esters was transferred into vials using Pasteur pipettes and promptly subjected to analysis.

2.4.1 Gas chromatographic condition

Gas chromatography using Agilent Technologies AGILENT 6890/7890 GC and ChemStation Operation with an FID detector was employed to analyze fatty acid methyl esters (FAMEs). The separation of FAMEs took place on a CP-Sil88 capillary column (a 100-m fused

silica capillary column with 0.25 mm internal diameter, coated with 0.2 µm cyano-propyl-polysiloxane as the stationary phase) provided by Supelco (Bellefonte, PA, USA). Chromatographic conditions involved 1 µL injections of the FAME mixture at a split ratio of 20:1. The split inlet conditions included an injector temperature of 250°C, an injector split flow of 20 mL/min, a pressure of 31,623 psi, and a total flow of 24 mL/min. The oven temperature program was initiated at 80°C, increased by 4°C/min up to 220°C (held for 5 min), then increased by 4°C/min up to 240°C, and held at 240°C for 10 min. Helium served as the carrier gas (constant flow of 1.0 mL/min), and nitrogen acted as the makeup gas with a flow of 25 mL/min. The FID detector operated at a temperature of 270°C, and the run time was 55 min. ChemStations was utilized for data collection and analysis, including the identification and quantification of peaks. Chromatographic peak identification was achieved by comparing retention times with an appropriate standard of FAMEs (Supelco FAME Mix, Bellefonte, PA). Quantification relied on the ratio between all peak areas and the corresponding peak, with results expressed as a percentage of individual fatty acids in total fatty acids. The column's efficiency, expressed as the number of theoretical plates of three standard fatty acids (palmitic, stearic, and oleic), ranged from 362,870 to 510,262. The reproducibility of the response, determined as a percentage of the relative standard deviation (RSD%) for successive measurements of the same reference solution, ranged from 2.3 to 4.6 for the same standard fatty acids.

TABLE 2 Fatty acid composition of fish oil (% w/w of total fatty acids).

SFA	16:0n	Palmitic	22.90
	18:0n	Stearic	2.23
MUFA	16:1n-7	Palmitoleic	11.90
	18:1n-7	Vaccenic	4.54
n-6	18:2n-6	Linoleic	1.67
	20:3n-6	Dihomo-gama-linolenic	0.29
	20:4n-6	Arachidonic	1.62
	22:4n-6	Adrenic	1.78
n-3	20:5n-3	EPA	25.51
	22:5n-3	DPA	1.82
	22:6n-3	DHA	15.49

SFA, saturated fatty acids; MUFA, mono-unsaturated fatty acids; n-6, omega-6 polyunsaturated fatty acids (PUFA); n-3, omega-3 polyunsaturated fatty acids; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

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

2.5.1 RNA isolation and reverse transcription

Total RNA was extracted from the eyes of both control and FO-treated animals ($N = 5–7$ per group) using the TRIzol isolation system, following the manufacturer's guidelines (Invitrogen Life Technologies, USA). The RNA pellet was dissolved in 20 mL of DEPC water, and the RNA concentration was determined using spectrophotometry. Additionally, RNA integrity was confirmed through 1% agarose gel electrophoresis. Subsequently, 6 mg of total RNA underwent treatment with RNase-free DNase I (Thermo Fisher Scientific, Waltham, MA, USA) and was reverse transcribed in the same tube using a High-Capacity cDNA Archive Kit (Applied Biosystems, USA), following the manufacturer's protocol. The resulting cDNA was stored at −20°C for future use.

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

PCR analysis utilized 20 ng of the resultant cDNA in a final volume of 10 µL with RT2SYBR Green qPCR Mastermix (Applied Biosystems). RT-PCR amplifications were conducted in an ABI 7500 thermal cycler (Applied Biosystems) following the default cycling mode (500°C for 30 min, 950°C for 15 min, followed by 40 cycles of 940°C for 60 s, 570°C for 60 s, 720°C for 60 s, and then incubation at 700°C for 10 min). The qRT-PCR results were analyzed using RQ Study add-on software for the 7,000 v 1.1 SDS instrument, with a confidence level of 95% ($p < 0.05$). Quantification was performed using the 2-DDCt method (57), and the change in mRNA levels was expressed relative to the control value. Primer sequences (Vivogen, Serbia) can be found in Table 3.

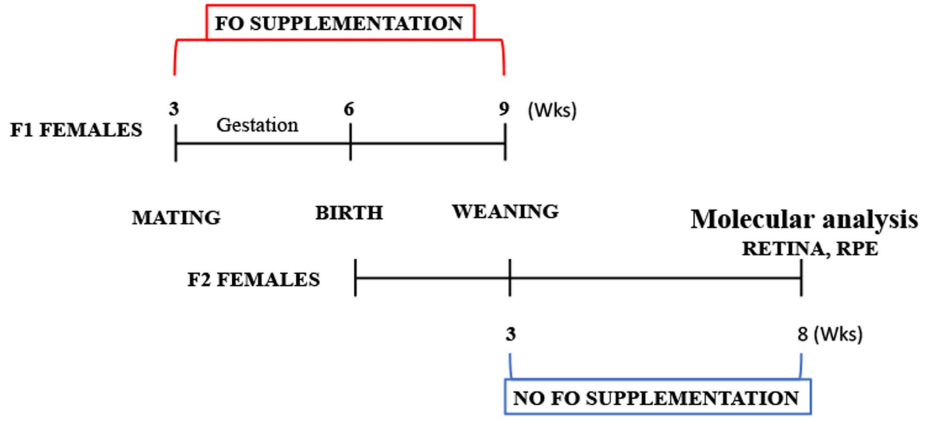

FIGURE 1
Schematic depiction of the study design.

TABLE 3 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
Srebf1	F	ACG GAG CCA TGG ATT GCA
	R	AAG TCA CTG TCT TGG TTG TTGATGA
Nr1h2 (*LXRB*)	F	AGC GTC CAT TCA GAG CAA GTG
	R	CAC TCG TGG ACA TCC CAG ATC T
Abca1	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 R	AGA AGC AGC AAC TGT CCA TTT. CTC GGC CCA CAA AAA GGA TAA T
Adipor1	F R	AAG CCA AGT CCC AGG AAC AC CAG TGG GAC CGG TT GC
Cyp46a1	F R	TGC AGT ATC TGT CGC AGG TC TAG GTG CTG AAC AGG AGA GG
Cyp27a1	F R	CGC TAG TCT CCC TAT GTC ACT ATG C AGC CGA AGG GAA GAG ATG C
Hprt1	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.

2.6 Statistical analysis

The Prism program (GraphPad Software) was employed for data analysis. The non-parametric Mann–Whitney *U* test was utilized to compare two experimental groups, given that the data did not adhere to the normal distribution criteria. Statistical significance was established at $p < 0.05$.

3 Results

3.1 The high-dose FO treatment during pregnancy and lactation altered the fatty acid content in the retinas and RPE of the 2-month-old offspring

We evaluated the alterations in the lipid composition, specifically n-6 and n-3 fatty acids, resulting from high-dose fish oil (FO) supplementation in the retinas and retinal pigmented epithelia (RPE) of the 2-month-old offspring (Figures 2A–E). In the offspring's retinas, there was a significant reduction in EPA levels (3.1-fold decrease, Figure 2A). Conversely, the levels of DHA and DPA remained unchanged (Figure 1A). However, in the RPE, all analyzed n-3 fatty acids, including EPA (2.95-fold decrease), DPA (2.18-fold decrease), and DHA (1.38-fold decrease), exhibited a reduction (Figure 2B). Regarding n-6 fatty acids, there was a decrease in dihomo-gamma-linoleic acid (DHGLA) levels (55%) in the FO-supplemented retinas (Figure 2C). In the RPE, along with a decrease in DHGLA levels (53%), there was a significant reduction in adrenic acid levels (32% decrease) (Figure 2D). While the n-6/n-3 ratio in the retina remained unchanged in FO-treated and control offspring, a significant decrease was observed in the RPE (65% decrease, Figure 2E).

3.2 The impact of fish oil treatment on the overall polyunsaturated fatty acids, mono-unsaturated fatty acids, and saturated fatty acids in both the retina and retinal pigmented epithelium

Given that the analysis of fish oil (FO) content revealed the presence of fatty acids other than essential PUFAs, particularly palmitic, palmitoleic, and oleic (Table 2), we investigated whether FO intake alters the levels of saturated fatty acids (SFAs) and mono-unsaturated fatty acids (MUFAs) in both the retinas and RPE. In the retinas of the FO-supplemented offspring, the sole observed change was a decrease in oleic acid levels (34%) (Table 4). However, in the FO-supplemented RPE, there was an increase in the levels of palmitic (12%), palmitoleic (91%), and oleic acid (41.5%) (Table 4). Despite this, total SFA and PUFA remained unchanged after FO treatment in both the retinas and RPE. Notably, n-3 levels were significantly reduced in the RPE (28%), whereas total MUFA showed an increase in the RPE exclusively (42%).

3.3 The high-dose FO supplementation during pregnancy and lactation resulted in an elevation of Mfsd2a expression in both the retinas and RPE of the 2-month-old offspring

Two specific proteins, Mfsd2a (48) and the adiponectin receptor 1 (Adipor1) (58), have been proposed as facilitators in the transport of DHA. Mfsd2a is a transporter highly specific for DHA, with lower specificity for palmitic and oleic acids. Adipor1 has recently been identified as a novel DHA transporter crucial for the proper functioning and maintenance of photoreceptors. The expression levels of *Adipor1* were unaffected by the FO treatment in both the retinas and RPE (Figure 3A). In contrast, the expression levels of *Mfsd2a* significantly increased in both the retina (Figure 2B, 84% increase) and the RPE (Figure 3B 3.5-fold increase) following FO supplementation.

Sterol regulatory element-binding protein 1-c (Srebp1-c) is among the suggested targets of Mfsd2a expression (59). Another function of Srebp1-c is to enhance the transcription of genes regulating fatty acid synthesis (60). However, contrary to the reported decrease in *Srebf1* in adult animals supplemented with FO associated with the increase in *Mfsd2a* (54), the expression levels of *Srebf1* remained unchanged in the retinas (Figure 3C) and increased in the RPE of FO-supplemented offspring (6.5-fold increase, Figure 3C).

3.4 The high-dose FO supplementation during pregnancy and lactation had opposite effects on the expression levels of *Hmgcr* in the retinas and RPE

Fish oil (FO) supplementation is known for its ability to lower cholesterol, yet the biochemical analyses of serum from 2-month-old

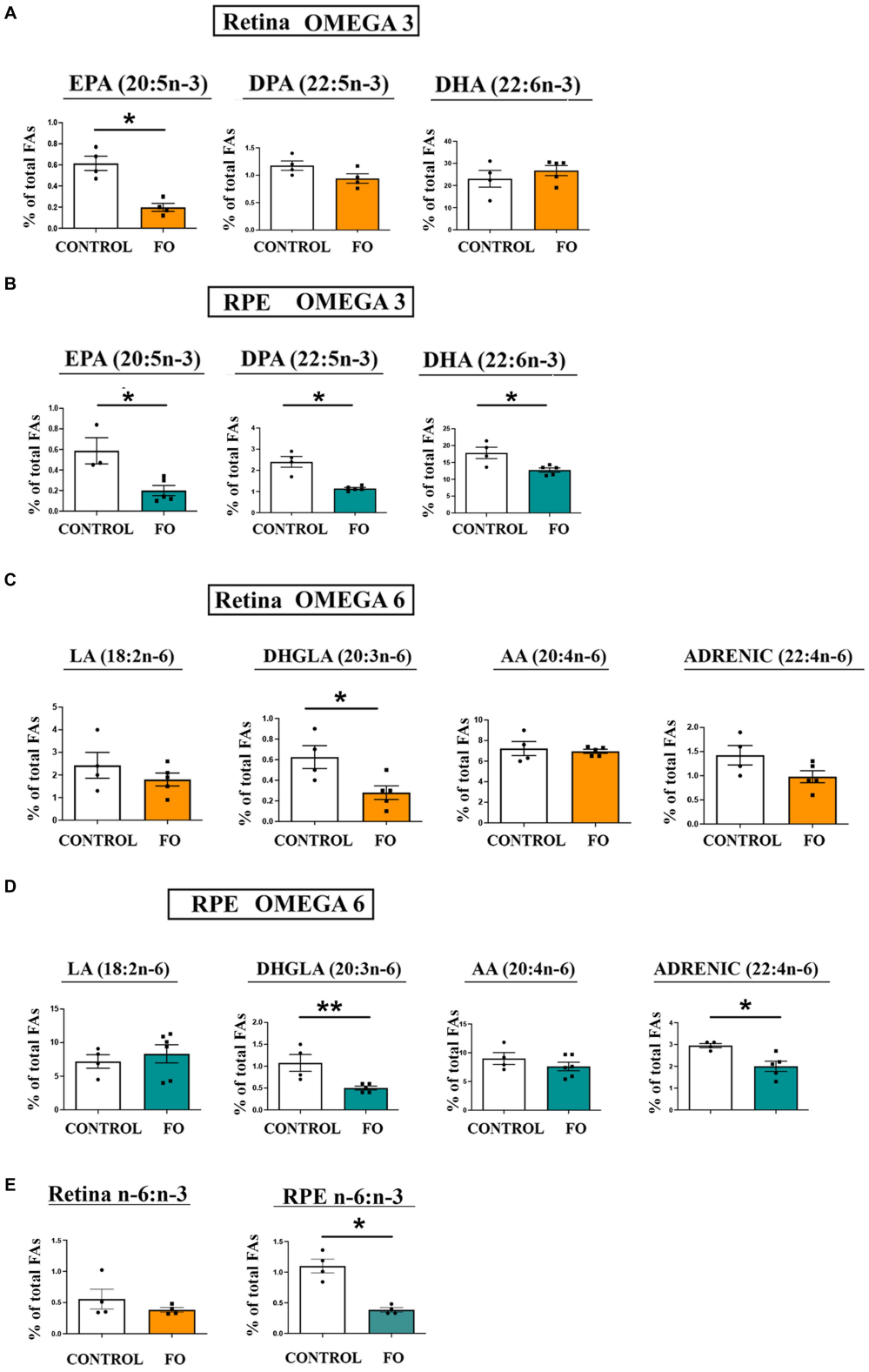

FIGURE 2
The impact of high-dose fish oil (FO) supplementation on the levels of n-3 and n-6 PUA in the retina and retinal pigmented epithelium (RPE) of both control and FO-supplemented offspring. **(A)** Displays the relative content of n-3 LC-PUFA in the retina, whereas panel **(B)** represents the same in the RPE of 2-month-old control and FO-treated offspring (FO). **(C)** Showcases the relative content of n-6 LC-PUFA in the retina, and **(D)** presents the equivalent in the RPE for both control and FO-treated offspring. The n-6/n-3 LC-PUFA ratio in the retinas and RPE for both groups is depicted in panel **(E)**. The abbreviations denote 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), and docosahexaenoic acid (DHA, 22:6n-3). The data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$.

TABLE 4 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)	19.93 ± 0.22	20.9 ± 0.28	18.58 ± 0.29	20.95 ± 0.724[b]
Stearic acid (18:0)	26.13 ± 1.84	25.74 ± 1.52	22.65 ± 0.56	19.57 ± 0.89
SFA	46.5 ± 1.97	47 ± 1.61	41.7 ± 0.71	41.2 ± 0.75
Palmitoleic acid (16:1n-7)	0.45 ± 0.06	0.34 ± 0.05	1.17 ± 0.26	2.25 ± 0.25[b]
Oleic acid (18:1n-9)	9.87 ± 0.54	6.48 ± 0.37[a]	10 ± 0.72	14.15 ± 0.65[c]
Vaccenic acid (18:1n-7)	1.53 ± 0.21	1.58 ± 0.18	2.03 ± 0.18	2.45 ± 0.07
MUFA	10.7 ± 1.23	8.46 ± 0.56	13.2 ± 0.81	18.95 ± 0.90[c]
n-6	12.2 ± 1.12	10 ± 0.02	22.5 ± 1.83	18.9 ± 0.08
n-3	24.8 ± 3.76	27.1 ± 2.79	20.2 ± 1.66	14.6 ± 0.59[c]
PUFA	32.7 ± 3.29	35.5 ± 2.39	34 ± 2.19	29.6 ± 0.935

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]$p < 0.05$, [c]$p < 0.001$.

FO-supplemented offspring revealed no differences in the levels of cholesterol, HDL, LDL, and triglyceride (Figure 4A). Considering that analyses of retinas from Mfsd2a$^{-/-}$ animals showed altered expression of genes regulating cholesterol and fatty acid synthesis (59, 61), we investigated whether FO supplementation affected the expression levels of genes regulating cholesterol synthesis. Specifically, we examined liver X receptor beta *Nr1h2* (*LXRB*) (62) and endoplasmic reticulum-bound 3-hydroxy-3-methylglutaryl-coenzyme-A reductase (*Hmgcr*) expression levels (63). The qRT-PCR analysis revealed that FO supplementation had no effect on the expression levels of *Nr1h2* (*LXRB*) in the retinas but induced a 2.48-fold increase in its expression in the RPE (Figure 4B). Simultaneously, the expression levels of *Hmgcr* decreased in the retinas (65% decrease) but increased in the RPE (2.65-fold increase) following FO treatment (Figure 4C).

3.5 The high-dose FO supplementation during pregnancy and lactation modified the expression levels of genes involved in regulating cholesterol transport

We investigated the impact of FO supplementation on cholesterol turnover. Cells efficiently recycle cholesterol through an apolipoprotein-dependent cascade, with apolipoprotein E (ApoE) being the most abundant member (64). The lipidation process is facilitated by the ATP-binding cassette transporter A1 (ABCA1), located in plasma membranes, which expels cholesterol and phospholipids from the cells (64). qRT-PCR analyses indicated that FO supplementation had no effect on *Apoe* and *Abca1* expression levels in the retinas (Figures 5A,B). However, in the RPE of FO-supplemented offspring, the expression levels of *Abca1* and *Apoe* transporters were significantly higher compared to the controls (8-fold and 2.85-fold, respectively) (Figures 5A,B).

Excess cholesterol is primarily eliminated by converting it into 24(S)-hydroxycholesterol (24S-OHC) through the enzyme cholesterol 24-hydroxylase (CYP46A1), predominantly found in the brain (65), and into 27-hydroxycholesterol through the enzyme cholesterol 27-hydroxylase (CYP27A1), a ubiquitous cholesterol 27-hydroxylase (66). Additionally, cytochrome (CYP27A1 and CYP46A1) levels regulate LXR. The qRT-PCR analysis revealed that FO supplementation during pregnancy and lactation had no impact on the expression levels of cholesterol degradation genes *Cyp27a1* and *Cyp46a1* in both retinas or RPE in the offspring (Figures 5C,D).

4 Discussion

Scientists and clinicians recommend supplementing with FO, rich in DHA and EPA, during pregnancy and lactation to support the proper development of neural and visual systems. High-dose omega-3 supplementation for pregnant women is strongly advocated (20, 34–36, 39). In this study, we examined the long-term effects of high-dose FO supplementation in healthy pregnant and lactating WT mice on the retinas and RPE of 2-month-old offspring (early adulthood). The key findings include a significant increase in Mfsd2a expression, a primary DHA transporter, in both the retinas and RPE, as well as notable changes in lipid profiles and expression levels of cholesterol metabolism-related genes in both tissues.

FO supplementation during pregnancy and lactation led to persistent changes in fatty acid content, even 5 weeks after supplementation cessation. In the retinas, there was a significant decrease in EPA levels (3.1-fold decrease) among n-3 LC-PUFAs, whereas DHA levels remained unchanged, ensuring proper photoreceptor function. On the other hand, in the RPE, all n-3 LC-PUFAs—EPA, DPA, and DHA—experienced significant decreases (2.95-fold, 2.18-fold, and 1.38-fold, respectively). These results can be explained by the significant lag between the end of the supplementation and the time of analysis (5 weeks). The retinas and RPE of the offspring were exposed to high levels of PUFA supplementation throughout the development, followed by the abrupt termination of the supplementation 3 weeks after birth, coinciding with the termination of lactation and subsequent weaning. The offspring were then switched to commercial chow with a substantially lower content of n-3 PUFAs. It is possible that the offspring that were exposed to the FO supplementation developed a different baseline for the n-3PUFA content and that the period between the end of the FO supplementation and analysis

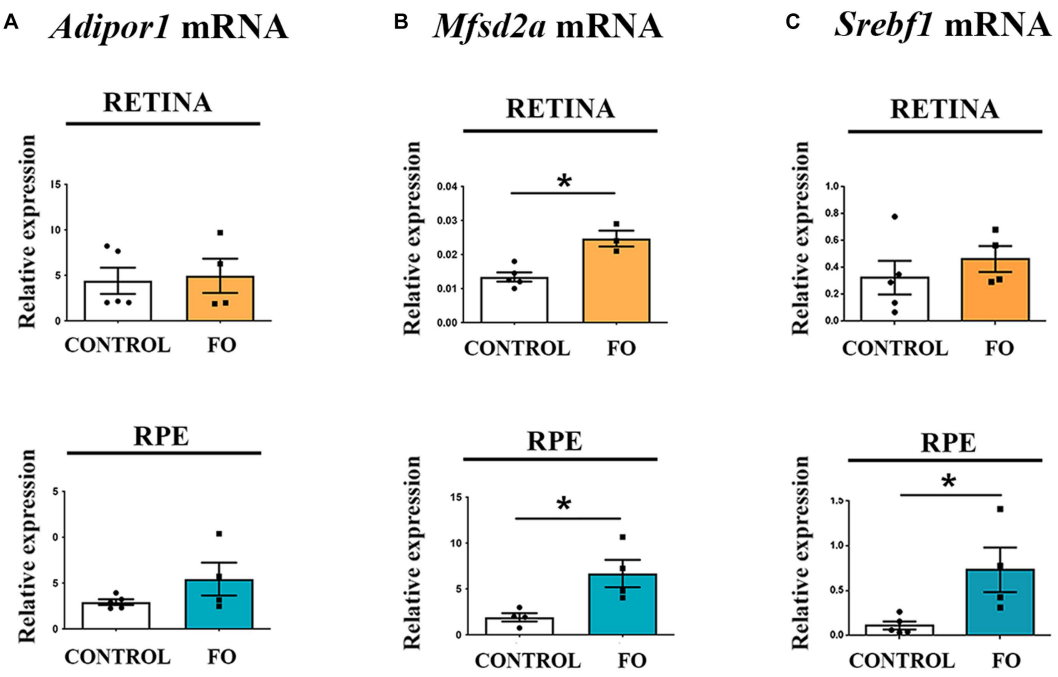

FIGURE 3
The expression levels of *Mfsd2a* were influenced by high-dose fish oil (FO) supplementation, whereas *Adipor1* levels remained unaffected in the retina and retinal pigmented epithelium (RPE) of 2-month-old offspring. **(A)** Real-time polymerase chain reaction (RT-PCR) was utilized to analyze the alterations in *Adipor1* expression in the retina (yellow) and RPE (blue). **(B)** RT-PCR was employed to assess changes in *Mfsd2a* expression in the retina (yellow) and RPE (blue). **(C)** RT-PCR was used to examine changes in *Srebf1* expression in the retina (yellow) and RPE (blue). The data are presented as mean ± SEM. *$p < 0.05$.

can be perceived as n-3 deprivation. The results suggest a potential period of n-3 deprivation in the offspring. Similarly, in the brains of the offspring that were FO-supplemented pre- and post-natally (67), a continual gradual decline in the levels of DHA was observed over the 8 weeks after the termination of the FO supplementation. The decline in the levels of DHA was similar to our findings in the RPE, while the DHA homeostasis in the retina was not disrupted. At the same time, the increased levels of DPA in the brain were not in correlation with the observed decrease in the levels of DPA in the RPE. These findings suggest a more plastic nature of the RPE considering its physiological role as a "gate-keeper" (68).

Recent studies suggest that EPA could serve as a readily available precursor for DHA synthesis in neuronal tissues, especially in diets enriched with ALA but deficient in DHA (69). The protective benefits of EPA supplementation, however, depend on its conversion to DHA through elongation and desaturation processes in neuronal cultures (70). Typically, elongase and desaturase activities are lower in the brain compared to the liver, where the primary source of brain and retinal DHA is synthesized from circulating ALA (71–73). While it is improbable for neuronal enzymatic activities to be solely responsible for high DHA levels in the retina, they might play a role in maintaining DHA levels in photoreceptors during transient decreases in plasma DHA. These enzymes could also be involved in responding to minor local changes in DHA levels in photoreceptor phospholipids. As DHA is released from these lipids, it activates the retinoid X receptor (RXR) and the ERK/MAPK pathway for photoreceptor protection (74, 75). Neuronal fatty acid elongase and desaturase enzymes may be activated to synthesize and replenish DHA in phospholipids, possibly from EPA, accounting for its decrease.

The impact of FO treatment on n-3 PUFAs in the RPE was more pronounced than in the retinas, leading to a significant decrease in all analyzed n-3 fatty acids—EPA, DPA, and DHA (2.95, 2.18, and 1.38-fold decrease, respectively). The protective role of RPE in maintaining retinal homeostasis could be one of the reasons for these changes, although other mechanisms cannot be ruled out. In a previous study, we demonstrated that high-dose fish oil (FO) supplementation maintained for 3 weeks in 3-month-old mice (adulthood) significantly altered n-3 PUFAs in the retinas and RPE but that the levels of DHA were also unaltered (54). These results confirm that the rigorous regulation of DHA homeostasis in the retina is necessary for the proper functioning of photoreceptors, regardless of the age of the supplementation.

FO supplementation also influenced n-6 PUFA levels, resulting in decreased DHGLA in the retinas and DHGLA and adrenic acid in the RPE. A similar decrease in DHGLA in the RPE was observed in adult animals that were supplemented with FO for a short period of time (3 weeks) (54). Although the n-6 PUFA changes were relatively small, they contributed to an increased n-6/n-3 ratio in the RPE of 2-month-old offspring, while the n-6/n-3 ratio in the retinas remained unaltered. These results strongly suggest that it would be beneficial to maintain the levels of FO supplementation in the late postnatal stages and in adolescence.

Notably, high-dose FO supplementation during pregnancy and lactation elevated *Mfsd2a* expression 5 weeks post-treatment (Figure 2). Increased *Mfsd2a* levels in the retina may contribute to DHA homeostasis, maintaining lipid saturation and cell membrane fluidity (48). In addition, the increase in the expression of the DHA transporter, *Mfsd2a*, was observed both in the adult supplementation and in the offspring that was supplemented during development (54) suggesting

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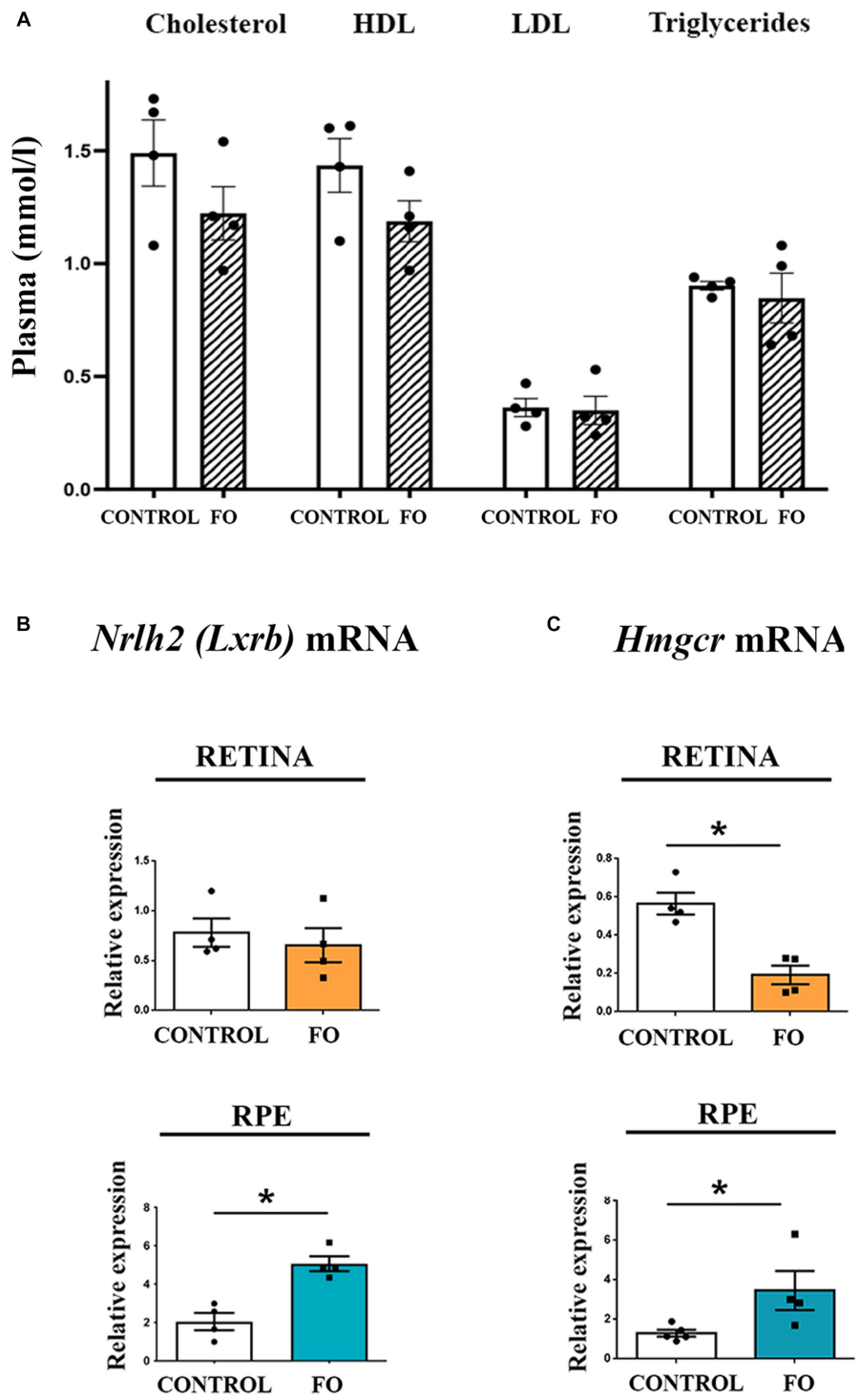

FIGURE 4
Examination of cholesterol levels in serum and the expression of genes regulating cholesterol synthesis in the retina and retinal pigmented epithelium (RPE) in control and fish oil (FO) supplemented mice. **(A)** Biochemical analyses of cholesterol, HDL, LDL, and triglyceride levels (mmol/l) in the plasma of control and FO-treated mice. Expression levels of *Nr1h2* (*LXRB*) **(B)** and *Hmgcr* **(C)** were assessed in the retina (yellow) and RPE (blue) of control and FO-supplemented offspring using real-time polymerase chain reaction (RT-PCR). The data are presented as mean ± SEM. *$p < 0.05$.

that FO supplementation is responsible for this increase, although it is possible that the mechanisms involved in the upregulation of *Mfsd2a* differ depending on the time of the supplementation.

Mfsd2a is specifically expressed in microvessels of the CNS that constitute the blood–brain barrier (BBB), playing crucial roles in both the formation and functioning of the BBB (43, 44). Studies in mice have shown that Mfsd2a expression begins at embryonic day 13.5 (E 13.5) in the BBB, which becomes functional by E15.5 (47). In Mfsd2a knockout (KO) animals, the absence of Mfsd2a resulted in a leaky BBB during embryogenesis, neonatal development, and adulthood without

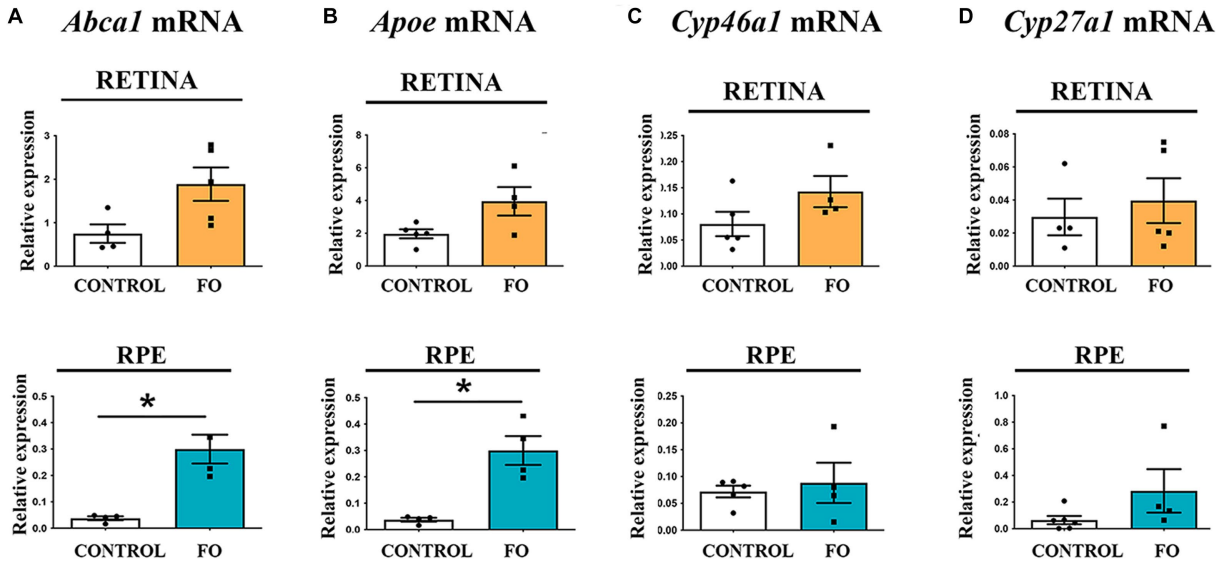

FIGURE 5
Gene expression profiles associated with cholesterol transport and elimination in the retinas and RPE of both control and FO-supplemented mice. The analysis focused on the expression levels of *Apoe* **(A)**, *Abca1* **(B)**, *Cyp46a1* **(C)**, and *Cyp27a1* **(D)** in the retina (highlighted in yellow) and RPE (highlighted in blue). Real-time polymerase chain reaction (RT-PCR) was employed for this assessment. The data are presented as mean ± SEM, and statistical significance is denoted by * when $p < 0.05$.

structural vascular abnormalities. Similarly, recent findings highlighted that during the formation of the blood–retinal barrier (BRB), functional tight junctions are present early in vessel ingression, but transcytosis is not yet suppressed (50). This contributes to the leakiness of the retinal vasculature during development, and the gradual suppression of transcytosis in BRB endothelial cells is required for the functional BRB to be established (50). Notably, Mfsd2a expression levels correlate with the functional formation of the BRB by regulating transcytosis. In addition, Mfsd2a deficiency leads to increased transcytosis and incomplete formation of a functional BRB, while premature suppression of transcytosis accelerates BRB development, indicating a time shift in impermeability during development (50).

Regulation of Mfsd2a expression may involve the Srebp pathway, a signaling pathway highly increased in the eyes of Mfsd2a knockout mice (76). Previous research has shown that the expression of *Srebf1* is upregulated concurrently with the decrease in *Mfsd2a* expression in 4-month-old 5xFAD retinas (77), and similarly, it is downregulated in parallel with the increase in *Mfsd2a* expression (54). However, in 2-month-old FO-supplemented offspring, where *Mfsd2a* expression levels were increased, Srebp-1-c levels were also elevated (Figure 2). Srebf1 expression is regulated by LXR, and Srebf1 promoter contains two LXR-responsive elements (LXREs). Interestingly, EPA and DHA have been shown to inhibit the LXR/RXR heterodimer binding to the LXREs in the Srebp-1-c promoter, decreasing Srebp-1-c mRNA levels (78). Therefore, the simultaneous upregulation of *Nr1h2* (*LXRB*) and the downregulation of n-3 PUFAs may contribute to the increased expression levels of *Srebf1* in the RPE.

There have been reports of a close interaction between proteins regulating cholesterol homeostasis and Mfsd2a. Our findings demonstrated increased Lxrβ expression in the RPE of 2-month-old offspring, suggesting an additional mechanism for regulating *Mfsd2a* expression. For example, treatment with the Lxr agonist T0901317 was shown to increase Mfsd2a expression in mice (76), and chromatin immunoprecipitation sequencing (ChIPseq) and gene array studies revealed Lxrβ binding sites in the mouse Mfsd2a intron (79). The upregulation of *Nr1h2* (*LXRB*) could, in turn, enhance the expression of *Hmgcr*, a key factor in cholesterol synthesis, as was observed in the RPE of 2-month-old FO-supplemented offspring (Figure 3).

As the lipid composition of CNS endothelial cells, particularly cholesterol content, plays a crucial role in regulating transcytosis and barrier permeability (51) the role of Mfsd2a as a lipid transporter delivering docosahexaenoic acid (DHA) into the brain (46) is highlighted. The proposed mechanism suggests that the inhibition of caveolae formation and subsequent suppression of transcytosis is maintained through the displacement of cholesterol with DHA and Cav-1 in the membrane (80). Consequently, the upregulation of genes involved in cholesterol synthesis in the RPE may act as a compensatory mechanism to maintain cholesterol homeostasis in the retina. However, the analysis of cholesterol content in the retinal endothelial cells of FO-supplemented offspring is currently lacking. Additionally, unraveling the exact role of proteins responsible for cholesterol synthesis in the regulation of Mfsd2a expression and transcytosis requires further studies.

There is a possibility that omega-3 regulates the expression of *Mfsd2a* through the activation of the Wnt signaling pathway. Disrupted Wnt signaling, as observed in mice lacking LRP5 or Norrin, led to an increase in retinal vascular leakage and, notably, demonstrated heightened transcytosis (81). The Wnt signaling pathway was shown to directly govern the transcription of Mfsd2a in a β-catenin-dependent manner (81), and recent research revealed that DHA supplementation is able to enhance Wnt signaling in a Wnt3a-dependent manner in human-induced pluripotent stem cell-derived neural progenitor cells (NPCs) (82). However, a more in-depth understanding of the interplay between Wnt signaling and omega-3 fatty acids is crucial for comprehending the mechanisms through which FO supplementation can regulate Mfsd2a expression during development.

5 Conclusion

The understanding of numerous diseases has been significantly advanced through the analysis of gene expression. While per-gene protein-to-mRNA ratios provide rough estimates of absolute protein abundances across genes, their ability to gauge changes in protein abundance for the same gene across samples is limited and relies on the extent of post-transcriptional, translational, and post-translational regulatory events during tissue development and homeostasis (83). Importantly, the integration of transcriptomic and proteomic data offers additional insights into the principles of gene expression control that cannot be gleaned from either type of data alone. Confirming the importance of transcriptomic analysis, nine databases have been identified that offer ocular transcriptome data from various developmental stages and diverse healthy and diseased ocular tissues. As a result, these databases contribute to the deepening of our knowledge about the molecular mediators involved, facilitate the formation of hypotheses, and assist in identifying novel diagnostic and therapeutic targets for a range of ocular diseases (84).

BRB dysfunction is a pathological characteristic observed in various ocular diseases (85–89). Thus, enhancing our understanding of ways to modulate transcytosis in BRB/BBB during development has the potential to enhance central nervous system (CNS) and retinal drug delivery by facilitating the transport of cargos of different sizes, ranging from compounds less than 1 kDa to large macromolecules. Moreover, targeting genes that regulate transcytosis could be a strategy for repairing CNS barriers in neurodegenerative diseases.

In conclusion, in the case of normal development, the recommended high-dose omega-3 (FO) supplementation may have enduring effects on lipid content and gene expression in the retina and retinal pigment epithelium (RPE) of the offspring. The findings from this study indicate that FO supplementation should be continued or gradually tapered after the lactation period.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by Ethical Committee for the Use of Laboratory Animals (resolution No. 01-06/13) of the Institute for Biological Research, University of Belgrade. The study was conducted in accordance with local legislation and institutional requirements.

Author contributions

IM: Visualization, Writing – original draft, Writing – review & editing, Data curation, Formal analysis, Methodology. ID: Formal analysis, Methodology, Visualization, Writing – review & editing. TM: Methodology, Writing – original draft, Writing – review & editing. DM: Formal analysis, Methodology, Visualization, Writing – original draft. SS: Funding acquisition, Writing – review & editing. SK: Funding acquisition, Writing – review & editing. SI: Conceptualization, Supervision, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor MT declared a past co-authorship with the authors IM and SI.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1330414/full#supplementary-material

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Effectiveness of omega-3 fatty acid supplementation for pruritus in patients undergoing hemodialysis

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Background: Patients undergoing hemodialysis (HD) frequently experience the chronic kidney disease-associated pruritus (CKD-aP).

Objective: The aim of this study was to investigate the effectiveness of omega-3 supplementation in the management of CKD-aP in patients undergoing hemodialysis.

Methods: In this triple blind, randomized clinical trial, the effect of the omega-3 supplement on uremic CKD-aP was assessed in 112 chronic hemodialysis patients at Caspian Hemodialysis Center in Rasht, Iran. Patients were randomly divided into the intervention group receiving omega-3 supplements (3 g/day) and the control group receiving placebo containing MCT oil for 2 months.

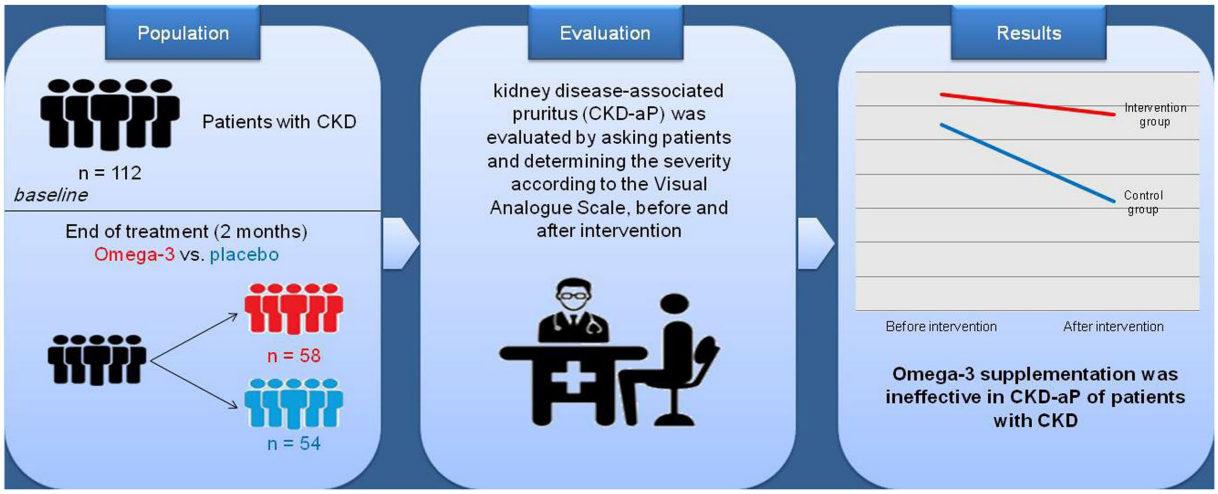
Results: Omega-3 supplementation had no effect on CKD-aP. The results did not change after adjusting for age and sex, additional adjustments for weight, height, physical activity, smoking, and alcohol use, additional adjustments for underlying diseases and weight, height, physical activity, smoking, and drinking alcohol, and further adjustments for underlying diseases and biochemical indices.

Discussion: Omega-3 supplementation for 2 months had no effect on CKD-aP in patients with CKD. Further studies with longer duration are warranted.

Clinical Trial Registration: https://www.irct.ir/trial/66638, IRCT20151226025699N6

KEYWORDS

chronic kidney disease, omega-3 fatty acids, pruritus, dialysis, CKD



GRAPHICAL ABSTRACT

Introduction

Chronic kidney disease-associated pruritus (CKD-aP), also previously known as uremic pruritus, is a common troublesome symptom in patients with advanced CKD or end-stage renal disease (ESRD) (1, 2). This complaint is most commonly described as daily or near-daily CKD-aP in large, symmetrical areas and is observed in up to 60% of dialysis patients (2–4) and affects 15–49% of pre-dialysis CKD patients and 50–90% of those undergoing dialysis, including peritoneal dialysis and hemodialysis (HD) (5).

The exact cause of pruritus in HD patients is not fully understood, but is believed to be multifactorial. Possible risk factors for uremic CKD-aP in HD patients are age, sex, calcium phosphate imbalance, prolonged dialysis duration, secondary hyperparathyroidism, concomitant cardiovascular diseases, heart failure, pulmonary diseases, liver diseases, and neurological diseases (6). Risk factors for developing pruritus in non-dialysis CKD patients include older age, female gender, advanced stage of CKD, lung disease, diabetes, and depression (1). CKD-aP dramatically influences the quality of life, so that about half of CKD patients experience from CKD-aP throughout the day and one third of them are most affected at night which causes sleep disturbances and depression (7). In particular, irritating symptoms of CKD-aP not only affect quality of life, but also lead to poor medical outcomes and patients with severe CKD-aP are also more likely to abandon or miss dialysis sessions (8). CKD-aP is also associated with worse clinical outcomes such as mortality, increased medication use (intravenous antibiotics, erythropoiesis-stimulating drugs, and iron supplementation), higher rate of infections, and hospitalizations (8–10).

Although the definite mechanisms for uremic CKD-aP have not been determined, some pathologic mechanisms are proposed for uremic CKD-aP such as increasing oxidants and inflammatory processes, loss of serum anti-oxidants in CKD patients during HD, imbalance of ions and electrolytes, and inability to excrete nitrogen products and other waste materials (11). Recent studies suggested using anti-inflammatory and antioxidant agents could lead to reduce pruritus (12). Several small studies have examined different dietary interventions against pruritus, but the efficacy and optimal treatment of these interventions are not yet well defined (13, 14).

Chronic kidney disease-associated pruritus may be influenced by essential fatty acids and their metabolites involved in the cyclooxygenase and lipoxygenase pathways, including prostaglandins and leukotrienes, respectively (15). Omega-3 fatty acid supplementation may provide many health benefits to dialysis patients by modulating the structure and function of cell membranes and the synthesis of lipid mediators such as eicosanoids. Omega-3 fatty acids have a key role in improving a variety of human body processes, including inflammatory and immune processes, atherosclerosis and cardiovascular diseases, arrhythmias, rheology, blood pressure, and lipid regulation (16). However, the effect of omega-3 fatty acids on CKD-aP in CKD patients is not clear. So, this study attempted to investigate the effectiveness of omega-3 fatty acids supplement for CKD-aP in HD patients undergoing dialysis.

Methods

Study design and participants

A randomized controlled triple blind trial was conducted on patients with CKD treated with HD in 2022–2023 at Caspian Hemodialysis Center in Rasht, Iran. A randomized block sampling method and the WinPepi program were used to assign the participants to the intervention and control groups. Finally, 16 blocks were determined and in each block, three persons were assigned in the control group and three persons were assigned in the intervention group in a random sequence. The intervention and control groups were randomly assigned using a web-based software.[1] Both groups were matched in terms of age and sex. This study was a triple blind clinical trial which ensures that neither the patients, the researchers, nor the statistical analyst are aware of the study components.

Inclusion criteria were completing written consent form, age over 20 years, KT/V (liters/min) higher than the standard range, no

1 https://www.randomizer.org

consumption of omega-3 fatty acid supplement during the last 3 months before starting the study, no history of peritoneal dialysis, no surgery in the previous 6 months, no history of hypersensitivity response to omega-3 fatty acid supplementation and/or medium chain triglycerides (MCTs) oil, no history of allergy to fish and fish products, and not to be pregnant. Exclusion criteria were refusal to continue the participation in the study, diagnosis of psychiatric conditions and intellectual disability, have active inflammatory, infection, pulmonary, cardiac, hemoglobinopathies and coagulopathy conditions, which may interfere with the research process, malignancy, and recent use of immunosuppressant, chemotherapeutic or anticoagulant drugs such as warfarin and the use of nonsteroidal anti-inflammatory medications, corticosteroids, incomplete medical documents, non-compliance with omega-3 supplementation program, patients not disciple to the hemodialysis program, disease aggravation, and the need for hospitalization and surgery.

Data on the demographic and socioeconomic status of individuals were collected using the medical records and face to face interviews. Also, medical information such as medical history, dialysis sessions per week, drug history, blood pressure, and serum biochemical indices including hemoglobin (HGB), hematocrit (HCT), and platelet count (PLT), were collected from the patients' files. The participants' height and weight were measured using a tape meter and a validated scale, respectively. The dialysis sheets were used to assess nutritional supplements received by the patients.

The intervention

Three capsules of omega-3 fatty acids supplement including 3 g of omega-3 fatty acids was given daily to the intervention group [each capsule contained about 180 mg eicosapentaenoic acid (EPA) and 120 mg dosahexaenoic acid (DHA) and 700 mg other omega-3 fatty acids; Zahravi Pharmaceutical Co, Tabriz, Iran] were given orally daily for 2 months to the patients in the intervention group. Previous studies reported high bioavailability of the fatty acids (20% of available EPA and DHA is absorbed from fish oil supplements) (17), which can increase the amount of omega-3 fatty acids in the serum (18, 19). The control group received three placebo capsules containing medium-chain triglyceride (MCT) (Zahravi Pharmaceutical Co, Tabriz, Iran). The participants were supplied with 21 capsules on a weekly basis. Both intervention and placebo consumptions were followed up with the patients through regular phone calls.

Pruritus measurements

Dialysis CKD-aP was evaluated by asking patients and after confirmation of CKD-aP diagnosis. The assessment of itch severity was performed by the Visual Analog Scale (the WI-NRS) (20). A score of 0–16 was considered as no CKD-aP and a score of 17–48 was considered as having pruritus.

Statistical analysis

The Kolmogorov–Smirnov test was used to determine the normal distribution. T-test and Chi-squared test were used to compare the quantitative and qualitative data between two groups, respectively. The logistic regression method was used to determine the effect of omega-3 fatty acids supplementation on CKD-aP after adjusting the confounding variables including age, sex, weight, height, physical activity, smoking, drinking alcohol, underlying diseases, and biochemical indices in different models. SPSS version 20 was used for all statistical analysis and a probability level of $p < 0.05$ was considered to be statistically significant.

Results

General characteristics of participants are presented in Table 1. Totally, 112 patients, with mean age of 61.17 ± 12.35 in the intervention group and 55.33 ± 12.6 in the placebo group were included ($p > 0.05$). All the demographic data or clinical characteristics at baseline were normally distributed and the groups were not significantly different. No significant difference was found between the groups regarding weight, height, amount of sleep, HGB, CBC, PLT, sex, marital status, history of diabetes, hypertension, metabolic disorder, heart disease, tobacco use, and alcohol use.

The data in Table 2 show that no significant difference was found between the frequency of CKD-aP between the intervention and control groups before and after the intervention. Furthermore, decrease in the frequency of CKD-aP in the intervention group (31.7–28.8%) was not statistically significant compared to the placebo group (27.3–16%) (Table 3). The effect of supplementation with omega-3 on CKD-aP in CKD patients was not significant after adjustments for age and sex (Model 1) (OR = 0.72; 95% CI: 0.27–1.92; $p = 0.51$), additional adjustments for weight, height, physical activity, smoking, and drinking alcohol (OR = 0.9; 95% CI: 0.3–2.72; $p = 0.87$) (Model 2) and further adjustments for underlying diseases and biochemical indices (OR = 0.79; 95% CI: 0.24–2.54; $p = 0.69$) (Model 3).

Discussion

In the present study, the effect of omega-3 supplementation on CKD-aP in HD patients was examined. The results showed that although the CKD-aP decreased after the intervention, there was no statistically significant difference between the intervention group and the placebo group after the intervention (Graphical abstract). Previous studies reported contradictory results on the efficacy of omega-3 fatty acid supplementation on CKD-aP in CKD patients. Several previous reports demonstrated the beneficial effects of omega-3 fatty acids supplementation in treatment of uremic CKD-aP in HD patients. For example, Ghanei et al. (13) by using omega-3 fatty acids in a double-blind randomized study found that CKD-aP was decreased up to 65% in HD patients suffering from pruritus. So it seems that omega-3 fatty acids could be used as an efficient strategy in the treatment of CKD-aP in uremic patients (13). In addition, Shayanpour et al. (14) concluded that the omega-3 supplement could reduce uremic pruritus in chronic HD patients. They found that the mean score of itching score in the intervention group decreased from 3.56 to 1.72 ($p < 0.001$) (14).

Moreover, in contrast with the present study, Begum et al. preformed a prospective, randomized, double-blinded, controlled study to compare

TABLE 1 The general characteristics of the participants.

	Intervention group ($N = 58$)	Placebo group ($N = 54$)	p*
Age (years)	61.17 ± 12.35	55.33 ± 12.6	0.14
Weight (kg)	67.87 ± 13.64	71.21 ± 14.0	0.204
Height (cm)	164.28 ± 9.32	167.19 ± 9.02	0.094
Amount of sleep (h)	8.0 ± 2.51	7.96 ± 2.52	0.951
HGB	11.0 ± 1.53	11.46 ± 1.42	0.100
HCT	34.98 ± 4.75	36.34 ± 4.49	0.118
PLT	197.33 ± 75.18	205.92 ± 86.58	0.577
Sex			
Females, *n* (%)	28 (48.3)	17 (31.5)	0.084
Male, *n* (%)	30 (51.7)	37 (68.5)	0.08
Married, *n* (%)	53 (94.6)	48 (92.3)	0.71
Diabetes, *n* (%)	26 (43.3)	26 (47.3)	0.71
Hypertension, *n* (%)	48 (81.4)	41 (74.5)	0.49
Heart Disease, *n* (%)	16 (26.7)	14 (25.5)	1.00
Tobacco use, *n* (%)	4 (6.7)	8 (14.5)	0.23
Alcohol use, *n* (%)	0 (0.0)	1 (1.8)	0.47

*Obtained using independent *t*-test and qui-squared test for quantitative and qualitative variables, respectively. $p < 0.05$ was considered as significant.
HGB, Hemoglobin; HCT, Hematocrit; and PLT, Platelet count.

TABLE 2 Frequency of pruritus among the control and intervention groups.

	Controls	Intervention	p*
Pruritus before intervention	15 (27.3%)	19 (31.7%)	0.68
Pruritus after intervention	4 (16%)	15 (28.8%)	0.27
p*	0.08	0.34	

*Obtained using qui-squared test. $p < 0.05$ was considered as significant.

TABLE 3 The association of omega-3 supplementation and pruritus in patients with CKD.

	OR (CI95%)	p*
Model 1	0.72 (0.27–1.92)	0.51
Model 2	0.91 (0.3–2.72)	0.87
Model 3	0.79 (0.24–2.54)	0.69

*Obtained using logistic regression. Model 1: Adjusted for age and sex, Model 2: Further adjustments for weight, height, physical activity, smoking, and drinking alcohol, and Model 3: Additional adjustments for underlying diseases and biochemical indices.

the effects of the supplementations with fish oil, rich in omega-3 fatty acids, compared with safflower oil, rich in omega-6 fatty acids, on symptoms of CKD-aP in HD patients. The intervention group received six fish oil capsules (728 mg omega-3 fatty acids in each capsule) and the control group received six safflower oil capsules (704 mg omega-6 fatty acids in each capsule) per day for 16 weeks. The results showed that despite the absence of a significant difference in the mean baseline of CKD-aP score between two groups, the frequency of CKD-aP decreased in the fish oil group compared to the safflower oil group after 16 weeks (15). However, the duration of this intervention was almost twice as long as the present study and it is possible that anti CKD-aP effects of omega-3 fatty acids appear after long term supplementation. Some studies have shown that it takes at least 3 months for omega-3 fatty acids to exert their anti-inflammatory effects (21). Furthermore, in our study, MCT was used as a placebo for the control group, and at the end of the study, the rate of CKD-aP decreased in both groups. It is possible that MCT oil, similar to omega-3 fatty acids, has beneficial effects in reducing CKD-aP. However, there are few studies on the effects of MCT on CKD-aP. A recent study by Abbasi et al. (22) reported that coconut oil, which is a rich source of MCT, was effective on reducing pruritus in ESRD patients. Also, the anti-itching effects of omega-3 fatty acids may be different according to their origin (animal or vegetable) (23, 24) or the composition of their fatty acids (25).

The exact mechanisms of the possible effects of omega-3 fatty acids on CKD-aP is not yet determined. Dysregulation of immune system and chronic inflammation are among the potential contributors of the development of CKD-aP through numerous mediators including interleukin-2 (IL-2), prostaglandin E2 (PGE2), serotonin, histamine, proteases, and platelet activating factor. Anti-inflammatory and immune regulatory properties of the omega-3 fatty acids might be responsible for

ameliorating CKD-aP (26). Other causes of the ineffectiveness of omega-3 fatty acids in CKD-aP is that other inflammatory mediators which are considered to be independent from the effects of omega-3 fatty acids (such as serotonin, histamine, protease, platelet-activating factor, etc.) may play a role in the clinical symptoms of CKD-aP. Moreover, CKD-aP may also be caused by non-inflammatory factors such as uremia, hyperparathyroidism, and calcium-phosphate imbalance (27).

However, the present study had some limitations. First, other predisposing factors for CKD-aP such as uremia and hyperparathyroidism were not controlled in this study. In addition, the duration of the intervention period was relatively short. Despite some study limitations, it should be noted that even mild relief of CKD-aP may have clinical significance in patients' condition. Therefore, considering the many health benefits of omega-3 fatty acids for CKD and the negligible risk profile, omega-3 intake may be widely applicable for CKD patients.

Conclusion

This randomized clinical trial did not support the efficacy of omega-3 fatty acid supplementation in CKD-aP of patients with CKD undergoing hemodialysis. Further clinical studies with different doses and types of omega-3 fatty acids, larger sample sizes, and longer durations along with the evaluation of potential pathophysiological pathways involved in CKD-aP are warranted to provide evidence-based recommendations and clinical guidelines regarding the effects of omega-3 fatty acids on CKD-aP.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

This study was approved by the ethical committee of the cancer research center, Guilan University of Medical Sciences (code: IR.GUMS.REC.1401.307). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

AR: Software, Writing – original draft. MT: Data curation, Writing – original draft. FA: Data curation, Writing – original draft. AT: Data curation, Writing – original draft. MM: Writing – original draft. AK: Data curation, Writing – original draft. HS: Data curation, Writing – review & editing. PB: Data curation, Writing – review & editing. MK: Project administration, Writing – original draft. SD: Methodology, Software, Writing – original draft. MG: Software, Writing – original draft. PM: Data curation, Writing – review & editing. ZM: Data collection, editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Optimal omegas – barriers and novel methods to narrow omega-3 gaps. A narrative review

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Dietary intakes of omega-3 long chain polyunsaturated fatty acids (O3LC-PUFAs) such as eicosapentaenoic and docosahexaenoic acid are central to development and health across the life course. O3LC-PUFAs have been linked to neurological development, maternal and child health and the etiology of certain non-communicable diseases including age-related cognitive decline, cardiovascular disease, and diabetes. However, dietary inadequacies exist in the United Kingdom and on a wider global scale. One predominant dietary source of O3LC-PUFAs is fish and fish oils. However, growing concerns about overfishing, oceanic contaminants such as dioxins and microplastics and the trend towards plant-based diets appear to be acting as cumulative barriers to O3LC-PUFAs from these food sources. Microalgae are an alternative provider of O3LC-PUFA-rich oils. The delivery of these into food systems is gaining interest. The present narrative review aims to discuss the present barriers to obtaining suitable levels of O3LC-PUFAs for health and wellbeing. It then discusses potential ways forward focusing on innovative delivery methods to utilize O3LC-PUFA-rich oils including the use of fortification strategies, bioengineered plants, microencapsulation, and microalgae.

KEYWORDS

bioavailability, delivery methods, docosahexaenoic acid, eicosapentaenoic acid, food fortification, long-chain polyunsaturated fatty acids, microalgae, microencapsulation

Introduction

Omega-3 long chain polyunsaturated fatty acids (O3LC-PUFAs) include α-linolenic acid (ALA; 18:3), stearidonic acid (SDA; 18:4), eicosatetraenoic acid (20:4), eicosapentaenoic acid (EPA; 20:5), docosapentaenoic acid (DPA; 22:5), and docosahexaenoic acid (DHA; 22:6) (1) (Figure 1). One means of assessing the conversion rate of ALA to the longer-chain products EPA, DPA and DHA is to determine the net rise in blood circulating levels of these fatty acids after increasing ALA intakes in controlled human trials. Isotope studies by Burdge and Calder (4) have demonstrated that ALA seems to be a limited source of O3LC-PUFAs, particularly for males although estrogen may improve the conversion of ALA to O3LC-PUFAs in women which could be a regulatory mechanism to meet fetal and neonate DHA needs. Other research using isotope labeled fatty acids in young women found that estimated ALA inter-conversion was 21% for EPA and just 6% for DPA and 9% for DHA (5).

Regarding omega-6 long chain polyunsaturated fatty acids linoleic acid (the parent omega-6 fatty acid) yields gamma-linolenic acid, dihomo-gamma-linolenic acid, arachidonic

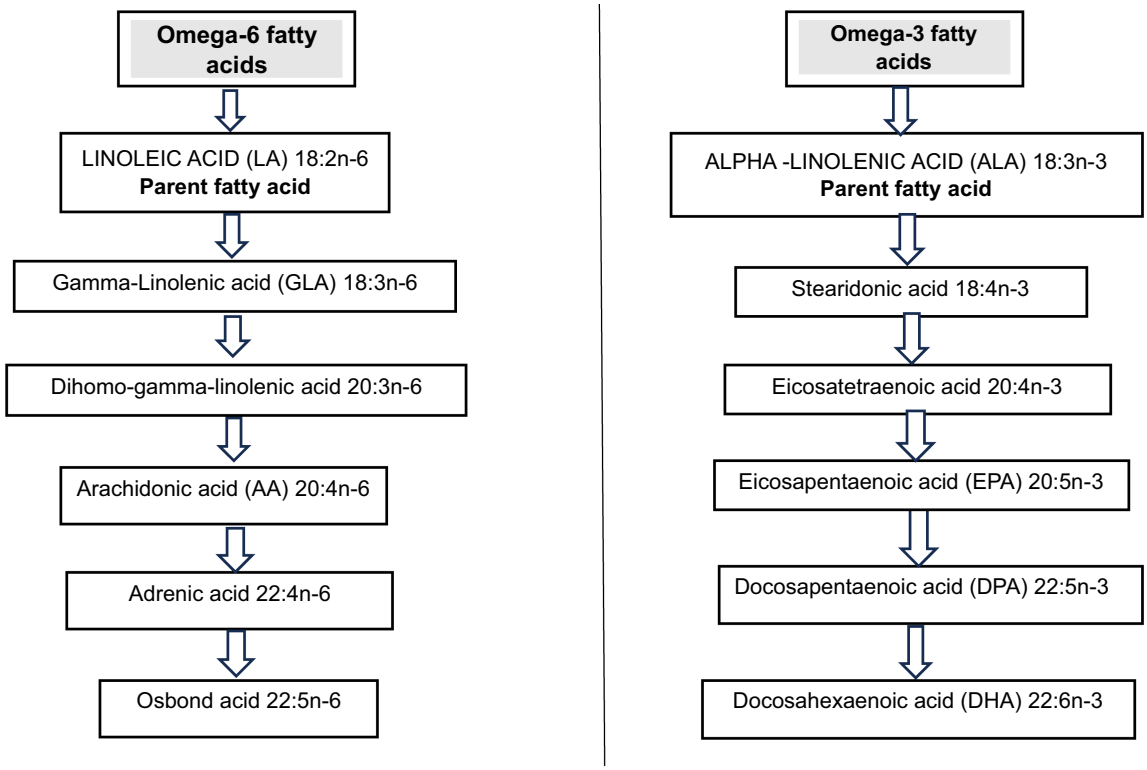

FIGURE 1
Omega-6 and omega-3 metabolic pathways. Source: Adapted from Miles and Calder (2) and Bird et al. (3).

acid, adrenic acid and then osbond acid (2). It was George and Mildred Burr in 1929 who first deciphered the "essentiality" of these fatty acids when rodent models were fed a fat-free diet (6). They identified that fatty acids were essential nutrients and found that the omega-6 fatty acid linoleic acid prevented disease thus was an 'essential' fatty acid (7). They also found that linolenic acid (the omega-3 analog of linoleic acid) was also an essential fatty acid (7). Linoleic acid and ALA acid cannot be produced endogenously by humans (mammals) thus are often termed 'essential' fatty acids although it should be considered that these fatty acids can be manufactured by plants and are found in plants tissues such as seeds, seed oils and nuts (2).

Physiologically, the omega-3 fatty acids are central building blocks of cell membranes (8). Around 50–60% of the brain weight consists of lipids and of this about 35% is derived from O3LC-PUFAs, with DHA accounting for over 40% of omega-3 fatty acids in the gray matter and neuronal tissue (9, 10). The function and structure of the human brain is dependent on an adequate and constant supply of EPA and DHA. O3LC-PUFAs have been found to play key roles in cell membrane fluidity, human growth and development, vision, reduced breast and colorectal cancer risk, lower metabolic syndrome risk and are known to exert cardio-protective actions (11–13). Meta-analytical evidence also shows that both O3LC-PUFAs and fish have been linked to reductions in the development of mild cognitive decline and Alzheimer's disease (14). DPA is also part of healthy nutrition with infants obtaining this from human milk and oily fish, fish oil supplements and grass-fed beef being some of the most predominant sources of DPA in the general population (15). Due to its similar chemical structure to EPA and DHA, DPA has also been linked to improvements in aspects of human health including neural health, lipid metabolism, reduced platelet aggregation and the attenuation of chronic inflammation (15). Evidence is accruing to suggest that EPA, DPA and DHA have both independent and shared effects, particularly in relation to neuroprotective effects (16). DHA remains to be the most quantitatively important O3LC-PUFAs in the human brain but DPA and EPA may act as useful anti-inflammatory mediators (16).

In 2022 the International Society for the Study of Fatty Acids and Lipids (ISSFAL) concluded that O3LC-PUFAs EPA and DHA play a central role in determining gestational length and adequate intakes, consistent with national guidelines, should be achieved in early pregnancy to lower preterm delivery risk (17). A Cochrane report compiled of 70 randomized controlled trials (RCTs) also concluded that preterm births (< 37 weeks) and early preterm births (< 34 weeks) were reduced in women receiving O3LC-PUFAs compared with no omega-3 (18). Possible reduced risks of neonatal care admission, perinatal death, low birthweight, and slight elevated risk of large for gestational age infants were also found for those receiving O3LC-PUFAs (18).

In the last 5 years several Cochrane reports have been published looking at the role of O3LC-PUFAs in relation to health (Table 1). Evidence appears to be stronger for certain health outcomes such as coronary heart disease and depressive symptomology than for others (20, 21). Overall, the health benefits of O3LC-PUFAs are becoming increasingly recognized. Despite this, O3LC-PUFA intakes in many regions and populations globally remain to be inadequate. The present narrative review describes this, discusses barriers to obtaining suitable levels of O3LC-PUFAs and reviews potential ways forward; focusing on innovative delivery methods to use O3LC-PUFA-rich oils including

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TABLE 1 Summary of evidence from Cochrane reports focusing on O3LC-PUFAs and aspects of health (last 5 years).

Reference	Aspect of Health/ Area of Focus	Number of RCTs/ studies reviewed	Sample size evaluated (*n*)	Main findings
Gillies et al. (19)	Attention deficit hyperactivity disorder	37	2,374	There was low-certainty evidence that children and adolescents receiving O3LC-PUFA were more likely to improve compared to those receiving placebo
Appleton et al. (20)	Major depressive disorder (adult focus)	35	1924	Analysis suggests a small-to-modest, non-clinically beneficial effect of O3LC-PUFA on depressive symptomology vs. placebo. The certainty of the evidence was low to very low
Abdelhamid et al. (21)	Primary and secondary prevention of cardiovascular disease	86	162,796	Moderate- and low-certainty evidence. Increasing O3LC-PUFAs slightly reduces risk of coronary heart disease mortality and events and reduces serum triglycerides (evidence from supplement trials). Increasing ALA slightly reduces risk of cardiovascular events and arrhythmia
Watson & Stackhouse (22)	Cystic fibrosis	23	106	Regular O3LC-PUFA supplements could provide some limited benefits for people with cystic fibrosis with few adverse effects. The evidence quality was very low
Dushianthan et al. (23)	Acute respiratory distress syndrome	10	1,015	Findings of this review were limited by lack of standardization among the included studies with regard to types of nutritional supplements given, methods and reporting of outcome measures. The evidence quality was low to very low.
Downie et al. (24)	Dry eye diseases	34	4,314	There is a possible role for O3LC-PUFA supplementation in managing dry eye disease, although the evidence is inconsistent and uncertain

the use of fortification strategies, bioengineered plants, microencapsulation, and microalgae.

Human recommendations

Several organizations and publications have established recommendations for O3LC-PUFAs. This advice is summarized in Table 2. The European Food Safety Authority (EFSA) in 2012 published guidance and recommendations in relation to Tolerable Upper Intake Levels for EPA, DHA and DPA (the maximum daily intake unlikely to cause adverse health effects) (25).

EFSA advised that EPA and DHA recommendations based on cardiovascular risk considerations for European adults are between 250 and 500 mg/day and that supplemental intakes of EPA and DHA combined at doses up to 5 g/day do not raise safety concerns for adults (25). More recently, ISSFAL (17) issued a statement reporting that there is now strong evidence that a proportion of preterm births could be prevented by increasing maternal dietary intakes of O3LC-PUFAs during pregnancy, advising that supplementation with 1,000 mg of DHA plus EPA could lower risk of early birth, ideally with supplementation commencing before 20 weeks' gestation.

Regarding oily fish consumption, in the United Kingdom it is advised that a healthy and balanced diet should provide 2 weekly

TABLE 2 Summary of O3LC-PUFAs recommendations.

Organization	Recommendation
EFSA (25)	Dietary recommendations for EPA and DHA based on cardiovascular risk considerations for European adults are between 250 and 500 mg/day
EFSA (25)	Supplemental intakes of DHA alone up to about 1 g/day do not raise safety concerns for the general population
EFSA (2012) (25)	Supplemental intakes of EPA alone up to 1.8 g/day, do not raise safety concerns for adults
EFSA (25)	Supplemental intakes of EPA and DHA combined at doses up to 5 g/day do not raise safety concerns for adults
Burns-Whitmore et al. (26)	Vegans should have separate AIs for LA and ALA than omnivores and utilize recommendations of between 2.2–4.4 g/g of ALA d (or 1.1 g/day/1000 Kcals)
ISFAAL (17)	Supplementation with a total of about 1,000 mg of DHA plus EPA is effective at reducing risk of early birth, preferably with supplementation commencing before 20 weeks' gestation

portions of fish, of which one should be oily (27). A portion is defined as about 140 g (4.9 oz) (27). Girls, those planning a pregnancy, or who are pregnant/breastfeeding should not eat more than 2 portions of oily fish a week (27). Young people aged 18–25 years (83%) appear to recognize to some extent that EPA and DHA are linked to brain and heart health (28) but they may be less familiar with the specifics, such as intake recommendations for omega-3 fatty acids and oily fish. As seen in studies in the next section, habitual omega-3 and oily fish intakes are generally lower than recommended.

Intakes of O3LC-PUFAs

Some recent publications have reported on habitual intakes of DHA/EPA and/or blood status. A systematic review of studies (12 reported on total n-3 intakes and eight on EPA and DHA intake) undertaken in Europe, North America and South/East Asia concluded that EPA and DHA intakes were lower amongst those following plant-based diets, defined as vegetarians and vegans in the analysis (29). Within the analysis 13 studies reported on EPA and/or DHA status, with most identifying lower EPA and DHA status in vegetarians and vegans compared to meat-consumers. Vegans also tended to have lower EPA and DHA status than vegetarians (29).

In the United Kingdom a secondary analysis of the National Diet and Nutrition Survey (NDNS), the country's largest cross-sectional dietary analysis, showed that only a quarter of the UK population were oily fish consumers – a predominant source of omega-3 fatty acids (30). Amongst those consuming oily fish only 7.3% of children, 12.8% of teenagers, and 15.6% of young adults aged 20–29 years fell in line and met oily fish recommendations (30). A further analysis of survey data within this publication (data from 10 publications) found that EPA and DHA intakes were lower than guidelines, with children, teenagers, females, and pregnant women presenting some of the largest dietary gaps (30). The PEAR (31) study conducted with 598 women before/during pregnancy found that women who ate fish prior to pregnancy reduced their intakes of both oily and white fish during pregnancy, with some avoiding it altogether. Overall intakes of fish were lower than intakes advised during pregnancy (36% compliance for consumers pre-pregnancy) (31).

In the United States (US) recent research measured EPA and DHA blood concentrations across pregnancy (32). Women self-reported their dietary intakes at enrolment (13–16 weeks into pregnancy) and at 36 weeks into gestation (32). It was found that polyunsaturated fatty acid consumption decreased from early to late pregnancy which was attributed to a decline in the nutritional quality of diets as pregnancy progressed (32). This is interesting given that sex hormones and physiological changes associated with pregnancy can increase liver enzymes involved in DHA synthesis (33). Levels of EPA and DHA are also 'more in demand' in the later stages of pregnancy and exposure to lower *in utero* ω-3 PUFA concentrations has been associated with reduced brain volume in childhood (34). These findings are therefore concerning and imply that EPA and DHA intakes were insufficient which could have extended ramification. Data from an earlier US National Health and Nutrition Examination Survey (NHANES) similarly found that 68% adults and 95% children had long-chain omega-3 levels below recommended thresholds (35).

Omega barriers

Endogenous synthesis & bioavailability

It has already been explained how linoleic acid and alpha-linolenic acid are not synthesized by mammals hence termed "essential fatty acids" but can be manufactured by plants (2). Although these fatty acids are not produced endogenously by humans they can be metabolize to other fatty acids (Figure 1) (2).

Some sex differences have been observed with regard to EPA/DHA status and incorporation into blood plasma, cells, and tissues, with this appearing to be slightly higher for females but not to a level that warrants movements towards sex-specific omega-3 dietary recommendations (36). It is thought that sex hormones such as estrogen and progesterone could, in part, be one mechanism behind this, with women tending to have heightened increases in EPA status after ALA supplementation when compared to men (33).

Other factors such as the lipid form in which the omega-3 fatty acid acts can also impact on bioavailability, nutrient delivery, and health. Research by Ghasemifard et al. (37) concluded that the bioavailability of different O3LC-PUFA forms appeared to be highest in the free fatty acid form and lowest in the ethyl ester form with no conclusions drawn from human data in relation to triacylglycerols or phospholipids. Other factors such as matrix effects (capsule ingestion with simultaneous intake of food, food fat content) or galenic form (i.e., emulsification, microencapsulation) can also influence the bioavailability of O3LC-PUFA (38).

Personal choice

There are an array of reasons why populations may not be obtaining suitable daily intakes of oily fish and omega-3 fatty acids.

Amongst pregnant mothers, risk aversion, availability, cost, smell/taste, family preferences and confusions over the number of advised weekly portions and finer details of public health guidance have all been found to act as barriers to fish consumption (31). Research undertaken with older Australian adults ($n = 854$, 51 years+) found that cost was the most frequently reported barrier to fresh finfish consumption (reported by 37%) (39). When sampling fish oils unpleasant after tastes have also been reported (40). In trials with oxidized fish oils, even when added at low levels to yogurt, they could be identified by consumers who reported negative impacts on acceptability. However, they remained interested in consuming such products if there were known health benefits (41). Alongside this, unfavorable aftertastes and challenges swallowing supplements may act as further barriers to suitable omega-3 intakes (42).

Dietary preferences such as vegetarianism or veganism can also impact on O3LC-PUFA intakes. A cross-sectional study of meat-eaters, vegetarians and vegans in the United Kingdom has shown that O3LC-PUFA intakes in non-fish-consumers were 57–80% that of fish-eaters (43). Similarly, the exclusion of oily fish has been has been associated with lower EPA and DHA status in vegetarian women, including the breast milk and infants of vegetarian mothers (44). The bioconversion of the omega-3 parent fatty acid ALA into EPA and then DHA is also inefficient therefore limiting the potential effects of ALA supplementation from vegetarian sources such as flaxseed oil (45, 46).

Fish source

The EPA and DHA profiles of oily fish can depend on where these are sourced from. For example, in farmed salmon the profile of saturated, monounsaturated and polyunsaturated fatty acids has been found to be 15.0, 55.4, and 29.6%, respectively and 26.3, 47.4, and 26.3% in wild salmon (47). A study feeding fish reared in sea cages with an average start weight of 275 g four diets containing different amounts of EPA and DHA found that fish fed on the higher 3.5% EPA and DHA diet had improved growth, filet visual color and quality compared with those on the three lower EPA and DHA diets (48). A study analyzing the DPA and EPA content of 39 Indian food dishes containing fish recognized that fish biodiversity can affect their nutritional (EPA/DHA) profile and found that *Tenualosa ilisha*, *Sardinella longiceps*, *Nemipterus japonicus*, and *Anabas testudineus* were some of the most abundant sources of DHA and EPA (49).

Sustainability

Over the past few decades fish has been regarded as key human health asset, contributing to more than 20% of animal protein intake for approximately 3 billion people, with this set to rise with growing populations (50). The promotion of oily fish consumption for health may be viewed as a somewhat 'antagonistic policy' where nutrition policies, i.e., eat more oily fish for health has subsequent environmental ramifications such as declining fish stocks. For example, over-fishing is responsible for the decline in freshwater fish and natural marine populations, so much so that since 1995 fishing has been banned for 2–3 months in specific periods of the year in China, to help replenish fish populations (51). Along the African coast near to the equator the dual effects of industrial fisheries and climate change have been hampering fish stocks and ecosystems (52). Concerningly, marine ecologists have projected that fish stocks could collapse by 2050 and emphasize that fishing restrictions and 'no-take' zones are of central importance to restore marine ecosystem health (50).

Contaminants

Oily seafood alongside providing O3LC-PUFAs, and micronutrients may also contain polychlorinated dibenzo-p-dioxins/polychlorinated dibenzofurans (PCDD/Fs), dioxin-like-polychlorinated biphenyls (dl-PCBs) and dioxin-like compounds (DLCs) which are known to pose health risks (53). For some populations, such as pregnant women balancing the benefits of fish consumption against mercury intake can be challenging (54). Rather concerningly, pollutants (erythrocyte mercury and urinary arsenobetaine) have been found to be an affective marker of seafood intake amongst pregnant women, more so than O3LC-PUFAs (55). Projected models have shown that methylmercury levels, a renowned neurotoxicant increased by up to 23% between the 1970s and 2000s, due to dietary shifts initiated by overfishing and are estimated to rise by 56% in Atlantic bluefin tuna due to temperatures rises which can increase organic-matter run off into ocean's (56).

Microplastics

The amount of literature now available related to microplastic in fish has been accruing. It is now known that microplastic contamination can occur in nearly all types of aquatic habitats around the globe, with fish being very vulnerable to the ingestion of microplastics (57). Over 690 marine species appear to have been affected by plastic debris, with this number set to rise (58). It has been found that fish unintentionally ingest microplastics, sucking these passively in microfibers whilst breathing, with these being found in the gills and gastrointestinal tracts of fish and having an increase presence in food (59).

Recent studies by Ragusa et al. (60, 61) have identified microplastics in human placental tissue including chorioamniotic membranes and within the syncytiotrophoblast, however, it is yet to be determined whether these microplastics derived from fish intake among pregnant females. These microplastics found in human placentas could contribute to the initiation of pathological processes, such as oxidative stress, apoptosis and inflammatory processes. In various environments globally microplastics (size around<5 mm) have been found to be present (59). From a human health stance certain microplastics such as bisphenol A (BPA), nonylphenol (NP), octylphenol (OP) and polybrominated diphenyl ethers (PBDE) may be potentially toxic to humans (62).

Potential ways forward

Fortification

Where there are nutritional shortfalls food fortification can be a cost-effective strategy that can also convey economic, health and

social benefits (63). Fortification *per se* has been defined by the 1987 Codex General Principles for the Addition of Essential Nutrients to Foods as "*the addition of one or more essential nutrients to a food whether or not it is normally contained in the food, for the purpose of preventing or correcting a demonstrated deficiency of one or more nutrients in the population or specific population groups*" (64). There are two predominant forms of mainstream fortification – targeted fortification for subpopulations, e.g., infant cereals and market-driven mass fortification with iodine fortification of salt being one example of this (65). Providing that specific food vehicles are used and explicit consumer needs addressed voluntary fortification can play a central role in contributing to nutritional requirements, particularly where gaps are evident (65). Biofortification is another form of fortification that stems back to agronomic practices and has been defined by the World Health Organization as "*the process by which the nutritional quality of food crops is improved through agronomic practices, conventional plant breeding, or modern biotechnology*" (66). These methods therefore aims to improve nutrient levels using agronomic practices rather than manual measures (66).

When it comes to O3LC-PUFAs, food fortification appears to be a growing market sector. In the United States when the Food and Drug Administration first authorized O3LC-PUFA use in supplements the market for such ingredients expanded by 24%, demonstrating their potential for popularity (67). Several past studies have fortified a range of foods (milk, margarines, sausages, luncheon meat, French onion dips, yoghurts) with fish oils (68–72). The majority of these methods demonstrate beneficial changes in omega-3 intakes, EPA and DHA pools and the ω-3 index of erythrocyte membranes (68–70, 72) although a degree of masking using flavors may be needed to override fishy tastes and flavors (68). It is worth mentioning that some studies use the terminology "enriched" rather than fortified.

Bioengineered plants

Alongside marine and plant oils genetic modifications of plants is being explored as a novel ways to produce and supply EPA and DHA (73). Scientists are now able to modify endogenous genes involved in the biosynthesis pathways, enabling the modifications of edible plant oils to upregulate and increase the content of desired components (such as omega-3 fatty acids) or reduce the content of undesirable components (74).

In particular, the transfer of certain genes into plants (such as oilseeds) from microalgae is being viewed as a promising and potentially effective way to yield these fatty acids (73). Omega-3 fish oil crops have also been produced although a range of challenges have also been presented which include underlying metabolic engineering, crop performance, intellectual property, regulatory and consumer acceptance difficulties (75).

Microencapsulation

Microencapsulation is a novel technology that has been developed with the intention of protecting sensitive compounds from environmental elements which also includes protecting compounds from digestive enzymes for enhanced delivery to the gut with minimal degradation (76). This method has been well used in pharmaceutical sectors and there is now rising interest in its application in food systems (76). Microencapsulation may have many potential uses, for example, the entrapment of nutrients and natural compounds such as probiotics which could aid and potentially protect their passage through the gastrointestinal tract (77). In the case of unsaturated fatty acids, microencapsulation can also help to improve their quality and shelf-life (78).

The unique process of microencapsulation may help to counteract traditional problems of strong odors/flavors (particularly those related to O3LC-PUFA delivery), reactions of other food matrix components which could reduce bioavailability and help to provide a form of targeted and controlled delivery and release (79). It is well recognized that food fortification with O3LC-PUFAs may be difficult due to their tendency to rapidly oxidize, variable bioavailability and poor water-solubility and encapsulation technologies may help to address some of these issues (80, 81). For example, research with flaxseed oil showed that the oxidative stability of encapsulated flaxseed oil was 13-fold higher than that in bulk oil form, demonstrating the stabilizing and protective effects of microencapsulation (82).

One mode of encapsulation involves integrating omega-3 oils into colloidal particles assembled from food-grade components such as emulsion droplets, liposomes, nanostructured lipid carriers, or microgels (80). Typical microencapsulation technologies can include approaches such as coacervation, extrusion, spray cooling and spray drying (78). Spray drying in particular is regarded as a flexible, simple and rapid microencapsulation method that is relatively easy to scale up (83). It is also regarded as a 'clean technology' as it does not utilize the use of organic solvents (83). Encapsulation has not been found to adversely affect the bioavailability of O3LC-PUFA compounds. In one double-blind trial 25 females were allocated to ingest 0.9 g n-3 PUFA/day in a capsule or microencapsulated fish-oil enriched foods (84). The microencapsulated fish-oil enriched foods were found to be as bioavailable as administration via a capsule thus regarded as an effective means of improving n-3 PUFA intakes to align with dietary recommendations (84). Foods such as ice cream and Indian yoghurt have also been found to administer microencapsulated flaxseed oils successfully (85, 86).

Microalgae

It has previously been explained how seafood was traditionally exploited as a prime O3LC-PUFA source. However, other food derivates will be needed to meet expanding populations and growing global demands. Subsequently alternative sustainable non-animal derived sources of O3LC-PUFAs are being sought and algae is being recognized as one of these (87). It is now recognized that certain microalgae yields have significant levels of EPA and/or DHA (45). Subsequently, alternative microalgae sources could be a novel means to bridging gaps between supply and demand for EPA and DHA in relation to achieving human requirements (88).

Several clinical trials with micro-algae oil have led to significant increases in blood erythrocyte and plasma DHA (89–91). For example, Yang et al. (90) found that lactating women ingesting algal oil (200 mg/d) over 8 weeks significantly improved DHA levels in breast milk compared with a placebo capsule. Arteburn et al. (89) found that

algal-oil capsules providing 600 mg/day DHA were bioequivalent in terms of providing similar levels of DHA to plasma and red blood cells when compared to cooked salmon. Earlier work by Geppert et al. (92) concluded that 0.94 g/d DHA over 8-weeks was well tolerated and could be a viable vegetarian O3LC-PUFA source. Sanders et al. (91) administered 1.5 g DHA + 0.6 g DPA to 79 healthy adults over 4 weeks finding that DHA and DPA erythrocyte phospholipid levels increased compared with the 4 g oil/d placebo.

Discussion

Given rising sustainability and toxicological concerns in the aquaculture sector research into possible alternative sources of O3LC-PUFAs has become a priority, to help plug the gap between supply and demand (42, 88). Dietary intakes of O3LC-PUFAs largely remain to be insufficient in relation to the optimisation of health and cognitive outcomes (29, 30, 43). Historical evidence shows that fresh aquatic plants and seaweed were chewed such as red, green, and brown seaweeds and most likely consumed during Mesolithic and Neolithic time periods indicating that wild food resources were ingested before a switch to domesticated resources (93). These at the time were likely to have been an important source of nutrients, including omega-3 fatty acids.

Giving the scale of aging populations and surging healthcare costs attributed to poor brain health, reflection on insufficient omega-3 intakes and how to achieve these is needed (94). There is also growing evidence that DHA is an important neuroprotective agent potentially helping to enhance brain development, function, and maintenance (synaptic plasticity and cognition), particularly alongside exercise (95). Unfortunately, many supplements available do not enrich 'brain' DHA, although they may enrich most other body tissues (96). Intriguingly, research has found that DHA from triacylglycerol which is released as free DHA or monoacylglycerol during digestion and absorbed as triacylglycerol in chylomicrons is preferentially integrated into heart and adipose tissue but not the brain (96). In contrast, LPC lysophosphatidylcholine DHA has been found to enhance DHA uptake in the brain by up to 100% but does not affect adipose tissue (96).

Recently, findings from the REDUCE-IT trial remarkably found that EPA at a dose of 4 g/day in patients at high cardiovascular risk with hypertriglyceridemia had a 25% relative reduction in risk of cardiovascular-related events (97, 98). In terms of sources, omega-3 s from both dietary and supplementation sources may have positive health benefits, additionally, supplementation studies appear to demonstrate more consistent reductions in inflammatory markers including IL-6 and TNF-α amongst populations with mild cognitive impairment (99).

There has been some question regarding the bioavailability of O3LC-PUFAs from supplements which could have resulted in negative or neutral outcomes in some studies (38). Subsequently, this has led to the question as to whether novel models of delivery such as microencapsulation could improve omega-3 fatty acid bioavailability (38). Provisional science indicates that this could be a promising way forward, (76–79, 82). Ongoing research specifically focusing on O3LC-PUFAs is now needed.

Finally, it is important to consider that studies investigating novel methods to narrow omega-3 gaps should consider several factors. Firstly, studies measuring EPA and/or DHA status should recognize that plasma levels can change due to diurnal rhythm (100). Research with 21 adults aged 25 to 44 years found that rhythmicity was strongest for DHA which peaked in the evening at 17:43 (100). Circulating levels of EPA and DHA fatty acids fell during the night and reached the lowest point in the morning (100). The wider implications of this research indicate that O3LC-PUFA consumption in the evenings could have a wider functional significance (100). Secondly, other work has found that there appear to be O3LC-PUFAs "responders" and "non-responders" which could affect the results of human intervention studies (101). For example, it is speculated that certain epigenetic/genetic variants and differences in dietary and gut microbiota composition could be behind such variations (101). Finally, novel biomarkers such as brain derived neurotrophic factor (BDNF) could be more highly regarded than the omega-3 index when measuring omega-3 fatty acid brain enrichment as DHA is known to increase the synthesis of BDNF in the brain and there is bidirectional BDNF transport through the blood brain barrier (102).

Alongside this it should be considered that findings from studies investigating O3LC-PUFAs can vary due to short follow-up periods, small samples sizes, withdrawal rates, different types, timings, dosages, and forms of interventions. Baseline population characteristics also need to be carefully controlled and considered when making comparisons between studies. Greater consistency is needed when reporting such components to synthesize future evidence more effectively. Greater standardization between studies would greatly improve and strength any future conclusions.

Conclusion

The present publication has identified modern-day challenges and shifting trends associated with inadequate O3LC-PUFA intakes and environmental challenges linked to meeting requirements for health via traditional means (oily fish consumption). The overarching conclusion is that novel models of EPA and DHA delivery could have a central role to play in helping to support future healthy and balanced diets whilst counteracting some of the sustainability and toxicological concerns that exist from present-day delivery methods.

Author contributions

ED: Conceptualization, Visualization, Writing – original draft. CB: Writing – review & editing. GB: Writing – review & editing. AE: Writing – review & editing. PM: Writing – review & editing. WL: Writing – review & editing.

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Conflict of interest

ED was employed by Nutritional Insight Limited. CB and GB were employed by AgriFood X Limited. AE was employed by HTC Group Limited. PM was employed by Efficiency Technologies Limited.

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Publisher's note

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Omega-3 eicosapentaenoic polar-lipid rich extract from microalgae *Nannochloropsis* decreases plasma triglycerides and cholesterol in a real-world normolipidemic supplement consumer population

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Introduction: AlmegaPL® is an oil rich in polar-lipid (> 15% w/w) derived from the microalga *Nannochloropsis,* that contains exclusively eicosapentaenoic acid (EPA > 25% w/w), without the DHA that is present in all other natural sources of omega-3. Previous findings from a randomized controlled clinical trial demonstrated the ability of AlmegaPL® supplementation to reduce cholesterol levels.

Methods: In this post-market cohort study, we built upon previous findings and targeted the actual end-users of the supplement. Participants were recruited from a new subscriber database of AlmegaPL® capsules (1000–1100 mg/day) to capture the complexity of real-world clinical and consumer settings. Changes in circulating triglycerides (TG), remnant cholesterol (RC), low-density lipoprotein cholesterol (LDL), high-density lipoprotein cholesterol (HDL), total cholesterol (TC), high-sensitivity C-reactive protein (hs-CRP), glucose and glycated hemoglobin (HbA1c) were monitored at baseline, Month 3, and Month 6 of supplementation using the at-home Baseline Heart Health Testing Kit by Imaware® (Houston, TX, USA).

Results: Participants, who had, on average, normal TG level at baseline (1.62 ± 0.60 mmol/L), experienced a significant and progressive decrease in TG at Month 3 (8.0%; −0.13 ± 0.59 mmol/L; $p < 0.001$) and Month 6 (14.2%; −0.23 ± 0.64 mmol/L; $p < 0.001$) (primary outcome). Furthermore, after 6 months of supplementation, TC and non-HDL-cholesterol decreased by 5.0% (−0.26 ± 0.98 mmol/L; $p < 0.001$) and 5.5% (−0.21 ± 0.86 mmol/L; $p < 0.001$) respectively, primarily driven by a 14.9% reduction in RC (−0.11 ± 0.29 mmol/L; $p < 0.001$).

Discussion: Consistent with our previous clinical trial, the decrease in RC was not coupled to an increase in LDL, which seems to be a benefit associated with EPA-only based formulations. In addition, this study demonstrated the AlmegaPL® capacity to maintain already healthy TG levels by further inducing

a 14.9% decrease. Collectively, these findings highlight AlmegaPL® uniqueness as a natural over-the-counter option for EPA-only polar lipid that appears particularly effective in maintaining blood lipid levels in a generally healthy, normolipidemic population.

Clinical trial registration: https://clinicaltrials.gov/, identifier NCT05267301

KEYWORDS

polar lipids, cardiovascular health, cholesterol, triglycerides, microalgae, *Nannochloropsis*, omega-3 eicosapentaenoic acid (EPA), dietary supplements

1 Introduction

Cardiovascular disease (CVD) continues to be the leading cause of mortality globally, as reported by the World Health Organization (1). During the pandemic, CVD accounted for 1 in 5 fatalities in the USA, which was almost twice the fatalities attributed to COVID-19 (2). Inadequate intake of omega-3 polyunsaturated fatty acids (LCn-3 PUFA), specifically eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3), is estimated to contribute to approximately half of all CVD-related deaths annually (3). Surprisingly, 95% of the USA population fails to consume enough DHA and EPA (4), emphasizing the importance of achieving the recommended daily intake of LCn-3 PUFA (5) to support cardiovascular health.

The connection between LCn-3 PUFA intake and CVD was first proposed in a seminal study conducted in 1971, focusing on the Greenland Inuit population (6). Since that groundbreaking publication, over 4,000 clinical trials have been conducted on this topic, indicating the extensive research interest in this area (7). Notably, the pharmaceutical industry has recently undertaken large-scale clinical trials, encompassing much larger sample sizes compared to studies conducted in the preceding five decades, with emphasis on key disease endpoints such as major adverse cardiovascular events (MACE) (7). Traditionally, LCn-3 PUFA clinical research predominantly utilized fish oil and other complex sources containing both EPA and DHA. However, some of the recent pharmaceutical trials specifically investigated EPA-only treatments (JELIS, REDUCE-IT, RESPECT-EPA) and demonstrated superior outcomes compared to trials using combined DHA and EPA treatments (VITAL, ASCEND, STRENGTH, OMEMI). This finding has ignited a significant debate regarding the distinct roles of each LCn-3 PUFA in cardiovascular protection, with EPA apparently emerging as a prominent player in this regard (8–10).

The microalgae *Nannochloropsis* produces exclusively EPA, unlike all other natural sources of LCn-3 PUFA (e.g., fish, krill, heterotrophic microalgal oils) that also contain DHA. Besides EPA, this photoautotrophic organism also produces many other bioactive molecules such as pigments and phytosterols that could provide further cardiovascular benefit (11). AlmegaPL® containing 25% w.w EPA is the first lipid extract derived from the *Nannochloropsis* made available in 2014 for human consumption (12). Besides being the only over-the-counter source of EPA-only, AlmegaPL® also includes 15% w/w of polar lipids with a distinctive profile (galactolipids, phospholipids, and sulfoquinovosyldiacylglycerol) that provides functional characteristics distinct from other forms of LCn-3 PUFAs (such as triacylglycerides, phospholipids and ethyl esters). These unique polar lipid properties confer surfactant properties that promote the spontaneous formation of micelles in the digestive tract, facilitating the digestion and delivery of LCn-3 PUFA while minimizing undesirable fishy burps and aftertaste. Studies have shown that LCn-3 PUFA in AlmegaPL® exhibit superior bioavailability compared to other forms of LCn-3 PUFA (13).

The cardioprotective benefits of AlmegaPL® were initially demonstrated in a randomized, double-blinded clinical trial, where it led to significant decreases in remnant cholesterol (RC) (25%; $p = 0.002$) and total cholesterol (TC) (5%; $p = 0.012$) compared to placebo (14). Typically, the decrease in atherogenic lipid levels induced by LCn-3 PUFA is driven by the reduction in triglycerides (TG) secretion by the liver, often signaled by the decrease in RC (15). These markers are considered independent causal risk factors for MACE (16, 17) and might even have a better predictive value than low-density lipoprotein cholesterol (LDL) (18).

The relationship between RC reduction and TC has been a subject of controversy, but recent reports suggest that it may depend on the type of LCn-3 PUFA used (19). While both DHA and EPA can decrease RC, formulations containing DHA have shown an increase in LDL levels in response to the RC decrease (19–21). In contrast, EPA-only formulations have been associated with a reduction in RC without an increase in LDL (22), a mechanism of action also observed with AlmegaPL® (14). Thus, LCn-3 PUFA supplementation affects blood lipids by decreasing TG, RC, and, in the case of EPA-only formulations, TC, all of which are associated with cardiovascular health (19). This is important because it implies that LCn-3 PUFA, particularly EPA-only formulations, are complementary to lipid lowering agents that primarily target LDL. Statins and phytosterols are successful at decreasing LDL, but even when LDL has been effectively controlled, there is substantial residual CVD risk associated to high TG and RC (8), which has been the focused of several pharma clinical trials (23). Therefore, in this dietary supplement trial TG was selected as the primary outcome because of the persistent risks present even in a generally healthy population (16).

Given the unique composition and positive results observed in our previous clinical trial conducted under controlled conditions, the aim of the present post-market cohort study was to confirm the cardiometabolic benefits of AlmegaPL® in real-world clinical

and costumer settings. By recruiting free-living adults who are consumers of this supplement, we sought to better reflect the complex conditions in which this supplement is intended to be effective.

2 Materials and methods

2.1 Clinical trial design, registration, and ethical approval

This clinical trial adhered to the International Conference on Harmonization (ICH) Guideline for Good Clinical Practice (GCP), the Notice for Guidance on Good Clinical Practice, and the Additional Ethical Considerations Guidelines. The trial received approval from the Argus Independent Review Board Committee (Tucson, Arizona) and is registered on the Clinicaltrials.gov Registry (NCT05267301).

This study followed an open-label, single arm design with a 6 month supplementation monitoring period. Its objective was to assess the effect of AlmegaPL® on cardio-metabolic parameters and inflammatory markers in men and women. This study was conducted between May and November 2022, with participants located across the USA.

2.2 Participants

Potential participants were recruited through email invitations using the new-subscriber database from the AlmegaPL® commercial website (24). Following a preliminary screening via the email questionnaire, participants were enrolled in the trial after providing written-informed consent.

A total of 480 otherwise healthy male and female volunteers over 18 years of age were enrolled from various regions in the USA. Exclusion criteria included unstable or serious illness (including but not limited to kidney, liver, and gastrointestinal disease, MACE, or diabetes), malignancy or treatment for malignancy within the previous 2 years, and allergic reactions to any of the supplement ingredients. Consequently, all participants were recruited regardless of their blood lipid levels.

2.3 Investigational product

The investigational product, supplied by Qualitas Health (Houston, TX, USA) under the brand name iwi, a vegetarian capsule containing 1000–1100 mg AlmegaPL® . AlmegaPL® is a lipid ethanol extract derived from whole-cell *Nannochloropsis oculata* QH5, a photoautotrophic microalga privately deposited at the University of Texas at Austin (UTEX) Culture Collection of Algae, which is rich in EPA conjugated to galactolipids and phospholipids. This marine microalga was grown in open pond raceways in Columbus, New Mexico and Imperial, Texas using brackish water and non-arable land. Participants were asked to consume a single capsule per day. Each capsule was standardized to contain a minimum of 1000 mg AlmegaPL®, which provided at least 250 mg EPA, 150 mg of polar lipids, 40 mg of arachidonic acid (ARA; 20:45 n-6), and 90 mg of palmitoleic acid (16:1 n-7). Additionally, it contained 23 mg of phytosterols and 15 mg of chlorophyll, 764 μg lutein, 387 μg zeaxanthin, and 541 μg beta-carotene analyzed according to Eurofins methods (Des Moines, IA, USA). The product is registered for use as a new dietary ingredient (NDIN) in the USA (12).

2.4 Intervention and study procedure

Upon enrollment, participants who provided signed consent forms were instructed to complete the Baseline Heart Health Testing Kit from Imaware® (Houston, TX, USA) before consuming the supplement. Participants performed finger prick blood sampling after an 8-h fast, and the resulting dried blood spot samples were sent to a CLIA/CAP certified laboratory for analysis using the return shipping label in the test kit. The aggregated results were then shared by Imaware via a secure HIPAA and SOC2 API in a protected data environment. Once baseline measures were obtained, participants were instructed to orally take 1000–1100 mg of encapsulated AlmegaPL® per day. This dosing regime was selected based on current standard dosing guidelines for the investigational product. Participants were then required to repeat this process after 3 months (mid-point) and 6 months (completion) of supplementation to monitor the progress from baseline.

Participants were advised to maintain their usual level of physical activity and diet throughout the study. Email questionnaires were used to assess any changes in activity level (exercising at least 150 min per week) and/or diet (2 servings of seafood intake/week, LCn-3 PUFA supplementation), which were considered during result analyses at each data point of the study. Compliance with the supplementation protocol was evaluated at the end of the study by counting the number of capsules used and determining the remaining product in the container. Participants with more than 20% of the assigned capsules remaining were considered non-compliant. Additionally, the authors monitored the participants by email or phone for any potential adverse reactions and adherence to the protocol at each data collection timepoint.

2.5 Outcome measures

Triglycerides (TG) (primary outcome), total cholesterol (TC), low-density lipoprotein cholesterol (LDL), high-density lipoprotein cholesterol (HDL), glycated hemoglobin (HbA1c), high-sensitivity C-reactive protein (hs-CRP), and glucose were analyzed using reagents for a clinical chemistry analyzer (Cobas 6000 and Cobas 8000, Roche Diagnostics, Indianapolis, IN). Remnant cholesterol (RC; calculates estimated following the VLDL calculation; VLDL = TC - LDL - HDL), non-high-density lipoprotein cholesterol (non-HDL-C; calculated as TC - HDL), and TG/HDL ratio (in mg/dL) were all calculated. Anthropometric measurements (height, weight, and body mass index) and lifestyle information (diet including seafood intake/week, LCn-3 PUFA supplementation, exercise, smoking, alcohol, and caffeine intake) were collected via email questionnaire. Participant safety was

assessed by recording adverse events (AE) and serious adverse events (SAE). Protocol adherence was monitored through emailed and phone questionnaires at each data collection timepoint.

2.6 Statistical analyses

A power and sample size calculation were performed on the primary outcome measure (TG) using G*Power 3.1 (Department of Psychology, University of Düsseldorf, Germany). Based on a two-tailed student *t*-test with an effect size of 0.5 and an allocation ratio N2/N1 of 1, the sample size was estimated to be 210 to achieve a power of 95%. Preliminary data helped us estimate a relatively high dropout rate of 65%, but financial compensation to decrease dropout was discharged to avoid influencing real-world consumer settings. Therefore, accounting for a 65% dropout rate, a total of 480 participants were enrolled, aiming to achieve a proposed power of >0.95 to detect a statistically significant difference between the two groups of data.

The outcome results were statistically analyzed using an intention-to-treat (ITT) approach, which included participants who followed the supplementation schedule and completed baseline and at least one additional test (n = 256). Additionally, the per protocol (PP) population was defined as participants who followed the AlmegaPL® supplementation schedule, completed baseline, and at least the Month 6 test (n = 223). Before conducting the analyses, all outcome data were checked for normality using the Kolmogorov-Smirnov Test and the Shapiro-Wilk Test. As the data did not follow a normal distribution, the non-parametric Wilcoxon Ranks sum test was used to compare two-tailed differences between baseline and either Month 3 or Month 6. To account for multiple variables, the thresholds for statistical significance of the 20 clinical outcomes were adjusted using the Bonferroni correction, resulting in a significance level of $p < 0.0025$.

Post hoc analyses were conducted to examine potential dropout bias associated with low baseline TG results. The Mann-Whitney test was used to compare two-tailed differences between the dropout population and the population that completed at least another test. Furthermore, dropout bias for underperforming participants was analyzed by comparing the TG delta at Month 3 between participants who also completed the Month 6 test and those who dropped out after completing the Month 3 test, using the Mann-Whitney Test. All statistical analyses were performed using SPSS Inc., Released in 2021, Version 28.0. Armonk, NY: IBM Corp.

3 Results

3.1 Participants

Of the initial 480 participants enrolled in the study, only 10 withdrawals were observed due to mild adverse effects, indicating a good tolerance to the supplement (Figure 1). Among these withdrawals, three participants reported increased bruising, including increased bleeding and petechiae, while seven participants reported abdominal discomfort, including symptoms such as abdominal pain, nausea, vomiting, and heartburn, following supplementation. Participants with adverse effect were advised to consult with their doctor. The vast majority of withdrawals were not related to adverse effects due to supplementation, but were primarily due to issues with adherence to the supplementation schedule, problems with the at-home testing user guide, cancelation of subscription for supplementation, or medical advice to discontinue all supplements due to an intervention or initiation of a new treatment plan which ultimately resulted in a failure to submit baseline (n = 89), Month 3 (n = 91), and Month 6 test kits (n = 67).

The all-baseline (n = 391) and ITT population (n = 292) were predominantly female in their early 50s, non-smokers and moderate alcohol consumers, who exercise frequently (Table 1). The ITT population was slightly overweight (BMI = 26.6 kg/m^2). There were no significant changes in exercise, diet, and other lifestyle parameters from baseline through 6 months of supplementation. The lifestyle changes included aerobic activity (not less than 150 min/week), smoking habits, seafood intake, intake of other sources of omega- 3, excess alcohol, or caffeine intake. Baseline lipid assessment of the cohort confirmed that AlmegaPL® consumers were normolipidemic (Table 2), with average TG (1.62 mmol/L), TC (5.14 mmol/L), and LDL (3.04 mmol/L) bellow their respective borderline limits (1.70, 5.17, and 3.36 mmol/L, respectively) (25). The supplementation compliance success rate was 81% among the ITT population.

3.2 Cardiometabolic markers

AlmegaPL® supplementation for 6 months significantly decreased TG by 14.2% (−0.23 ± 0.64 mmol/L; $p < 0.001$), TC by 5.0% (−0.26 ± 0.98 mmol/L; $p < 0.001$), RC by 14.9% (−0.11 ± 0.29 mmol/L; $p < 0.001$), non-HDL-C by 5.5% (−0.21 ± 0.86 mmol/L; $p < 0.001$) and TG to HDL cholesterol ratio by 11.3% (−0.33 ± 1.30 mmol/L; $p < 0.001$) (Table 2). hs-CRP decreased by 25.3% after 3 months and 30.2% after 6 months from baseline, albeit the decrease at the end of the study was not statistically significant (−0.92 ± 5.85 mg/L; $p = 0.146$).

The baseline TG results of the dropout population (1.54 ± 0.61 mmol/L; n = 99) were not statistically significantly different ($p = 0.234$) from the population that completed at least one of the subsequent tests (1.62 ± 0.60 mmol/L; n = 292), suggesting that there was no dropout bias associated with low baseline TG levels. Likewise, the Month 3 TG change (Δ0M–3M) of participants who also completed Month 6 (−0.10 ± 0.56 mmol/L; n = 187) was not statistically significantly different (p = 0.442) from the Δ0M–3M of those participants who dropped out after completing the Month 3 blood sample (−0.22 ± 0.66 mmol/L; n = 69), suggesting we had no dropout bias for underperforming participants.

4 Discussion

The participants recruited in the present post-market cohort study are reflective of the health-conscious consumers of this supplement, with the majority being non-smokers (99%), moderate alcohol consumers (96.2%), predominantly female (67%), in their early 50s, marginally overweight (BMI = 26.6 kg/m2), but active (65.7%) (Table 1). The mean baseline TG (1.62 ± 0.60 mmol/L) and

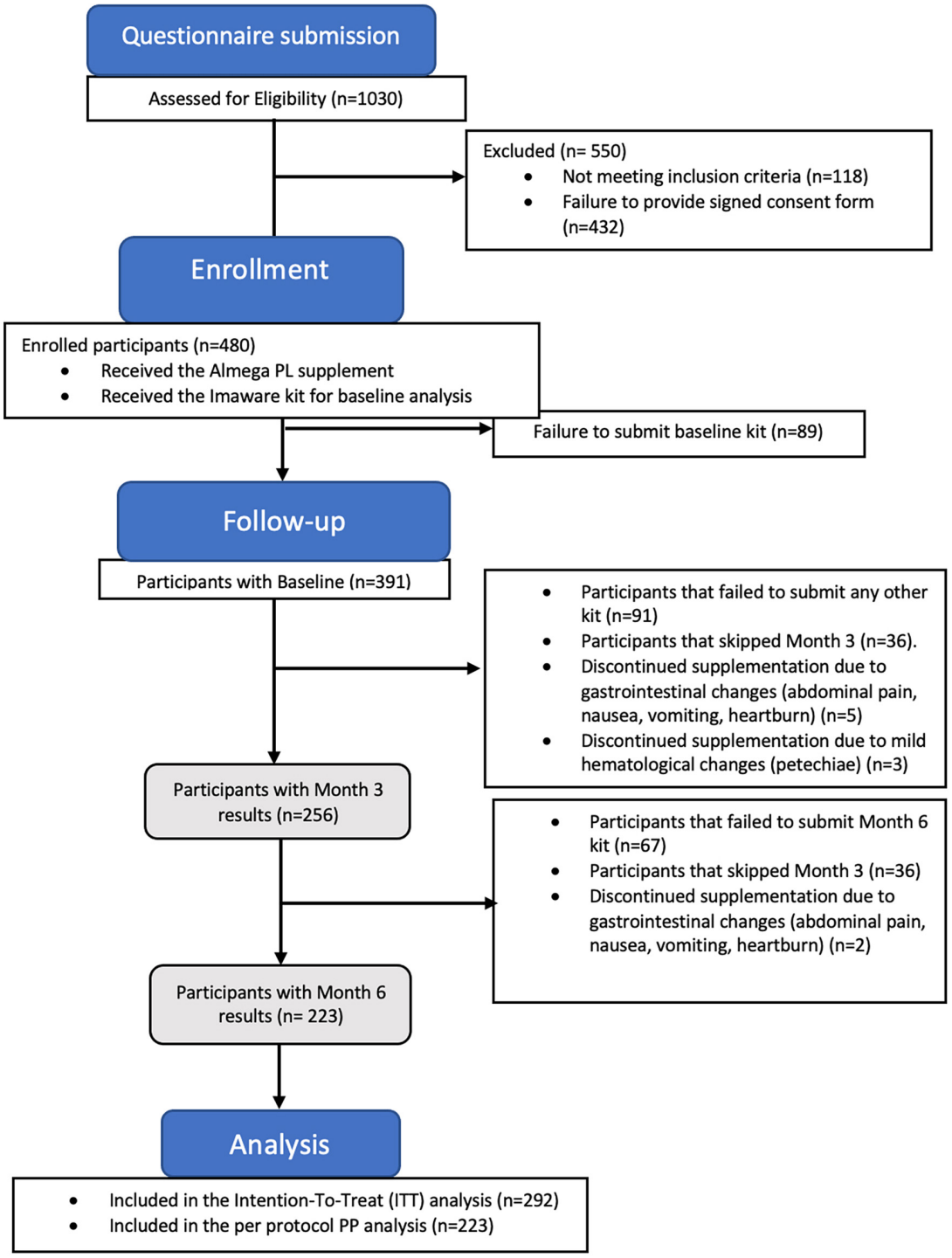

FIGURE 1
The study flow CONSORT diagram illustrates the number participants who completed the study after submitting the results at Month 6 (*n* = 223 per protocol population), along with the participants who completed at least 2 tests (*n* = 292 intention-to-treat population).

TC (5.14 ± 0.91 mmol/L) concentrations were within the normal range according to the Adult Treatment Panel Guidelines (ATP III) (25), confirming the overall health of the real-world population that consumed the AlmegaPL® supplement. Although the population participating in this study represented a normolipidemic population (only 12.3% of the population was pharmaceutically medicated for dyslipidemia), it's important to note that lower TG levels, even within the normal range < 1.7 mmol/L), are still associated with better cardiovascular health (16). For this reason, the ability of AlmegaPL® supplementation to significantly reduce TG levels already in the normal range offers valuable cardiometabolic support for healthy, active adults. Precisely, the population participating in this study represents a broad non-diseased target for primordial CVD prevention that could

TABLE 1 Baseline anthropometric and lifestyle parameters of participants.

Parameter	All baseline (n = 391)	ITT population (n = 292)
Female (n)	265	199
Male (n)	126	93
Age (years)	50 ± 10	51 ± 10
Weight (kg)	76 ± 18	78 ± 19
Height (m)	1.7 ± 0.1	1.7 ± 0.1
BMI (kg/m^2)	26.6 ± 5.2	27.1 ± 6.2
Smokers (%)	1.3	0.7
CVD diagnosed excluding MACE (%)	31.8	32.3
CVD family history (%)	51.6	50.0
Diabetes diagnosed (%)	8.8	7.8
Diabetes family history (%)	38.8	35.8
Use of fish oil or LCn-3 PUFA supplementation (%)	36.6	39.0
Use of Statin or LCn-3 PUFA containing medication (%)	11.8	12.3
Use of other cholesterol or TG lowering supplement (%)	12.8	13.7
Exercising at least 150 min per week (%)	65.7	66.1
At least 2 seafood servings/week (%)	33.0	32.5
At least 14 alcoholic drinks/week (%)	3.8	4.5
At least 4 caffeinated drinks/day (%)	4.6	5.5

CVD, cardiovascular disease; MACE, major adverse cardiovascular events. Values represented as mean ± SD. No significant differences were observed between the All Baseline group and the intention-to-treat ITT population ($p < 0.05$).

improve their cardiovascular health through over-the-counter dietary supplementation, prior to reaching clinically-indicated levels requiring pharmaceutical intervention (25).

The daily intake of LCn-3 PUFA in AlmegaPL® (250 mg/day), while consistent with the promotion of cardiovascular health in a generally healthy population (26), is well below the intake levels (2000–4000 mg/day) recommended to treat CVD in diseased participants (23). Thus, AlmegaPL® is intended for healthy adults seeking cardiometabolic support rather than for treatment of CVD. The impact of LCn-3 PUFA intake on the TG levels follows a dose-response relationship, even at relatively low supplementation levels (200–500 mg/day), where the TG decrease is estimated at 3.1–7.2% relative to baseline (27). In the present study, AlmegaPL® decreased TG levels by 14.2% (−0.23 ± 0.64 mmol/L; $p < 0.001$) after 6 months (primary outcome) of supplementation, a response equivalent to 4-times the DHA + EPA dose reported for other LCn-3 PUFA sources (27). This suggests that LCn-3 PUFAs alone might not explain the high response obtained with AlmegaPL®, and some other factors might be at play. While EPA-only composition outperformed DHA + EPA in terms of decreasing MACE (8), there is no consensus on whether the EPA-only composition truly increases the LC-3 PUFA capacity to lower TG (28, 29). In turn, the polar form (glycolipids and phospholipids) to which the EPA is conjugated in AlmegaPL® may partially explain the greater reductions observed for TG. Polar lipids extracted from soybean have been shown to decrease both TG and TC independently from their fatty acid profile (30), suggesting AlmegaPL® polar lipids may also contribute to the observed lipid-lowering effect. Furthermore, minor constituents of the algal oil, such as phytosterols (31) and palmitoleic acid (32), may reinforce the observed lipid homeostasis.

Participants, who had, on average, normal TG level at baseline (1.62 ± 0.60 mmol/L), experienced a significant and progressive decrease in TG at Month 3 (8.0%; −0.13 ± 0.59 mmol/L; $p < 0.001$) and Month 6 (14.2%; −0.23 ± 0.64 mmol/L; $p < 0.001$) (primary outcome). The magnitude of TG lowering is influenced by the baseline TG level, with lower baseline TG levels associated with lower reductions (19). This illustrates the difficulty of demonstrating supplementation efficacy in an already healthy population compared to a population with dyslipidaemia, a condition with more room for improvement. The baseline TG level (1.6 ± 0.60 mmol/L) in the present post-market cohort study is by design more reflective of the real-world population taking this supplement than in the previous clinical trial (1.0 ± 0.59 mmol/L) (14). While both studies targeted a normolipidemic population (TG < 1.7 mmol/L), the baseline TG of the clinically standardized population selected previously was surprisingly low, which explains why AlmegaPL® supplementation did not significantly decrease TG in that trial (14).

RC is the cholesterol content of triglyceride-rich lipoproteins, which correlates closely with plasma TG, as these lipoproteins are predominantly responsible for TG transport. The liver responds to LCn-3 PUFA supplementation by increasing TG oxidation, which contributes to the decrease in RC (15). This trend was observed in the present study, where, along with abovementioned TG, RC also decreased by 14.9% (−0.11 ± 0.29 mmol/L; $p < 0.001$) after 6 months of AlmegaPL® supplementation. Consistent with our previous study (14) and with EPA-only formulations in general, LDL remains unchanged, ultimately resulting in significant decreases in TC (5.0%; −0.26 ± 0.98 mmol/L; $p < 0.001$) and non-HDL-C (5.5%), both of which are well-characterized risk factors for CVD. According to Varvo (33), RC, but not LDL, causes low grade inflammation, which explains why the inflammatory marker hs-CRP (high-sensitivity C-reactive protein) decreased by 30.2% (−0.92 ± 5.85 mg/L), albeit not significantly ($p = 0.146$). The lack of significance might be explained by the non-specific nature of the acute-phase hs-CRP response. This response can be dramatically triggered by many disorders unrelated to cardiovascular disease (e.g., infection) and may interfere with the interpretation of results in numerous CVD studies (34).

This is the second clinical trial that supports the use of AlmegaPL® to promote cardiovascular health in a generally healthy adult population. The previous clinical trial (12), a randomized, placebo-controlled study, demonstrated in a controlled clinical setting, that AlmegaPL® supplementation for 3 months significantly decreased TC, RC, and non-HDL-C compared to placebo. While placebo-controlled studies are considered the gold standard design for assessing the efficacy

TABLE 2 The effect of AlmegaPL® supplementation for 6 months on cardiometabolic and inflammatory markers for the intention-to-treat (ITT) (n = 292) and per protocol (PP) population (n = 223).

	Baseline	Month 3	Δ 0M–3M	Month 6	Δ 0M–6M
Number of participants	**292**	**256**	**256**	**223**	**223**
TG	1.62 ± 0.60	1.48 ± 0.55*	−0.13 ± 0.59	1.39 ± 0.53*	−0.23 ± 0.64
TC	5.14 ± 0.91	5.02 ± 0.93	−0.12 ± 1.02	4.92 ± 0.95*	−0.26 ± 0.98
RC	0.74 ± 0.28	0.68 ± 0.25*	−0.06 ± 0.27	0.64 ± 0.24*	−0.11 ± 0.29
LDL	3.04 ± 0.78	2.99 ± 0.75	−0.04 ± 0.81	2.94 ± 0.78	−0.12 ± 0.77
HDL	1.37 ± 0.34	1.35 ± 0.34	−0.02 ± 0.30	1.34 ± 0.35	−0.05 ± 0.31
Non-HDL-C	3.78 ± 0.81	3.67 ± 0.79	−0.10 ± 0.87	3.58 ± 0.86*	−0.21 ± 0.86
TG:HDL ratio	2.94 ± 1.50	2.70 ± 1.34	−0.20 ± 1.24	2.60 ± 1.33*	−0.33 ± 1.30
hs-CRP	2.84 ± 5.60	1.98 ± 2.43	−0.67 ± 4.98	2.13 ± 1.84	−0.92 ± 5.85
GLU	4.57 ± 1.22	4.52 ± 0.93	−0.07 ± 1.46	4.79 ± 1.10	0.21 ± 1.46
HbA1c	5.58 ± 0.60	5.56 ± 0.39	−0.02 ± 0.45	5.58 ± 0.44	0.01 ± 0.53

Values represented as mean ± SD, *significantly different from baseline, $p < 0.0025$, triglycerides (TG; mmol/L), total cholesterol (TC; mmol/L), remnant cholesterol (RC; mmol/L), low-density lipoprotein cholesterol (LDL; mmol/L), high-density lipoprotein cholesterol (HDL; mmol/L), non-HDL-cholesterol (non-HDL-C; mmol/L), TG:HDL ratio, high-sensitivity C-reactive protein (hs-CRP; mg/L), glucose in plasma (GLU; mmol/L), hemoglobin A1c (HbA1c;%), change between baseline and month 3 (Δ0M–3M), change between baseline and month 6 (Δ 0M–6M).

of new drugs and ingredients, they may create standardized conditions that deviate from the real-world complexity of supplementation use. These discrepancies in patient selection or treatment conditions may alter effectiveness of the supplement in the end users. Consequently, this second clinical trial was designed as a post-market cohort study that targeted actual consumers of this dietary supplement, accounting for the complexity associated with real-world clinical and consumer settings. The supplementation period was doubled from three (previous trial) to 6 months, and sample size increased from 120 to 480 participants. This second trial reinforced the previously observed decrease in TC, RL, and non-HDL-C, and also demonstrated a remarkable (14.2%) decrease in TG. In line with the dietary supplement's health-supporting role, major disease hard endpoints were not measured. Despite these limitations, both studies observed the same mechanisms intrinsic to EPA-only drugs. This is important because, thus far, large pharmaceutical trials evaluating the MACE risk of mixed (DHA-containing) LCn-3 PUFAs have not shown the benefits of EPA ethyl esters, as seen in the REDUCE-IT and the JELIS trials (10, 21). Therefore, as EPA is emerging as the leading LCn-3 PUFA for the treatment of CVD in diseased patients, these clinical trials provide strong evidence for AlmegaPL® supplementation to support cardiometabolic health in healthy adults by helping maintain blood lipids already within the normal range. AlmegaPL® is the only natural source of EPA-only that is currently available over the counter for dietary supplementation. This novel ingredient provides a less processed and more affordable source of this fatty acid that fits the needs of the general population. In conclusion, AlmegaPL® provides a natural EPA-only supplementation option, previously unavailable, to help maintain cardiovascular health in the general population.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by the Argus Independent Review Board Committee (Tucson, Arizona). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

EG: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review and editing. EE: Data curation, Formal analysis, Investigation, Methodology, Writing – review and editing. MO: Data curation, Formal analysis, Investigation, Methodology, Writing – review and editing. CW: Formal analysis, Investigation, Methodology, Writing – review and editing.

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Conflict of interest

EG and EE are employees of Qualitas Health Inc. This study received funding from Qualitas Health Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Mussel oil is superior to fish oil in preventing atherosclerosis of ApoE$^{-/-}$ mice

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Objectives: The present study aimed to explore the preventive effect of mussel oil (MO) on atherosclerosis and the potential mechanism in apolipoprotein E-null (ApoE$^{-/-}$) mice.

Methods: ApoE$^{-/-}$ mice were fed with a high-fat and high-cholesterol chow and given corn oil (CO), fish oil (FO), MO, or aspirin (ASP, dissolved in CO) by gavage for 12 weeks. The total n-3 polyunsaturated fatty acids (PUFAs) in MO (51.01%) and FO (46.82%) were comparable (mainly C22:6n-3 and C20:5n-3). Wild-type mice were fed with a normal chow and given equivalent CO as health control (CON).

Results: Compared with the CON group, obvious atherosclerotic plaque appeared at aorta and aortic sinus in the CO group. Compared with the CO group, MO but not FO had a significantly smaller atherosclerotic plaque area in the aorta. The aortic atherosclerotic plaque area was comparable in the MO, CON, and ASP groups. The MO group had a significantly smaller atherosclerotic plaque area, lower lipid deposition, lower contents of smooth muscle cell (SMC), and slightly lower contents of macrophage at the aortic sinus than the FO group. Serum concentrations of IL-1β, NF-κB, and VCAM-1 were comparable in the MO and FO groups and were significantly lower than the CO group. Compared with the CO group, the MO group but not FO group had significantly lower aortic protein levels of p65NF-κB, p38MAPK, and VCAM-1. The aortic protein levels of p-p65NF-κB and p-p38MAPK were significantly lower in the MO group than the FO group.

Conclusion: In conclusion, MO is more potent than FO in preventing atherosclerosis, and the possible mechanism may be by downregulating p38MAPK/NF-κB signaling pathway, decreasing VCAM-1 and macrophage, and inhibiting proliferation and migration of SMC.

KEYWORDS

atherosclerosis, mussel, lipids, inflammation, smooth muscle cell, NF-κB, p38MAPK, VCAM-1

1 Introduction

Atherosclerosis is one of the most important causes of coronary artery disease, carotid artery disease, and peripheral arterial disease (1). Dysregulation of lipid metabolism and chronic inflammation are key triggers of atherosclerosis (2, 3).

In recent years, the beneficial effect of functional lipids on atherosclerosis has been paid much attention, and one of the most representatives is n-3 polyunsaturated fatty acid

(PUFA)-enriched oil, such as fish oil (FO). In low density lipoprotein (LDL) receptor knock-out mice, FO supplementation led to a significantly lower atherosclerotic lesion area (4). Dietary intake of C20:5n-3 decreased the area of atherosclerosis lesions in apolipoprotein E-null (ApoE$^{-/-}$) mice (5), one of the most widely used atherosclerosis models with lesions comparable to human lesions (6). A prospective cohort study observed a negative association between n-3 PUFA intake and the risk of carotid intima-media thickness (7). Another cohort study found a negative association between plasma C20:5n-3 and risk of cardiovascular disease events, and this association is more apparent in subjects with a higher score of coronary artery calcium (8). Potential mechanism is conducted by anti-inflammation and improvement in lipid metabolism (9–11).

Mussel oil (MO) contains a high content of n-3 PUFA (mainly C20:5n-3 and C22:6n-3) (12). Our latest studies found that MO had a beneficial effect on glycemic traits in both humans and mice and was superior to FO having comparable content of total n-3 PUFA (12, 13). It is noteworthy that MO also has a terrific anti-inflammatory effect. Our previous randomized controlled trials (RCTs) observed that MO improved clinical conditions of patients with rheumatoid arthritis and decreased serum levels of pro-inflammatory cytokines and eicosanoids (14). A better anti-inflammatory effect of MO than FO was observed in patients with type 2 diabetes mellitus (T2DM) (13). Another study extracted furan fatty acids from MO and found that they had a much better anti-inflammatory effect than C20:5n-3 in adjuvant-induced arthritis rats (15). In addition, MO also has a better lowering effect than FO on serum triacylglycerol (TG) (13).

Considering the beneficial role of MO in inflammation and lipid metabolism, we speculate that MO may have an anti-atherosclerosis effect, but this has not been verified in previous studies. Therefore, the aim of the present study was to explore the effect of MO on atherosclerosis and the potential mechanism by using ApoE$^{-/-}$ mice.

2 Materials and methods

2.1 Ethical approval

The study was approved by the Ethics Committee of Medical College of Qingdao University (QDU-AEC-2022369). All animal experimental procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Qingdao University.

2.2 Treatment oils

Mussel meat was vacuum freeze-dried and homogenized into powder, and then, MO was separated by supercritical fluid extraction (China Harbin Essen Biotechnology Co., Ltd.) (16). FO was purchased from Longzhou Biotechnology Co., Ltd., Xi'an, China. Corn oil (CO) was purchased from a local supermarket (Brand: LONGEVITY FLOWER).

2.3 Study design

The study design is shown in Figure 1. In brief, 6-week-old male wild-type C57BL/6J mice ($n = 6$) and ApoE$^{-/-}$ C57BL/6J mice ($n = 24$; Beijing Vital River Laboratory Animal Technology Co., Ltd) were housed in standard laboratory cages in a specific pathogen-free room under standard conditions (21 ± 1°C, 60% humidity, and 12 h light/dark cycle). After 1 week of adaption, ApoE$^{-/-}$ mice were randomly divided into four groups and fed with a high-fat and high-cholesterol (HFHC) chow (21% fat and 0.15% cholesterol, w/w), given CO, FO, MO, or aspirin (ASP, 0.5 mg/mL, and dissolved in CO) by gavage. The dosage of each oil is 0.1 mL/10 g/day. Wild-type mice were fed with a normal chow (AIN-93G) and given equivalent CO by gavage as health control (CON). The detailed fatty acid compositions of treatment oils are shown in Supplementary Table S1. In brief, the total n-3 PUFA content in MO and FO was 51.01 and 46.82%; MO had a slightly higher C22:6n-3 (26.72% vs. 19.34%) and a slightly lower C20:5n-3 (20.66% vs. 25.27%) than FO; the content of C18:2n-6 in CO was 53.47%. After 12 weeks of treatment, mice were sacrificed to collect tissues and blood samples for subsequent detection.

2.4 Atherosclerotic plaque quantitation and histologic analysis

To detect atherosclerotic plaque in the aorta, the entire aorta was put in PBS solution (0.01 M, pH 7.2–7.4), carefully stripped of perivascular fat under stereoscopic microscope, fixed with 4% paraformaldehyde, and cut open longitudinally and stained by Oil-red O (17, 18). Serial cryosections were obtained at the aortic sinus and aortic arch for histological analysis. In brief, the tissues were embedded, and serial 8–10 μm thick sections were cut from the aortic root for observation under microscope. Once three valves were observed, the sections were retained for staining. The plaque and lipid deposition of aortic arch and aortic sinus was quantified by staining sections with hematoxylin–eosin (H&E) and Oil-red O, respectively. The macrophage and the smooth muscle cell (SMC) of aortic sinus were detected by immunohistochemically staining with CD68 and anti-a-smooth muscle actin (a-SMA) antibodies, respectively (17, 19). Collagen of aortic sinus was detected by Sirius red staining (17). IPWIN32 software was used to quantify the plaque area, lipid deposition, macrophage, SMC, and collagen. The atherosclerotic lesion area in the aorta en face was quantified as a percent of the aortic surface area (20). The outline of the atherosclerotic lesions in aortic sinus and aortic arch was marked with a black dashed line. The positive regions of CD68 and α-SMA are brownish yellow, and the positive regions of Oil-red O and Sirius red staining are red. Lipid deposition in the aortic sinus and aortic arch and CD68, α-SMA, and Sirius red staining in the aortic sinus were expressed as a ratio of positive area versus atherosclerotic plaque area (21).

2.5 Fatty acid determination

We analyzed erythrocyte membrane phospholipid (PL) fatty acid composition, which can reflect the changes in response to long-term dietary fat intake (22, 23). The fatty acid compositions (% in total fatty acids) in the treatment oil were detected according to our previous study (12). Moreover, the erythrocyte membrane was separated and washed according to our another previous study (24). In brief, lipids of erythrocyte membrane were extracted by chloroform/methanol (1:1), and the phospholipid (PL) fraction was separated by thin-layer

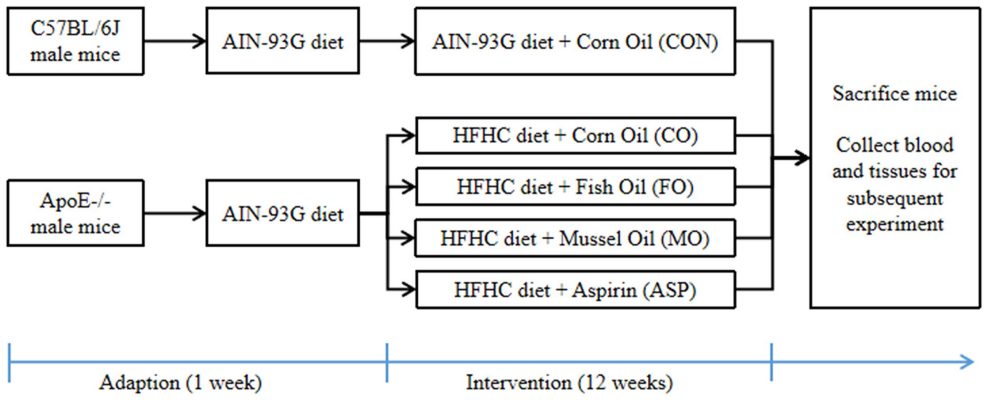

FIGURE 1
Diagram of study design. HFHC, high-fat and high-cholesterol. Aspirin was dissolved in corn oil (0.5 mg/mL). The dosage of each oil was 0.1 mL/10 g/day.

chromatography. The lipids within the treatment oils and the PL fraction of erythrocyte membrane were blended with toluene and 0.9 mol/L H_2SO_4/methanol (1:3, v:v). Fatty acid methyl esters were generated by incubating the mixture at 70°C for 120 min. They were extracted using n-hexane and then purified using a Sep-Pak silica column. Subsequently, the solution was dried under N_2 and redissolved in n-hexane. Fatty acid methyl esters of the treatment oil and erythrocyte membrane PL were separated by a gas chromatography (GC) equipped with an Agilent DB-23 column (60 m, 0.25 mm*0.25 μm). The sample inlet temperature was maintained at 260°C, while the pressure of N_2 and H_2 was set to 50 and 75 kPa, respectively. The temperature program of the GC was as follows: 0–2 min at 140°C; 2–3 min ramping to 160°C; 3–8 min at 160°C; 8–9 min ramping to 180°C; 9–21 min at 180°C; 21–22 min ramping to 200°C; 22–30 min at 200°C; 30–30.25 min ramping to 205°C; and 30.25–41.25 min at 205°C. A standard of fatty acid mixture (cat. no. 18919-1AMP SUPELCO, Sigma–Aldrich) was used to identify individual fatty acids according to retention time.

2.6 Determination of serum lipids and inflammatory factors

Serum lipids were detected by biochemical kits (Nanjing Jiancheng Bioengineering Institute): TG (A110-1-1), total cholesterol (TC; A111-1-1), high density lipoprotein cholesterol (HDL-C; A112-1-1), and low density lipoprotein cholesterol (LDL-C; A113-1-1). Serum inflammatory factors were detected by ELISA kits (Shanghai Jining Industrial Co, Ltd): interleukin-6 (IL-6; JN16894), IL-10 (JN17305), IL-1β (JN16939), vascular cellular adhesion molecule-1 (VCAM-1; HN20565), nuclear factor kappa-B (NF-κB; JN20529), tumor necrosis factor-α (TNF-α; JN17113), and Monocyte Chemoattractant Protein-1 (MCP-1; JN17005).

2.7 Determination of protein levels in the aorta

Protein levels of genes in the aorta were detected by Western blotting analysis. In brief, aortic tissue lysates were separated by 10% SDS-PAGE and transferred to 0.45 μm PVDF membranes (IPVH00010, Merck Millipore). After blocking in 5% skim milk (Cat#D8340, Solarbio; for non-phosphorylated protein) or 5% BSA (G5001-5G, Servicebio; for phosphorylated protein) for 2 h, the membranes were incubated overnight at 4°C with primary antibodies: p65NF-κB (1:1000, AF5006, Affinity Biosciences); p-p65NF-κB (1:1000, AB76302, Abcam); p38 mitogen-activated protein kinase (p38MAPK; 1:2500, ab170099, Abcam); p-p38MAPK (1:1000, AB195049, Abcam); VCAM-1 (1:5000, ab134047, Abcam); and *β*-actin (1:1000, GB15003, Servicebio). The membranes were then incubated with HRP-conjugated secondary antibody for 90 min at room temperature. Protein bands were visualized using ECL solution on Ultra Sensitive Multifunctional Imager (AI680RGB, GE, Japan) and analyzed using the ImageJ software.

2.8 Determination of furan fatty acids and astaxanthin

The contents of 11-(3,4-dimethyl-5-propylfuran-2-yl)undecanoic acid (11D3) and 11-(3,4-dimethyl-5-pentylfuran-2-yl)undecanoic acid (11D5) were quantified by Agilent Technologies 6530C Q-TOF UPLC-MS/MS, according to previous studies (25, 26). In brief, serum (20 μL), internal standard (100 ng), and 5% KOH-ethanol (500 μL) were added to a centrifuge tube and kept at 60°C for 2 h. Then, the pH was adjusted to 4 with 1 M HCl. The reaction products were extracted with n-hexane (300 μL) three times. After evaporation of the solvent, 11D3 and 11D5 were derived into 11D3-3-acyl-oxymethyl-1-methylpyridinium iodide (AMMP) and 11D5-AMMP using 20 μLBMP (2-bromo-1-methyliodopyridine, 7.5 mg/mL in acetonitrile), 20 μl CMP (3-methanol-1-methyliodopyridine, 10 mg/mL in acetonitrile), and 1 μL triethylamine. After derivatization, the solution was dried under N_2 again and redissolved in 100 μL of acetonitrile/H_2O (7:3, v/v). The derivatized furan fatty acids were separated by UPLC equipped with a Sepax Opalshell C18 column (2.1 mm x 100 mm, i.d.2.6 μm) in positive mode. The mobile phase consisted of H_2O (0.1%HCOOH; A) and acetonitrile (0.1%HCOOH; B). The gradient used was (min/% B): 0:10; 1:20; 4:30; 7:40; 13:50; 20:50; 21:60; 25:60; 26:70; 27:70; 28:100; 32:100; and 33:10. A flow rate of 0.4 mL min^{-1} was used, and the injection volume was 2 μL. The main

fragmentations were: 11D3-AMMP (m/z 428–107, 428–124, 428–178) and 11D5-AMMP (m/z 456–107, 456–124, 456–178). The MS scan range was set at m/z 100–600 and the MS/MS analysis at collision energy of 42 V.

Astaxanthin content was quantified by high-performance liquid chromatography (HPLC) (26, 27). A certain amount of the sample was dissolved in 1 mL dichloromethane: methanol (1:3, v:v), saponified, and transferred into a capped test tube. The samples were determined by HPLC equipped with a C30 column (250 mm x 4.6 mm, 5 μm) at 25°C. The mobile phase consisted of methanol (A), tert-butyl methyl ether (B), and 1% phosphoric acid solution (C). The gradient used was (min/% B): 0:15; 15:30; 23:80; 27:80; 30:15; and 35:15. The mobile phase C remained at 4% throughout the process. A flow rate of 1.0 mL min^{-1} was used, and the column was monitored at 474 nm.

2.9 Statistical analysis

Data were expressed as mean ± SEM unless otherwise specified. One-way ANOVA was used for significance test, and an LSD post-hoc test was used for multiple comparisons between groups. Spearman correlation analysis was used to evaluate the linear relationship between continuous variables. Partial correlation was used to analyze the relationship between serum furan fatty acids and atherosclerosis-related parameters. A value of $p < 0.05$ was considered to be statistically significant. All statistical analyses were conducted using SPSS 26.0. Figures were generated using GraphPad Prism 8.02.

3 Results

3.1 Effect of mussel oil on atherosclerotic lesion formation

Compared with the CON group, significantly greater atherosclerotic plaque area in the aorta and aortic sinus was observed in the CO group ($p < 0.05$; Figure 2). The MO group had a significantly smaller atherosclerotic lesion area of the aorta than the CO group ($p < 0.05$), and it was comparable with that in the ASP and CON groups ($p > 0.05$). The atherosclerotic lesion area in the aorta of the FO group was slightly smaller than the CO group, but this difference was non-significant ($p > 0.05$). The atherosclerotic plaque area and lipid deposition of the aortic sinus were significantly lower in the MO group than in the FO group ($p < 0.05$). There was no significant difference in atherosclerotic lesion area of the aortic arch in the CON, CO, FO, MO, and ASP groups ($p > 0.05$; Figure 3). Significantly higher lipid deposition of the aortic arch was observed in the CO group than in the CON and MO groups ($p < 0.05$). The lipid deposition of the aortic arch in the MO group was slightly lower than in the FO and ASP groups, but this difference was non-significant ($p > 0.05$).

There was significantly fewer SMC (indicated by α-SMA) in the aortic sinus in the MO and ASP groups than in the CO and FO groups ($p < 0.05$; Figure 4). The CO, MO, and ASP groups had a significantly higher content of collagen (indicated by Sirius red staining) in the aortic sinus than in the CON group ($p < 0.05$). No significant difference in collagen was observed between the CO, FO, MO, and ASP groups ($p > 0.05$). The content of macrophages (indicated by CD68) in the aortic sinus of the CO and FO groups was significantly higher than in the CON group ($p < 0.05$). The ASP group had significantly lower content of macrophages than in the FO group ($p < 0.05$), and the MO group had slightly lower content of macrophages than in the FO group ($p = 0.077$).

3.2 Effect of mussel oil on serum lipids and inflammatory factors

The serum concentration of TG in the CO group was significantly higher than in the CON group but was significantly lower than in the FO group ($p < 0.05$; Figure 5). The serum concentration of TG in MO had no significant difference with the CO, FO, and ASP groups ($p > 0.05$). Compared with the CON group, serum TC and LDL-C were significantly higher, but HDL-C was significantly lower in the CO, FO, MO, and ASP groups ($p < 0.05$). No significant difference was observed in TC, LDL-C, and HDL-C between the CO, FO, MO, and ASP groups ($p > 0.05$).

Compared with the CON group, the CO group had a significantly higher serum IL-6, IL-1β, IL-10, TNF-α, NF-κB, VCAM-1, and MCP-1 ($p < 0.05$; Figure 5). The MO and FO groups had a significantly lower serum IL-1β, NF-κB, and VCAM-1 than in the CO group ($p < 0.05$). No significant difference was observed in serum inflammatory factors between the MO and FO groups ($p > 0.05$).

3.3 Effect of mussel oil on inflammatory factors in the aorta

The protein content of VCAM-1, p65NF-κB, and p-p38MAPK in the aorta was significantly higher in the CO group than in the CON group ($p < 0.05$; Figure 6). Compared with the CO group, the MO group but not the FO or ASP group had significantly lower VCAM-1 content in the aorta ($p < 0.05$), and this content in the MO group was comparable to the CON group ($p > 0.05$). The MO group but not the FO or ASP group had a significantly lower protein content of p65NF-κB and p38MAPK in the aorta than in the CO group ($p < 0.05$). The MO and ASP groups had a significantly lower protein level of p-p65NF-κB and the ratio of p-p65NF-κB/p65NF-κB than in the FO group and a lower protein level of p-p38MAPK than in the CO and FO groups ($p < 0.05$), and these contents in the MO and ASP groups were comparable to the CON group ($p > 0.05$). Aortic p65NF-κB was positively correlated with p38MAPK ($r = 0.718$, $p = 0.003$) and VCAM-1 ($r = 0.611$, $p = 0.016$); p-p65NF-κB was positively correlated with p-p38MAPK ($r = 0.821$, $p < 0.001$); the ratio of p-p65NF-κB/p65NF-κB was positively correlated with p-p38MAPK ($r = 0.586$, $p = 0.022$).

3.4 Fatty acid composition of erythrocyte membrane phospholipids

C18:2n-6, C20:4n-6, and total n-6 PUFA of erythrocyte membrane PL were significantly lower in the FO and MO groups than in the CON, CO, and ASP groups ($p < 0.05$; Supplementary Figure S1). C20:5n-3, C22:6n-3, and total n-3 PUFA and the ratio of n-3/n-6 PUFA were significantly higher in the FO and MO groups than in the CON, CO, and ASP groups ($p < 0.05$). No significant difference was observed in these PL PUFA contents between the FO and MO groups ($p > 0.05$).

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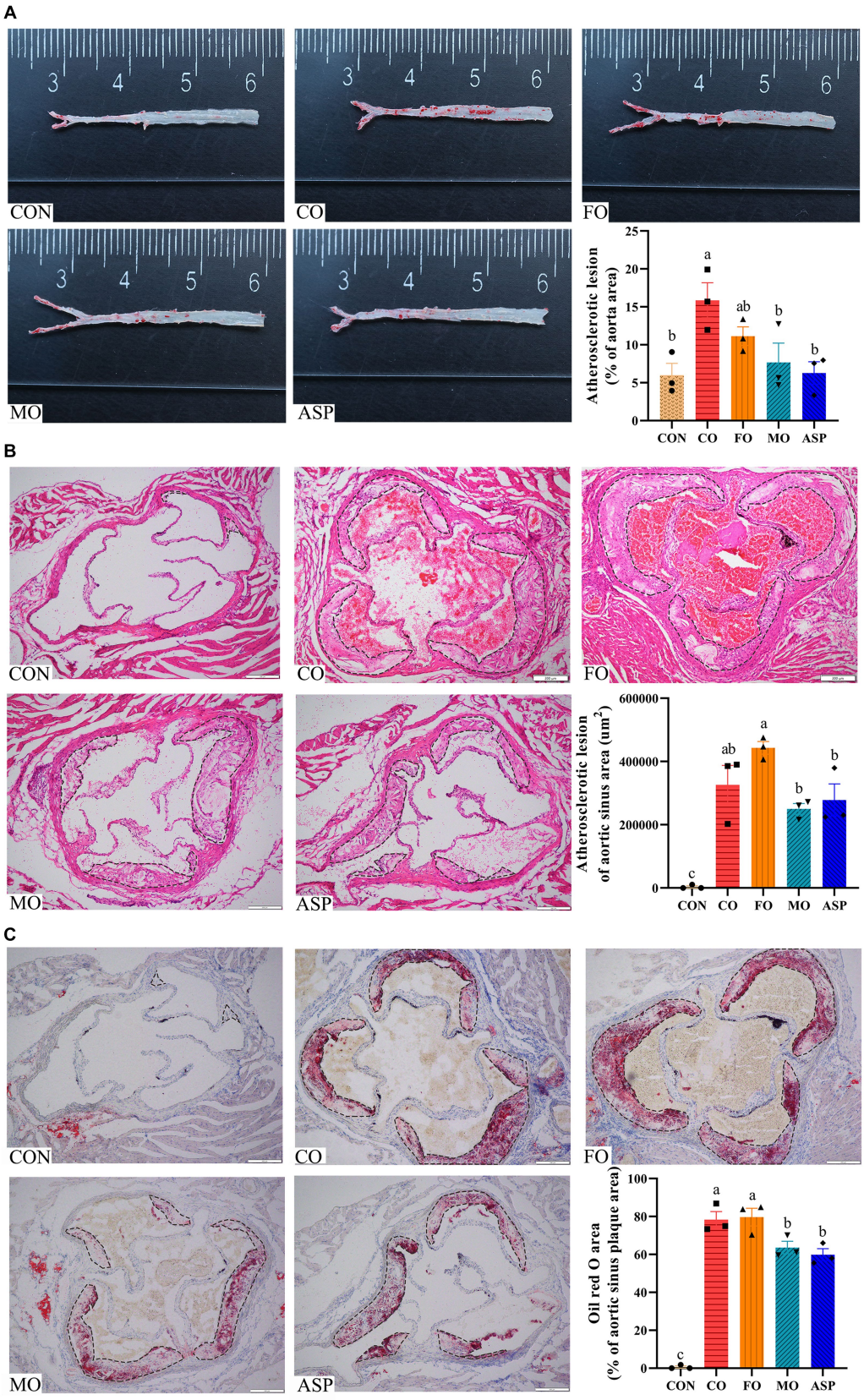

FIGURE 2
Effect of treatment oils on atherosclerotic plaque in the aorta **(A)** and aortic sinus **(B,C)**. Three mice in each group were included for analysis. For B–C, 2 serial sections of each mice were used, and the mean of two sections from one mice was included in the final analysis. Atherosclerotic plaque in the aorta was detected by Oil-red O staining **(A)**. Atherosclerotic plaque and lipid deposition in the aortic sinus was detected by H&E **(B)** and Oil-red O staining **(C)**, respectively. The outline of the atherosclerotic lesions in the aortic sinus was marked with a black dashed line. The result of Oil red O staining in the aortic sinus was normalized by atherosclerotic plaque area. Data were expressed as mean ± SEM. There was significance if groups did not share the same letter ($p < 0.05$). CON, health control; CO, corn oil; FO, fish oil; MO, mussel oil; ASP, aspirin.

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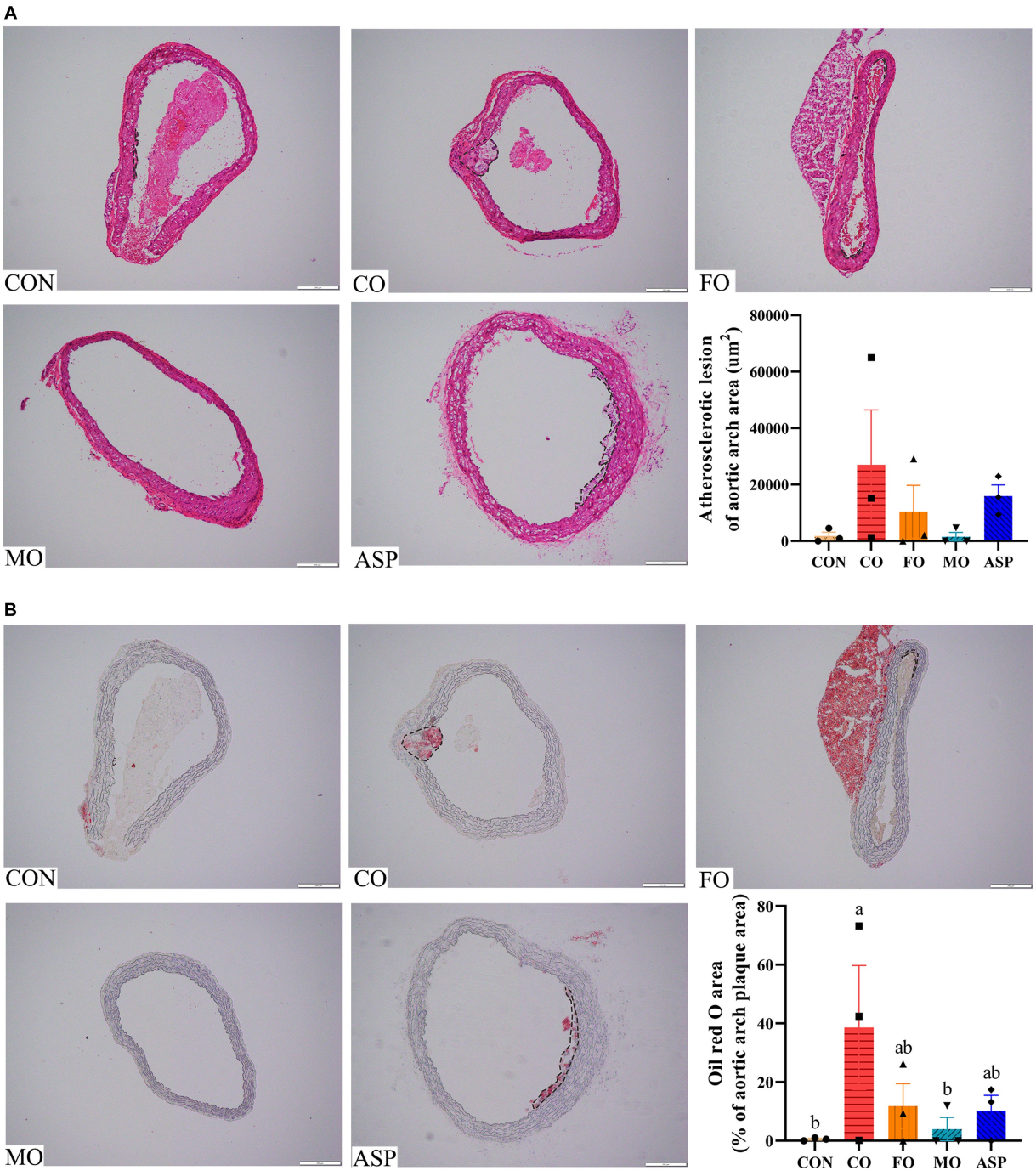

FIGURE 3
Effect of treatment oils on atherosclerotic plaque in the aortic arch. Three mice in each group were included for analysis, and two serial sections of each mice were used. The mean of two serial sections of each mice were used for analysis. **(A)** Results of H&E staining. **(B)** Results of Oil-red O staining. The outline of the atherosclerotic lesions in the aortic arch was marked with a black dashed line. The result of Oil red O staining was normalized by atherosclerotic plaque area. Data were expressed as mean ± SEM. There was significance if groups did not share the same letter ($p < 0.05$). CON, health control; CO, corn oil; FO, fish oil; MO, mussel oil; ASP, aspirin.

3.5 Correlation between erythrocyte membrane phospholipid fatty acids and atherosclerosis-related parameters

Erythrocyte membrane PL C20:5n-3 and total n-3 PUFA were negatively correlated with atherosclerotic lesion area of the aorta ($r = -0.717$, $p = 0.030$; $r = -0.783$, $p = 0.013$) and SMC of the aortic sinus (indicated by α-SMA; $r = -0.733$, $p = 0.025$; $r = -0.817$, $p = 0.007$; Figure 7). PL C20:5n-3 was negatively correlated with lipid deposition (Oil red O staining) of the aortic arch ($r = -0.763$, $p = 0.017$), and PL total n-3 PUFA was negatively correlated with lipid deposition of the aortic sinus ($r = -0.733$, $p = 0.025$). PL C22:6n-3 was negatively correlated with

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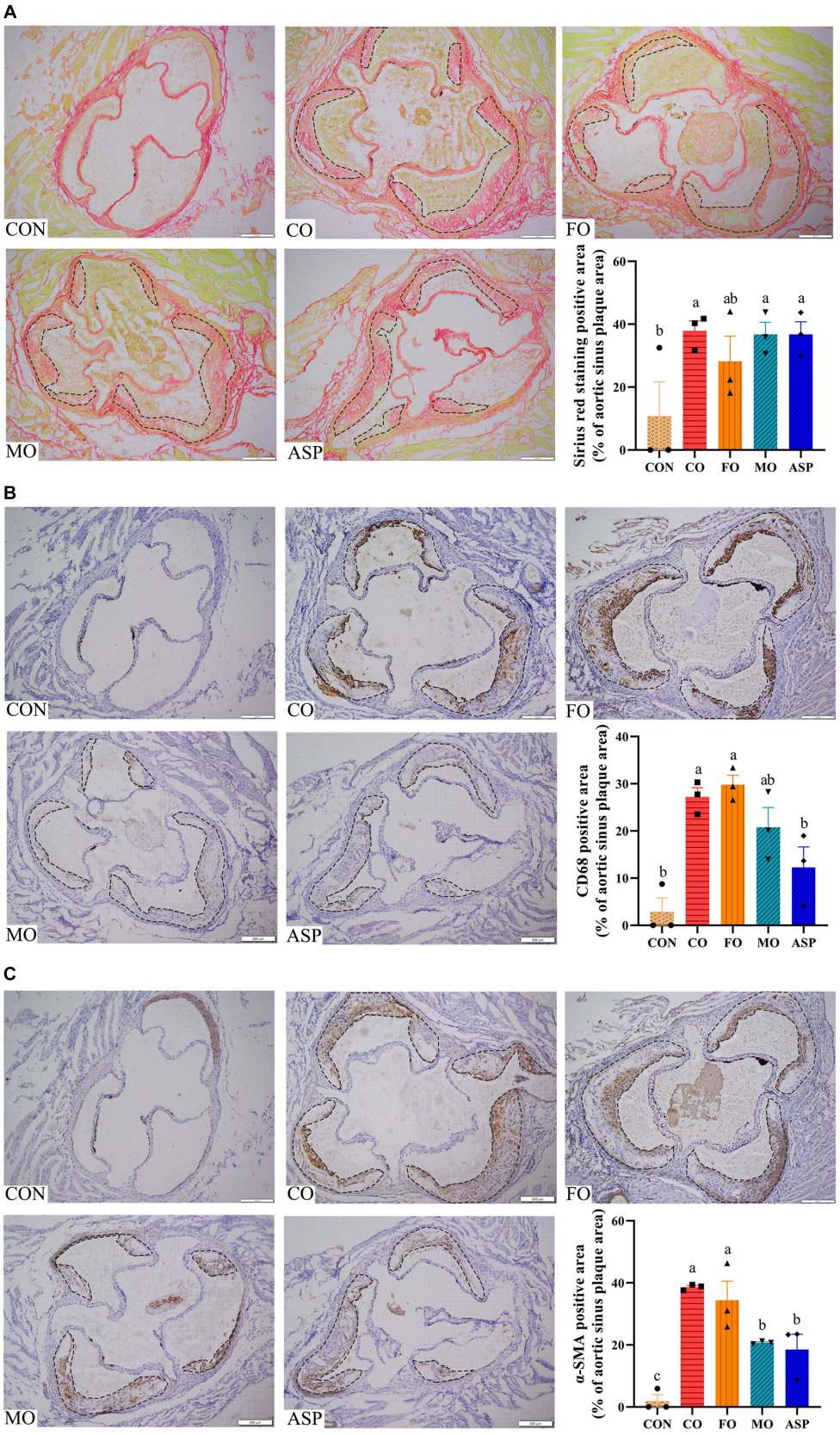

FIGURE 4
Effect of treatment oils on collagen **(A)**, macrophage **(B)**, and SMC **(C)** in the aortic sinus. Three mice in each group were included for analysis, and two serial sections of each mice were used. The mean of two serial sections of each mice were used for analysis. The collagen was detected by Sirius red staining. The macrophage and SMC were detected by immunohistochemically staining with CD68 and anti-a-smooth muscle actin (a-SMA) antibody, respectively. The positive areas were quantified within the range of plaques circled by the black dashed line. The results were normalized by atherosclerotic plaque area. Data were expressed as mean ± SEM. There was significance if groups did not share the same letter ($p < 0.05$). SMC, smooth muscle cell; CON, health control; CO, corn oil; FO, fish oil; MO, mussel oil; ASP, aspirin.

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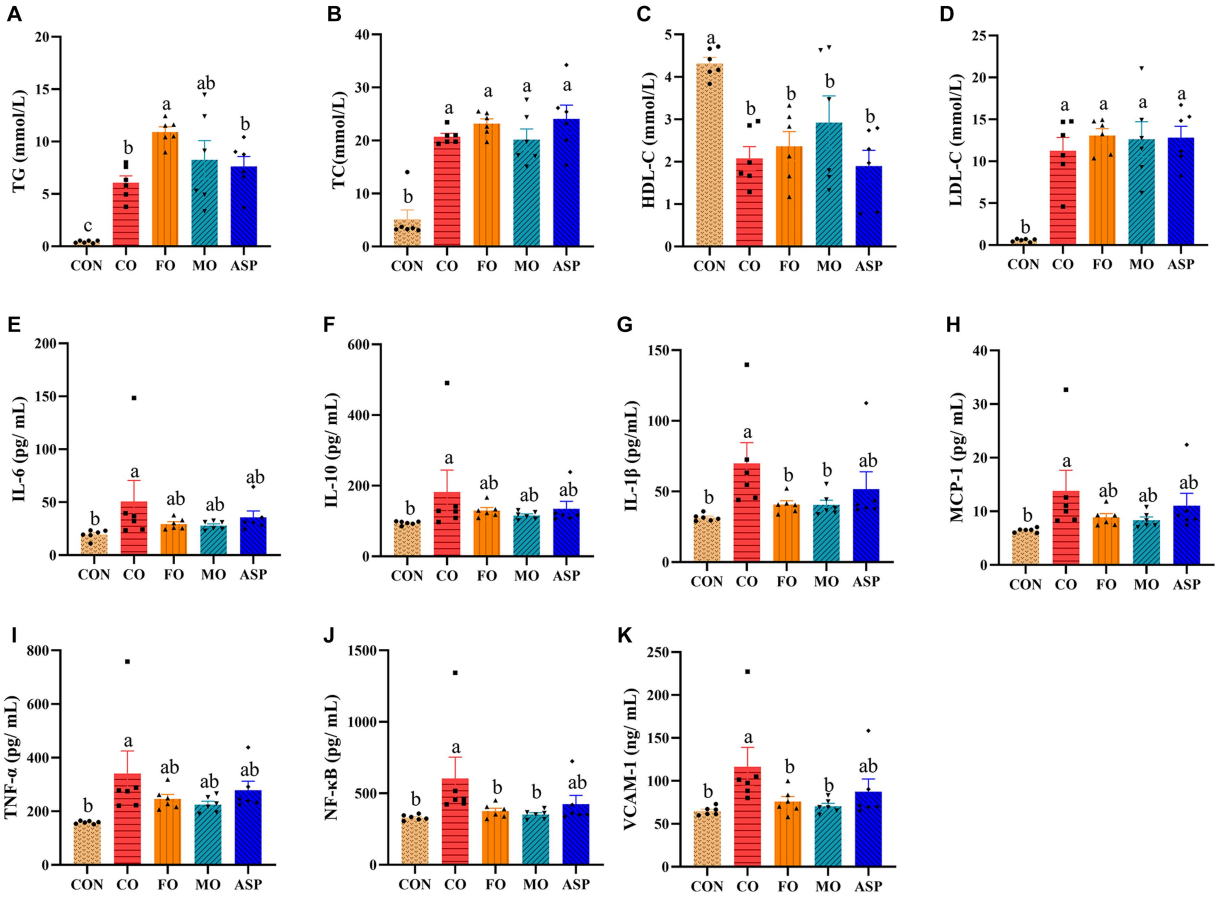

FIGURE 5
Effect of treatment oils on serum lipids and inflammatory factors (n = 6 in each group). The results of TG **(A)**, TC **(B)**, HDL-C **(C)**, LDL-C **(D)**, IL-6 **(E)**, IL-10 **(F)**, IL-1β **(G)**, MCP-1 **(H)**, TNF-β **(I)**, NF-βB **(J)** and VCAM-1 **(K)**. Data were expressed as mean ± SEM. There was significance if groups did not share the same letter ($p < 0.05$). CON, health control; CO, corn oil; FO, fish oil; MO, mussel oil; ASP, aspirin.

atherosclerotic lesion area of the aortic arch ($r = -0.729$, $p = 0.026$) and SMC of the aortic sinus ($r = -0.817$, $p = 0.007$). p-p38MAPK was negatively correlated with PL C20:5n-3 ($r = -0.650$, $p = 0.058$), PL n-3 PUFA ($r = -0.750$, $p = 0.020$), and the ratio of PL n-3/n-6 PUFA ($r = -0.800$, $p = 0.010$). p65NF-κB was negatively correlated with the ratio of PL n-3/n-6 PUFA ($r = -0.650$, $p = 0.058$). VCAM-1 was negatively correlated with PL n-3 PUFA ($r = -0.683$, $p = 0.042$) and the ratio of PL n-3/n-6 PUFA ($r = -0.667$, $p = 0.050$).

Erythrocyte membrane PL C20:5n-3 was negatively correlated with serum IL-1β ($r = -0.623$, $p = 0.006$), NF-κB ($r = -0.641$, $p = 0.004$), and VCAM-1 ($r = -0.536$, $p = 0.022$; Supplementary Table S2). PL C22:6n-3 was positively correlated with serum TG ($r = 0.569$, $p = 0.014$). PL C22:6n-3 and total n-3 PUFA were negatively correlated with serum IL-1β ($r = -0.663$, $p = 0.003$; $r = -0.657$, $p = 0.003$), MCP-1 ($r = -0.536$, $p = 0.022$; $r = -0.581$, $p = 0.011$), NF-κB ($r = -0.806$, $p < 0.001$; $r = -0.773$, $p < 0.001$), TNF-α ($r = -0.470$, $p = 0.049$; $r = -0.517$, $p = 0.028$), and VCAM-1 ($r = -0.692$, $p = 0.001$; $r = -0.672$, $p = 0.002$). The ratio of PL n-3/n-6 PUFA was positively correlated with serum HDL-C ($r = 0.523$, $p = 0.026$) and negatively correlated with serum IL-1β ($r = -0.599$, $p = 0.009$), MCP-1 ($r = -0.511$, $p = 0.030$), NF-κB ($r = -0.711$, $p = 0.001$), and VCAM-1 ($r = -0.550$, $p = 0.018$).

3.6 The content of furan fatty acids and astaxanthin in treatment oils

The contents of 11D3 and 11D5 in FO were 3906.91 ng/mg and 1971.60 ng/mg. The content of astaxanthin was under detection limit in FO. The MO and CO used in the present study were the same as those used in our previous study (26). The contents of 11D3 and 11D5 in MO were 2828.70 ng/mg and 1582.10 ng/mg, the contents of 11D3 and 11D5 in CO were 210.92 ng/mg and 167.79 ng/mg, the content of astaxanthin was 191 mg/kg in MO and was under detection limit in CO, according to the previous study (26).

3.7 Serum furan fatty acids and its correlation with atherosclerosis-related parameters

The contents of 11D3 and 11D5 in serum are shown in Figure 8. No significant difference in 11D3 was found between the FO and MO groups ($p > 0.05$). The content of 11D3 in the serum of the CON, CO, and ASP groups did not reach the detection

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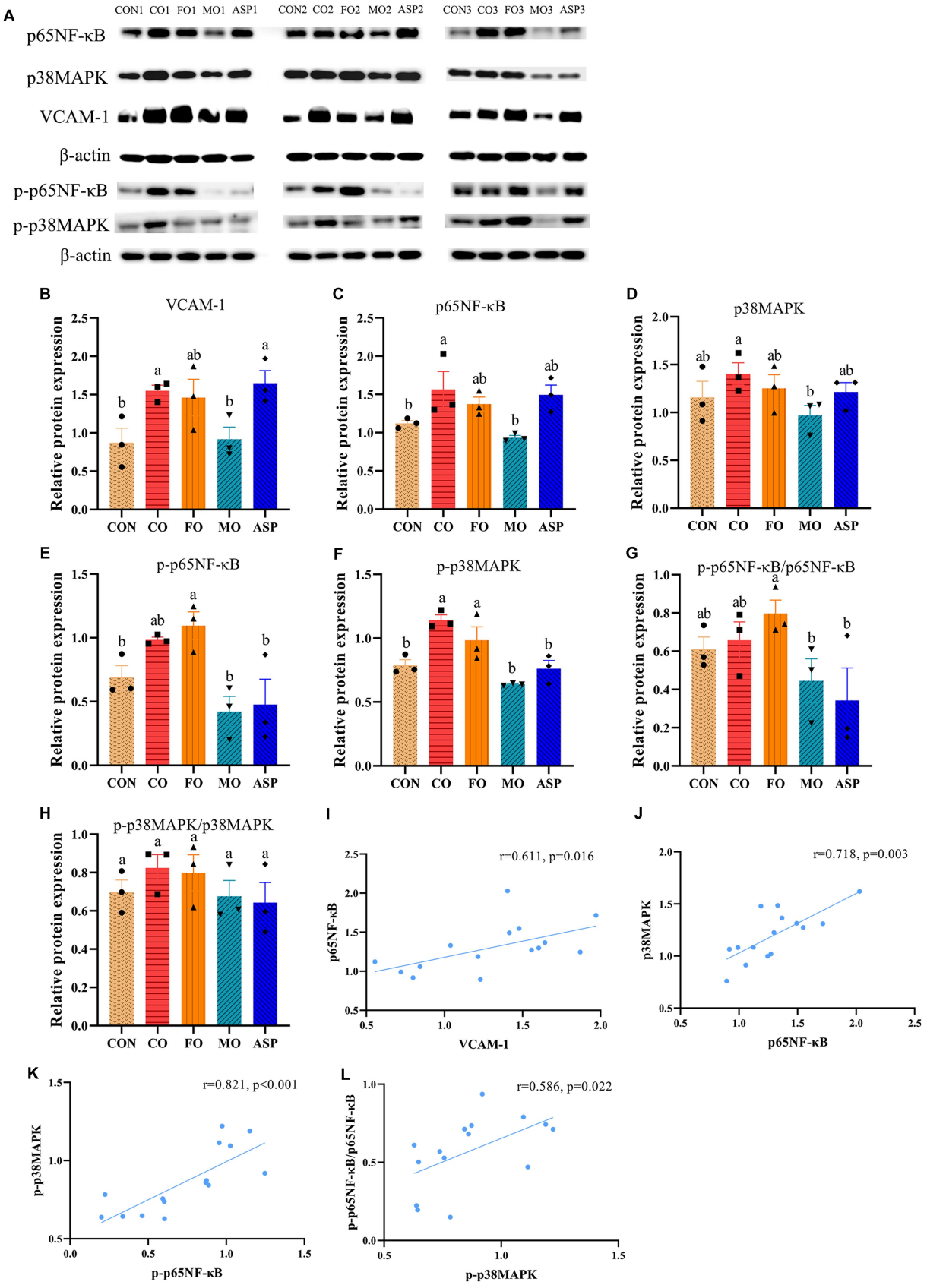

FIGURE 6
Effect of treatment oils on inflammatory factors in the aorta ($n = 3$ in each group). **(A)** Representative bands of Western blotting assay. **(B–F)** Quantitative results of VCAM-1, p65NF-κB, p38MAPK, p-p65NF-κB, and p-p38MAPK, respectively (mean ± SEM). **(G,H)** Ratios of p-p65NF-κB/p65NF-κB and p-p38MAPK/p38MAPK (mean ± SEM). **(I–L)** Significant correlations between inflammatory factors in the aorta (Spearman correlation). There was significance if groups did not share the same letter ($p < 0.05$). CON, health control; CO, corn oil; FO, fish oil; MO, mussel oil; ASP, aspirin.

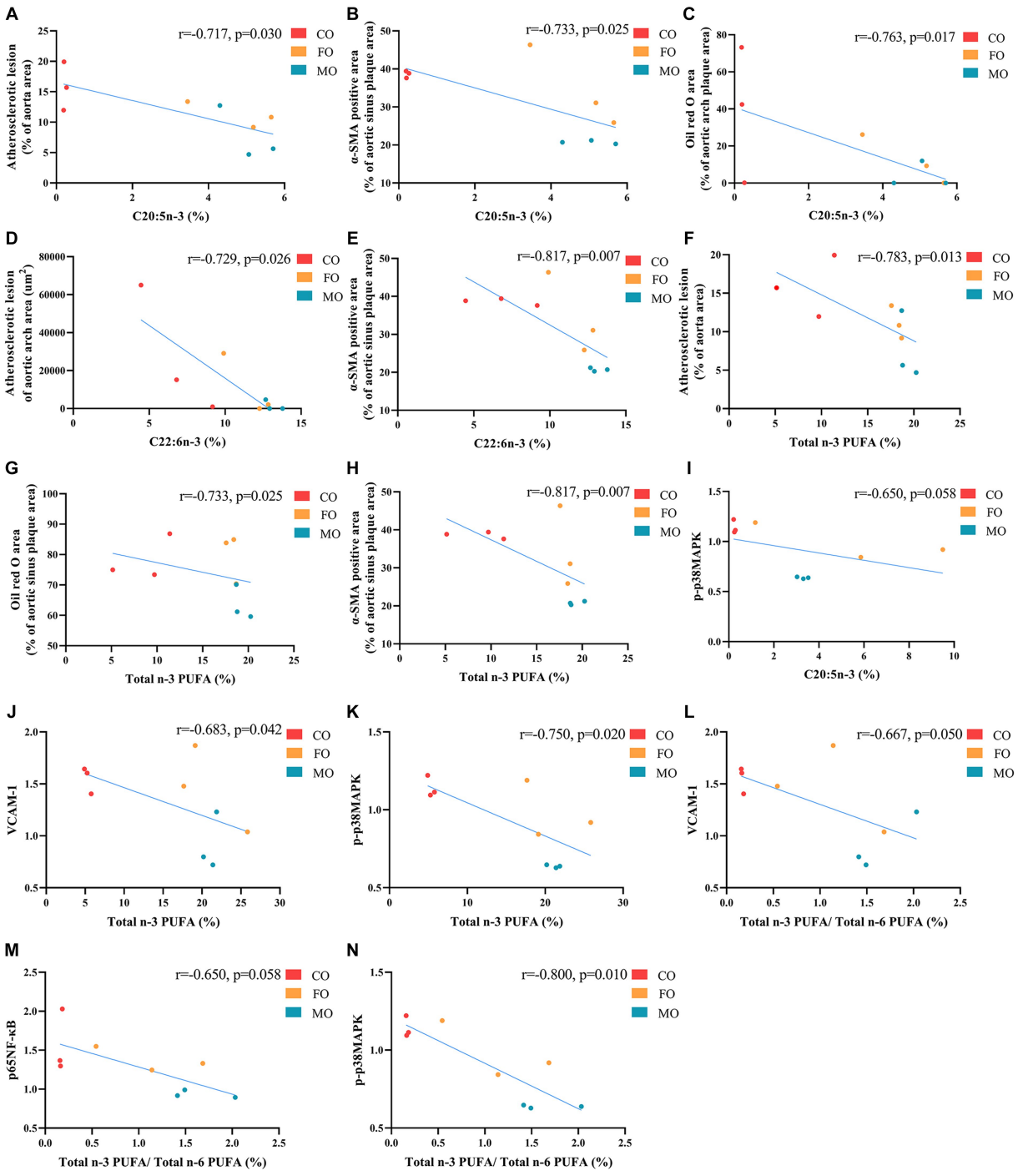

FIGURE 7
Correlation of n-3 polyunsaturated fatty acids between erythrocyte membrane phospholipids and atherosclerosis-related parameters (n = 9). **(A-C, I)** The correlation between C20:5n-3 (%) and atherosclerotic lesion (% of aorta area), α-SMA positive area (% of aortic sinus plaque area), Oil red O area (% of aortic arch plaque area) and p-p38MAPK respectively. **(D-E)** The correlation between C22:6n-3 (%) and atherosclerotic lesion of aortic arch area and α-SMA positive area (% of aortic sinus plaque area) respectively. **(F,H,J,K)** The correlation between Total n-3 PUFA (%) and atherosclerotic lesion (% of aorta area), Oil red O area (% of aortic sinus plaque area), α-SMA positive area (% of aortic sinus plaque area), VCAM-1 and p-p38MAPK respectively. **(L-N)** The correlation between Total n-3 PUFA/ Total n-6 PUFA (%) and VCAM-1, p65NF-κB and p-p38MAPK. Spearman correlation analysis was used for data analysis. Mice in CO, FO and MO were included in correlation analysis.

limit. The content of 11D5 in the MO group was significantly lower than that in the FO group ($p < 0.05$) but significantly higher than that in the CON, CO, and ASP groups ($p < 0.05$). Serum 11D5 was positively correlated with aortic p-p38MAPK/p38MAPK ($r = 0.803$, $p = 0.016$), aortic VACM-1 ($r = 0.805$, $p = 0.016$), and serum TG ($r = 0.669$, $p = 0.003$) after adjusting for

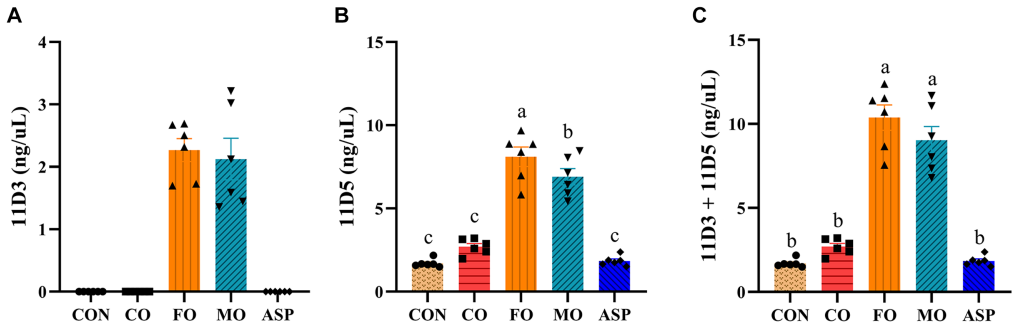

FIGURE 8
Effect of treatment oils on furan fatty acids in serum (n = 6 in each group). The results of 11D3 **(A)**, 11D5 **(B)** and 11D3+11D5 **(C)**. Data were expressed as mean ± SEM. There was significance if groups did not share the same letter ($p < 0.05$). CON, health control; CO, corn oil; FO, fish oil; MO, mussel oil; ASP, aspirin.

erythrocyte membrane PL n-3 PUFA. The mass spectrum of 11D3-AMMP and 11D5-AMMP in serum is shown in Supplementary Figures S2, S3.

4 Discussion

In the present study, MO was more potent than FO in preventing atherosclerosis of ApoE$^{-/-}$ mice, which provided a new strategy for nutritional prevention of atherogenesis. Commercial products of MO already existed, such as Lyprinol (PCSO-524), a lipid extract of the green-lipped mussel. Several clinical trials of MO have been conducted and found its beneficial effect on rheumatoid arthritis (14), osteoarthritis (28), asthma (29), and attention-deficit hyperactivity disorder (ADHD) (30). No obvious adverse effect of MO was reported during clinical trials (14, 28), indicating its safety. Future RCT can be conducted to clarify whether MO has an anti-atherosclerosis effect on humans and thus provide a basis for its application as a nutritional product for prevention and treatment of atherosclerosis.

The major fatty acids in both MO and FO are long-chain n-3 PUFA (mainly C20:5n-3 and C22:6n-3). The beneficial effect of n-3 PUFA on atherosclerosis has been reported in cohort studies (7, 8). A previous study showed that FO led to reduced aortic root lesions in ApoE$^{-/-}$mice (31). In the present study, we observed that total n-3 PUFA content in erythrocyte membrane phospholipids was negatively correlated with the Oil Red O area of the aortic sinus (Figure 7G). However, no significant difference was observed in atherosclerotic area and Oil Red O area of the aortic sinus between the FO and CO groups. Possible reasons for this inconsistency are as follows. ApoE$^{-/-}$ mice develop arterial lesions in a time-dependent manner (32). The age of mice in the present study for starting intervention was 7 weeks, but the age in the study by Wang et al. was 10 weeks (31). Difference in the age of mice can lead to variation in severity of atherosclerosis and may influence the treatment effect. In addition, during adaption, all mice were fed with AIN-93G diet for 1 week in the present study. As reported in our previous study (33), this diet contains 7% soybean oils (w/w) and 6% C18:3n-3 (% in total fatty acids). C18:3n-3 can be converted to C20:5n-3, C22:5n-3, and C22:6n-3 *in vivo* after desaturation and elongation. However, the fat of diet in the control group of the study by Wang et al. was provided by CO and did not contain C18:3n-3 (31). Therefore, the C18:3n-3 in the adaption diet may narrow the gap of n-3 PUFA content in mice tissue between the FO and CO groups and thus led to non-significant results. Conflicting results were also observed in clinical trials. An RCT found that pure C20:5n-3 supplementation (1.8 g/d) significantly decreased carotid intima-media thickness (IMT) of T2DM patients (34). However, another RCT found that the supplementation of fish oil (2.4 g/d, containing 35% C20:5n-3 and 20% C22:6n-3) had no effect on carotid IMT, plaque score, and plaque area in subjects with hypercholesterolaemia (35). Non-significant result was also observed in an RCT with fish oil treatment (3–6 g/d, containing 55% C20:5n-3 and C22:6n-3) (36). The anti-atherosclerosis effect of n-3 PUFA was supported by a recent meta-analysis of clinical trials with high dose of pure n-3 PUFA (≥1.8 g/d), but subgroup analysis indicated that a significant effect was only observed for pure C20:5n-3 but not mixture of C20:5n-3 and C22:6n-3 (37). These results indicated that type of n-3 PUFA, purity, and dosage may influence its anti-atherosclerosis effect. Therefore, for fish oil (a common source of n-3 PUFA), sources of fish and different preparation methods may also influence its beneficial effect on atherosclerosis. In the present study, lipid deposition of the aorta and aortic arch in the MO group was significantly lower than in the CO group; aortic atherosclerotic plaque area in the FO group was also slightly lower than in the CO group, although this difference was non-significant. The present study found that PL n-3 PUFA of erythrocyte membrane was negatively correlated with atherosclerotic plaque area of the aorta and aortic arch, lipid deposition of the aortic arch and sinus, and SMC content of the aortic sinus, indicating that n-3 PUFA is a bioactive compound which is responsible for the beneficial effect of MO on atherogenesis. However, n-3 PUFA cannot completely explain why MO was more potent than FO in preventing atherogenesis. In the present study, the content of total n-3 PUFA in MO and FO was comparable. Although MO had a slightly higher C22:6n-3 and a slightly lower C20:5n-3 than FO, there was no significant difference in PL C20:5n-3, C22:6n-3, and total n-3 PUFA in erythrocyte membrane between the MO and FO groups. Therefore, other bioactive components, except for n-3 PUFA in MO, may also have a strong anti-atherogenesis property.

In the present study, there was limited atherosclerotic lesion in the aortic arch. According to previous studies, ApoE$^{-/-}$ mice aged 18 weeks (38) and ApoE$^{-/-}$ mice fed a high-fat diet for 12 weeks also showed limited lipid deposition in the aortic arch (39). Aortic arch is a vulnerable site for atherosclerotic plaque formation in ApoE$^{-/-}$ mice (40). However, one

study observed that lipid deposition in the abdominal aorta was more than that in the aortic arch (20). Another study found that atherosclerotic plaque was higher in the aortic sinus than in the aortic arch (39). These were consistent with the present study.

The better anti-atherogenesis effect of MO than FO may be attributed to its good anti-inflammatory property. Our previous randomized controlled trial and animal study found that MO could improve arthritis by decreasing pro-inflammatory factors (such as TNF-α and PGE_2) and increasing anti-inflammatory factors (such as IL-10) (14, 16). The anti-inflammatory effect of MO was also observed in mice with inflammatory bowel disease (41). In the present study, although the lowering effect of MO and FO on serum pro-inflammatory factors was comparable, only MO but not FO led to significantly lower aortic NF-κB, p-NF-κB, p38MAPK, and p-p38MAPK levels than CO. NF-κB and p38MAPK can upregulate the expression of pro-inflammatory factors, such as TNF-α, IL-6, and IL-1β (42–44). Both NF-κB and p38MAPK play a key role in atherosclerotic lesion formation (44, 45).

The oil composition of MO and FO differs in two aspects, which can help explain the better anti-atherogenesis effect of MO than FO. On one hand, in the present study, only MO but not FO contains a high level of astaxanthin. Overwhelming evidence suggests that astaxanthin may have a preventive effect on atherosclerotic cardiovascular disease through its potential to improve inflammation, lipid metabolism, and oxidative stress (46). Astaxanthin remarkably suppressed the expression of various inflammatory mediators, such as TNF-α, IL-1β, and IL-6 (47). It can also reduce the release of inflammatory cytokines in mice through the MAPK pathway and NF-κB pathway (48, 49). In addition, astaxanthin has a strong antioxidant ability, which has been shown to reduce cellular oxidative stress, DNA damage, and cell death through Nrf2-antioxidant response element pathway (50). On the other hand, the content of furan fatty acids in MO was lower than FO. Although several studies suggested potential benefits of furan fatty acids in inhibiting lipid peroxidation and reducing inflammation, including reducing risk factors associated with cardiovascular disease (15, 51), the contribution of furan fatty acids to human health remains uncertain. Some studies even reported its unfavorable effects. One study demonstrated that 11D3 exacerbated hepatic steatosis and acute kidney injury in diabetic mice and may also increase the risk of coronary heart disease in T2DM patients (52). Furan fatty acid 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF), a metabolite of furan fatty acids and n-3 PUFA (53), has been reported to be enriched in the plasma of patients with chronic renal failure, gestational diabetes mellitus, and T2DM (54–56). However, an opposite result was observed in a recent study, which found that CMPF in T2DM patients was lower than in healthy people (57). CMPF also acts as a pro-oxidant leading to renal cellular damage (58). In the present study, serum 11D5 levels were significantly higher in the FO group than in the MO group, and 11D5 was found to be positively correlated with aortic p-p38MAPK/p38MAPK, VCAM-1, and serum TG. Therefore, we speculate that MO has better anti-atherosclerotic effects than FO, which may be due to the fact that MO contains more astaxanthin and less 11D5.

NF-κB is a transcription factor and plays an essential role in inflammation and immunity (59). It is necessary for cytokine-induced transcription of VCAM-1 (60), and p38MAPK can upregulate VCAM-1 expression at post-transcriptional level (61). p65 and p50 are two members of the NF-κB family (62). In the steady state, the dimers composed of p65 and p50 are retained within the cytoplasm by the IκB proteins. When the cell is stimulated, it leads to the ubiquitination of IκBs and subsequent degradation, inducing the phosphorylation of IκB protein and resulting in the release of p-p65NF-κB from cytoplasmic restraint. Released pp65NF- κB can drive target gene transcription and induce proinflammatory cytokine expression (63). In the present study, steady-state NF-κB was measured in serum, and activated p-p65NF-κB was measured in the aorta. The content of p-NF-κB in the aorta was significantly lower in the MO group than in the FO group, while there was no significant difference in serum NF-κB in the present study. Some previous studies have also shown inconsistent results for NF-κB and p-NF-κB (64, 65), indicating that there is no necessary connection between the levels of NF-κB and p-NF-κB. In the present study, aortic protein content of VCAM-1 was positively correlated with aortic protein contents of NF-κB. VCAM-1 can recruit monocytes to activate vascular endothelium (1). The recruited monocytes can differentiate into macrophages and further transform into foam cells by the uptake of modified LDL (such as oxidized LDL), leading to the formation of lipid plaque (45). In the present study, MO but not FO significantly decreased the aortic protein level of VCAM-1, and the MO group had a slightly lower level of macrophages in the aortic sinus than in the FO group. This can help explain why MO was more potent than FO in the prevention of atherogenesis. In addition, oxidized LDL can enhance the uptake of macrophages and lead to cholesterol ester accumulation and foam cell formation (66). One previous study found that p38MAPK is necessary for oxidized LDL-induced lipid uptake of macrophages and foam cell formation (67). In the present study, both p38MAPK and p-p38MAPK in the aorta were decreased by MO but not FO, implying that MO may also inhibit the influx of cholesterol into macrophages and foam cell formation. However, this has not been verified in the present study and future study is needed to clarify this point.

SMC plays a dual role in atherosclerosis progression (45): On one hand, abnormal proliferation, migration, and cell growth of SMC are driving factors of atherosclerosis during the development of this disease, leading to thickened intima layer of the blood vessel wall and thus reduced blood flow; on the other hand, after atherosclerotic plaque was formed, SMC can protect against plaque rupture by forming a protective layer around lipid cores. In the present study, the SMC content in the MO and ASP groups was significantly lower than in the CO and FO groups. The present study aimed to evaluate the preventive effect of MO on atherosclerosis (intervention was started before serious atherosclerotic plaque was formed) rather than the treatment effect after serious plaque was formed. Therefore, the lowering effect of MO on SMC content reflected a protective role of MO against the development of atherosclerosis. The p38MAPK can accelerate the proliferation of pulmonary artery SMC (68) and is involved in airway SMC migration (69). The important role of p38MAPK in proliferation and migration of vascular SMC was observed *in vitro* study (70). In addition, NF-κB is also involved in migration of SMC (71, 72). Therefore, inhibiting proliferation and migration of SMC by downregulating the p38MAPK/NF-κB signaling pathway is another possible mechanism for the anti-atherogenesis effect of MO.

The present study had several limitations. First, serum astaxanthin was not detected in the present study because the serum was used for detecting other parameters. Future studies are necessary to clarify the relationship between astaxanthin and atherosclerosis-related

parameters in biological samples. Second, the results have not been verified in human studies, and thus, caution should be exercised when extending the results to humans. Third, in the present study, the results about the underlying mechanism are preliminary, and future study is warranted to further dig into the potential mechanism, such as the influence of MO on influx and efflux of cholesterol in macrophages. Fourth, the sample size of the present study was small. We will verify the results with larger sample size in future studies.

In conclusion, the MO group had significantly smaller atherosclerotic plaque area, lower lipid deposition, lower contents of SMC, and slightly lower contents of macrophage at the aortic sinus than in the FO group. Compared with the CO group, MO but not FO had significantly lower lipid deposition in the aortic arch, smaller atherosclerotic plaque area, and lower inflammatory factors in the aorta. Therefore, MO is more potent than FO in preventing atherosclerosis. The possible mechanism may be by downregulating p38MAPK/NF-κB signaling pathway, decreasing VCAM-1 and macrophages, and inhibiting proliferation and migration of SMC. Considering that the total n-3 PUFA in MO and FO was comparable, the anti-atherosclerotic effect of MO was better than FO because MO contains more astaxanthin and less 11D5.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by the Ethics Committee of Medical College of Qingdao University (QDU-AEC-2022369). All animal experimental procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Qingdao University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

KL: Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. XS: Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing – review & editing. HL: Project administration, Validation, Writing – review & editing. XK: Project administration, Validation, Writing – review & editing. SL: Investigation, Project administration, Validation, Writing – review & editing. RL: Funding acquisition, Project administration, Writing – review & editing. DL: Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2024.1326421/full#supplementary-material

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The relationship between dietary intake of ω-3 and ω-6 fatty acids and frailty risk in middle-aged and elderly individuals: a cross-sectional study from NHANES

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Background: Frailty is a complex clinical syndrome characterized by a decline in the functioning of multiple body systems and reduced adaptability to external stressors. Dietary ω-3 fatty acids are considered beneficial dietary nutrients for preventing frailty due to their anti-inflammatory and immune-regulating properties. However, previous research has yielded conflicting results, and the association between ω-6 fatty acids, the ω-6: ω-3 ratio, and frailty remains unclear. This study aims to explore the relationship between these factors using the National Health and Nutrition Examination Survey (NHANES) database.

Materials and methods: Specialized weighted complex survey design analysis software was employed to analyze data from the 2005–2014 NHANES, which included 12,315 participants. Multivariate logistic regression models and restricted cubic splines (RCS) were utilized to assess the relationship between omega intake and frailty risk in all participants. Additionally, a nomogram model for predicting frailty risk was developed based on risk factors. The reliability of the clinical model was determined by the area under the receiver operating characteristic (ROC) curve, calibration curves, and decision curve analysis (DCA).

Results: In dietary ω-3 intake, compared to the T1 group (≤1.175 g/d), the T3 group's intake level (>2.050 g/d) was associated with approximately 17% reduction in frailty risk in model 3, after rigorous covariate adjustments (odds ratio (OR) = 0.83, 95% confidence interval (CI): (0.70, 0.99)). In dietary ω-6 intake, the T2 group's intake level (>11.423, ≤19.160 g/d) was associated with a 14% reduction in frailty risk compared to the T1 group (≤11.423 g/d) (OR: 0.86, 95% CI: 0.75, 1.00, $p = 0.044$). RCS results indicated a non-linear association between ω-3 and ω-6 intake and frailty risk. Both ROC and DCA curves demonstrated the stability of the constructed model and the effectiveness of an omega-rich diet in reducing frailty risk. However, we did not find a significant association between the ω-6: ω-3 ratio and frailty.

Conclusion: This study provides support for the notion that a high intake of ω-3 and a moderate intake of ω-6 may contribute to reducing frailty risk in middle-aged and elderly individuals.

KEYWORDS

dietary ω-3 fatty acids, dietary ω-6 fatty acids, frailty, national health and nutrition examination survey, cross-sectional study

Introduction

Frailty is a comprehensive syndrome characterized by a decline in physiological reserves when confronted with external stressors. Individuals in a frail state experience decreased capabilities in areas such as musculoskeletal function, nutritional intake, metabolism, cognition, and the nervous system. Frailty is also associated with a higher risk of adverse outcomes, including falls, fractures, hospitalization, and disability (1). Heterogeneity exists among individuals in terms of frailty, and it is not simply a linear function of age. Frailty shows a significant association with Disability-adjusted life years (DALYs) compared to age alone (2), making it advantageous for assessing an individual's health status (3). Currently, due to societal stress and unhealthy lifestyles, many middle-aged individuals are also experiencing frailty, which is no longer exclusively considered a complex condition associated only with the elderly. It is now viewed as a manifestation of physical decline, thereby increasing the urgency for frailty prevention strategies (4). To date, there is no cure for frailty, making prevention and symptom management the desired goals. The most effective interventions involve increased physical activity and maintaining a balanced nutritional diet (5, 6).

Polyunsaturated fatty acids (PUFAs) are essential fats that must be obtained from external sources (7). Among these, ω-3 and ω-6 fatty acids (omega-3 and omega-6) are the two main families of PUFAs and play crucial roles in heart health, brain development and function, inflammation regulation, immune system support, mental health, and cancer prevention, among other aspects (8, 9). ω-3, in particular, is considered to have a direct impact on factors closely associated with frailty due to its anti-inflammatory properties and its role in preserving muscle and bone health (10, 11), making it a potential risk reducer for frailty (6, 12). Studies by León-Muñoz (13) and Hutchins-Wiese (14) have confirmed the benefits of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), the primary components of ω-3, in improving frailty phenotypic symptoms. Additionally, a meta-analysis is working to provide guidance in dietary supplementation recommendations of omega for frailty prevention in the elderly (15). However, some large-scale cohort studies have contradictory findings (16, 17), and the data regarding ω-6 interventions for frailty are limited, impeding strong recommendations for the use of long-chain polyunsaturated fatty acids in frailty prevention.

There is currently no research clearly defining the specific intake levels of ω-3 and ω-6 and their association with frailty risk. Therefore, the aim of this study is to explore the correlation between dietary ω-3 and ω-6, as well as their ratio, and the risk of frailty using data from the National Health and Nutrition Examination Survey (NHANES). The NHANES study is a multi-stage, stratified, and nationally representative investigation of the US population conducted by the National Center for Health Statistics of the Centers for Disease Control and Prevention. Its objective is to assess the nutritional and health status of Americans, gathering data on demographics, dietary habits, physical examinations, laboratory tests, and questionnaires. This article is presented in accordance with the STROBE reporting checklist.

Materials and methods

Study population in NHANES

For our study, we specifically examined data collected between 2005 and 2014. Subjects were excluded from our study for the following reasons: (1) missing data on the frailty index (FI); (2) missing data on ω-3 and ω-6 intake; (3) aged under 45 years old (Non-Elderly Population); (4) missing covariate data (such as hypertension, hyperlipidemia, diabetes, etc.) (Figure 1).

Frailty

Building upon the standards for frailty established by Searle (18), Hakeem (19) further expanded the FI to include 49 deficits, encompassing various systems. These systems include cognitive function (related to confusion and memory issues), dependence (difficulties with activities of daily living), depression (assessed with the Patient Health Questionnaire-9), comorbidities (various chronic diseases), hospital utilization, and self-rated health status (healthcare utilization frequency and prescription medication counts compared to the past year), physical performance and anthropometric measurements (grip strength and body mass index), and laboratory values (including complete blood counts and blood glucose levels). The FI is expressed as the number of deficits acquired by participants divided by the total potential deficits (with a numerical range from 0–1) (Supplementary Table S1). For example, an individual with 10 deficits would have an FI score of 0.20 (10/49 = 0.20). Consistent with previous research, we categorized individuals as "robust" (FI $\leq$ 0.21) and "frail" (FI > 0.21) (19).

Dietary ω-3 and ω-6 intake

Dietary ω-3 and ω-6 intake was based on data obtained from 24-h dietary recall interviews. The primary dietary interview was conducted by trained interviewers at the Mobile Examination Center (MEC) using an automated data collection system. Detailed information on data processing procedures can be found in the NHANES website's dietary interview component [The examination protocol and data collection methods. https://wwwn.cdc.gov/nchs/data/nhanes/2011-2012/manuals/mec_in_person_dietary_procedures_manual_jan_2012.pdf].

In this study, we aimed to capture as many components of ω-3 and ω-6 as possible. Dietary ω-3 included alpha-linolenic acid (ALA, 18:3), EPA (20:5), docosapentaenoic acid (DPA, 22:5), and docosahexaenoic acid (DHA, 22:6), in addition to other forms [stearidonic acid (SDA), 18:4]. Dietary ω-6 included linoleic acid (LA, 18:2) and arachidonic acid (AA, 20:4) (20). Daily average intake

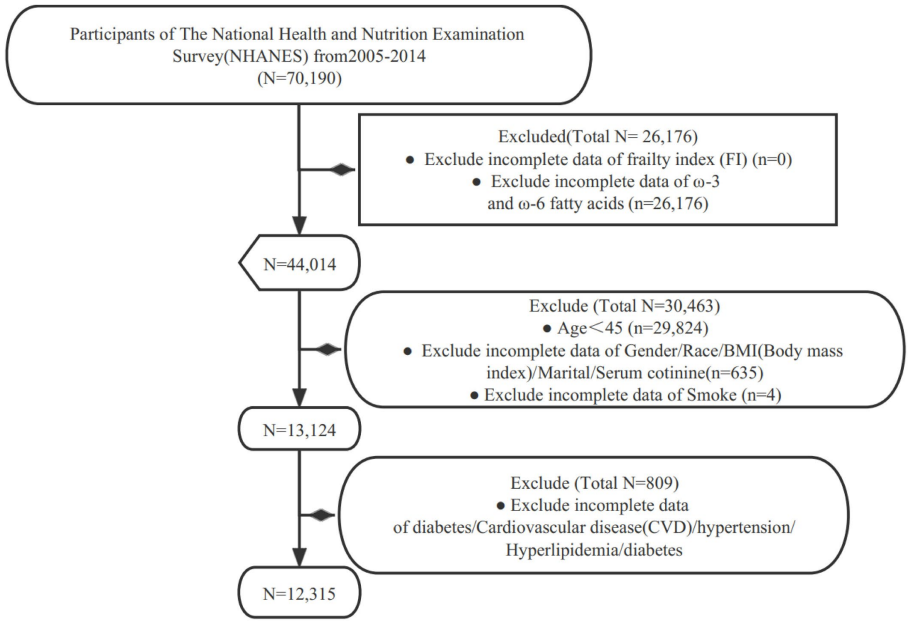

FIGURE 1
Inclusion flow diagram.

of ω-3 and ω-6 was calculated from dietary intake based on the USDA Food and Nutrient Database for Dietary Studies, and only dietary intake of these fatty acids was considered, with supplements not being taken into account [U.S. Department of Agriculture, Agricultural Research Service. Usda Food and Nutrient Database for Dietary Studies. https://www.ars.usda.gov/northeast-area/beltsville-md-bhnrc/beltsville-human-nutrition-research-center/food-surveys-research-group/docs/wweianhanes-overview/].

Furthermore, the low-fat dietary pattern is defined based on the total lipid intake and total calorie intake of the respondents. Specifically, this dietary pattern is characterized by a total lipid intake of less than or equal to 30% of the daily intake. Since the NHANES questionnaire does not provide a detailed breakdown of all lipid energies, we used a fat energy coefficient for conversion, where approximately 1 g of fat intake equals 9 kcal.

Covariates

Covariates included age, sex, race/ethnicity (Mexican American, other Hispanic, non-Hispanic white, non-Hispanic black, non-Hispanic Asian, other race), BMI, marital status, smoking status, cotinine, hypertension, hyperlipidemia, diabetes and coronary artery disease (CVD). BMI was divided into three categories: Normal (<25 kg/m^2), Overweight (≥25 kg/m^2, <30 kg/m^2), and Obese (≥30 kg/m^2). Smoking status was classified as Current Smoker (Defined as having smoked more than 100 cigarettes in a lifetime and still smoking), Former Smoker (Defined as having smoked more than 100 cigarettes in a lifetime but no longer smoking), Never smoke (defined as smoking less than 100 cigarettes in their lifetime). Hypertension was defined according to the American Heart Association/American College of Cardiology (AHA/ACC) 2017 guidelines as systolic blood pressure ≥ 130 mmHg or diastolic blood pressure ≥ 80 mmHg and self-reported diagnosis or use of antihypertensive medication. As per the guidelines set by the Adult Treatment Panel III (ATP 3) of the National Cholesterol Education Program (NCEP), hyperlipidemia is defined by the following criteria: total cholesterol levels equal to or exceeding 200 mg/dL, triglyceride levels equal to or exceeding 150 mg/dL, HDL cholesterol levels below 40 mg/dL for men and below 50 mg/dL for women, or LDL cholesterol levels equal to or exceeding 130 mg/dL (21). Diabete as any of the following: (1) HbA1c levels equal to or greater than 6.5%; (2) serum glucose levels exceeding 200 mg/dL at 2 h after a 75 g glucose load (OGTT); (3) fasting glucose levels equal to or greater than 126 mg/dL; (4) self-reported diagnosis of diabetes; (5) self-reported use of insulin or other diabetes medication. The duration of diabetes was determined by subtracting the participant's current age from the self-reported age at diagnosis, or zero for individuals diagnosed during the NHANES examination. For CVD, a positive response to any of the following questions was defined as CVD: "Has a doctor or other health professional ever told you that you have congestive heart failure (CHF)/coronary heart disease (CHD)/angina/heart attack/stroke?"

Considering drugs affecting lipid metabolism, we defined lipid-lowering drugs and drug categories (such as statins, fibric acid derivatives, ezetimibe, cholesterol absorption inhibitors, and PCSK9 inhibitors, etc.) using the Multum Lexicon standardized drug codes or therapeutic classification schemes. Specifically, we confirmed the use of prescription drugs in the past month, with primary treatment category drugs classified as "metabolism modifiers" (code "358") and secondary treatment drug category classified as anti-hyperlipidemic agents (code "19"), and associated them with respondents' individual identification symbols "SEQN" through the unique identifier "RXDDRGID" (https://wwwn.cdc.gov/Nchs/Nhanes/1999-2000/RXQ_DRUG.htm#Component_Description).

NHANES analysis

A complex sampling design was implemented to ensure nationally representative estimates. All analyses were adjusted for survey design and weighted variables, with new sample weights calculated as the original 2-year sample weights divided by 2. Dietary ω-3 and ω-6 fatty acids intake were categorized into tertiles (with 1/3, 2/3, and 1 as cut-off points). The ω-6: ω-3 ratio was divided into four groups: recommended (≤4), mildly high (>4, ≤10), high (>10, ≤15), and very high (>15). Continuous variables were expressed as mean ± standard deviation (SD), while categorical variables were presented as counts (N) and percentages (%). Weighted t-tests (for continuous variables) and weighted chi-square tests (for categorical variables) were used to assess differences between robust and frailty subjects. Kruskal-Wallis tests (for continuous variables) or weighted chi-square tests (for categorical variables) were employed to evaluate differences among the three groups based on omega intake.

Initially, a crude model was fitted, followed by stepwise adjustment for covariates. Model 1 adjusted for age, sex, and race; Model 2 further adjusted for BMI, marital status, serum cotinine, and smoking status based on Model 1; Model 3 additionally adjusted for hyperlipidemia, hypertension, diabetes, and CVD based on Model 2. Results were presented as odds ratios (OR) with corresponding 95% confidence intervals (95% CI). Subgroup analyses were conducted for significant results. Furthermore, a logistic regression model was used to assess the significance of the interaction between omega intake and covariates on frailty. Regression models and restricted cubic splines (RCS) flexibly model the relationship between independent and dependent variables, especially in regression analysis. When the relationship between independent and dependent variables is not a simple linear one, RCS can help capture this non-linear relationship. They allow researchers to approximate the relationship using different polynomial functions within different ranges of independent variables, thereby providing a more accurate description of the data's trend. RCS with three knots, at the 10^{th}, 50^{th}, and 90^{th} percentages, were used to explore the non-linear relationships of Omega intake levels and frailty in the linear terms model.

In addition, the risk magnitude of all variables on frailty was evaluated by constructing a nomogram. Subsequently, calibration curves were plotted to assess the fit between the predicted probabilities from the nomogram and the actual proportions. To further evaluate the sensitivity of the constructed model, the performance of the model was assessed using the area under the curve (AUC) of the receiver operating characteristic (ROC) curve. Decision curve analysis (DCA) was employed to estimate the net benefit at different threshold probabilities, determining the clinical utility of the model (22). Statistical significance was considered at a p-value <0.05, and all reported probability tests were two-sided.

Results

The baseline characteristics of the participants

In this study, a total of 12,315 individuals were finally included. Based on the exclusion criteria, 3,568 participants were classified as "Frail." Compared to the "Robust" group, which consisted of 8,747 individuals, the "Frail" individuals were older, had a higher proportion of females, obesity, divorced individuals, and smokers (smoking history population). Additionally, they had a higher prevalence of underlying conditions such as hypertension and hyperlipidemia. Their intake of ω-3 was notably inadequate, and their intake of ω-6 was slightly lower. However, the ω-6:ω-3 ratio was significantly high at 10.1, indicating a deficiency of ω-3 intake and an imbalance in the ω-6 to ω-3 ratio compared to the recommended values in the human evolutionary diet (Table 1). These findings align with the current health challenges faced by frail individuals, especially in terms of their dietary habits. These risk factors are visualized through a nomogram (Figure 2A). The daily intake of ω-3 was divided into three equal parts using the tertiles method: T1 (≤1.175 g/day), T2 (>1.175, ≤2.050 g/day), and T3 (>2.050 g/day). Similarly, the daily intake of ω-6 was divided into three parts: T1 (≤11.423 g/day), T2 (>11.423, ≤19.160 g/day), and T3 (>19.160 g/day). It was observed that female individuals, divorced individuals, smokers, and those with hypertension were less attentive to ω-3 and ω-6 intake. In contrast, individuals with normal BMI did not pay much attention to the intake of polyunsaturated fatty acids. Additionally, we also investigated the use of ω-3 dietary supplements, with approximately 5.3% of respondents reporting their intake. There was no statistically significant difference in the intake of supplements between the frail and robust groups, indicating that supplements are unlikely to have influenced the results of this study. It's interesting to note that an increased intake of ω-3 fatty acids often goes hand in hand with an increased intake of ω-6 fatty acids, and the proportion of vulnerable populations is also on the decline. When grouping individuals based on the ω-6: ω-3 ratio, there was no significant difference in the proportion of frail individuals in these groups, and most variables showed no significant differences (Supplementary Tables S2–S5).

Associations between omega intake and frailty outcomes

Through the construction of multiple linear regression models, we found that for ω-3, using the daily intake level T1 (≤1.175 g/day) as the reference, at the T3 level of ω-3 intake (>2.050 g/day), there was a significant negative correlation with the risk of frailty in all models ($p < 0.05$). This result remained robust even after adjusting for covariates, especially after adjusting for variables such as age, gender, race, marital status, serum cotinine, smoking, hypertension, hyperlipidemia, CVD, and diabetes (Model 3). The risk reduction was up to 17% (OR: 0.83, 95% CI: 0.70–0.99, $p = 0.035$). Similarly, for ω-6, using the daily intake level T1 (≤11.423 g/day) as the reference, at the T2 level of ω-6 intake (>11.423, ≤19.160 g/day), there was a significant negative correlation with the risk of frailty in all models. In Model 3, the risk reduction was up to 14% (OR: 0.86, 95% CI: 0.75–1.00, $p = 0.044$). These results showed little variation across different models (Table 2).

The ROC curves for ω-3 and ω-6 in Model 3 both had an AUC of 0.80, indicating that the constructed Model 3 had good

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TABLE 1 Characteristics of participants by Robust or Frail. (NHANES 2005–2014, *N* = 12,315).

Characteristic	Overall, *N* = 12,315 (100%)[1]	Robust, *N* = 8,747 (77%)[1]	Frail, *N* = 3,568 (23%)[2]	*P* Value
Age (years)	59.9 (10.6)	58.9 (10.2)	62.9 (11.5)	**<0.001**
Sex				**<0.001**
Female	6,305 (53%)	4,250 (51%)	2,055 (61%)	
Male	6,010 (47%)	4,497 (49%)	1,513 (39%)	
Race				**<0.001**
Non-Hispanic White	6,155 (76%)	4,445 (78%)	1,710 (71%)	
Non-Hispanic Black	2,606 (9.7%)	1,698 (8.3%)	908 (14%)	
Mexican American	1,678 (5.2%)	1,203 (5.0%)	475 (5.8%)	
Other Hispanic	1,064 (3.7%)	770 (3.6%)	294 (4.0%)	
Other Race - Including Multi-Racial	812 (5.1%)	631 (8.1%)	181 (5.2%)	
BMI				**<0.001**
Normal (≥18.5,<25)	3,067 (26%)	2,390 (29%)	677 (19%)	
Obese (≥30)	4,837 (38%)	3,045 (34%)	1,792 (51%)	
Overweight (≥25,<30)	4,411 (36%)	3,312 (37%)	1,099 (31%)	
Marital				**<0.001**
Divorced	7,129 (64%)	5,446 (68%)	1,683 (52%)	
Married	4,764 (34%)	3,049 (30%)	1,715 (44%)	
Never married	422 (2.2%)	252 (1.8%)	170 (3.6%)	
Serum cotinine	53 (127)	49 (123)	67 (136)	**<0.001**
Smoking status				**<0.001**
Current	2,123 (17%)	1,352 (15%)	771 (22%)	
Former	4,037 (32%)	2,777 (31%)	1,260 (35%)	
Never	6,155 (51%)	4,618 (54%)	1,537 (42%)	
Hypertension				**<0.001**
Yes	7,274 (53%)	4,476 (46%)	2,798 (76%)	
No	5,041 (47%)	4,271 (54%)	770 (24%)	
Hyperlipidemia				**<0.001**
Yes	10,074 (82%)	7,005 (80%)	3,069 (87%)	
No	2,241 (18%)	1,742 (20%)	499 (13%)	
Diabetes				**<0.001**
Yes	3,311 (20%)	1,605 (13%)	1,706 (43%)	
No	9,004 (80%)	7,142 (87%)	1,862 (57%)	
CVD				**<0.001**
Yes	10,203 (86%)	8,027 (93%)	2,176 (64%)	
No	2,112 (14%)	720 (7.1%)	1,392 (36%)	
ω-3	1.90 (1.37)	1.95 (1.39)	1.73 (1.30)	**<0.001**
ALA	1.65 (1.25)	1.69 (1.27)	1.53 (1.19)	**<0.001**
SDA	0.01 (0.04)	0.01 (0.04)	0.01 (0.03)	**<0.001**
EPA	0.09 (0.13)	0.09 (0.13)	0.06 (0.10)	**<0.001**
DPA	0.08 (0.04)	0.09 (0.04)	0.06 (0.04)	**<0.001**
DHA	0.07 (0.20)	0.08 (0.20)	0.05 (0.17)	**<0.001**
T1 (≤1.175 g/d)	4,487 (33%)	2,953 (31%)	1,534 (40%)	
T2 (>1.175, ≤2.050 g/d)	4,054 (33%)	2,952 (34%)	1,102 (32%)	
T3 (>2.050 g/d)	3,774 (33%)	2,842 (35%)	932 (28%)	

(Continued)

TABLE 1 (Continued)

Characteristic	Overall, *N* = 12,315 (100%)[1]	Robust, *N* = 8,747 (77%)[1]	Frail, *N* = 3,568 (23%)[2]	*P* Value
ω-6	17 (11)	17 (11)	16 (11)	**<0.001**
LA	16 (11)	16 (11)	15 (11)	**<0.001**
AA	0.84 (0.13)	0.94 (0.13)	0.73 (0.13)	**0.001**
T1 (≤11.423 g/d)	4,663 (33%)	3,106 (31%)	1,557 (40%)	
T2 (>11.423, ≤19.160 g/d)	3,956 (33%)	2,882 (34%)	1,074 (31%)	
T3 (>19.160 g/d)	3,696 (33%)	2,759 (35%)	937 (29%)	
ω-6/ω-3	10.1 (5.1)	10.1 (5.2)	10.1 (4.8)	0.8
Recommended (≤4)	264 (1.9%)	202 (2.0%)	62 (1.6%)	
Mildly high (>4, ≤10)	7,474 (60%)	5,316 (60%)	2,158 (61%)	
High (>10, ≤15)	3,534 (30%)	2,500 (30%)	1,034 (29%)	
Very high (>15)	1,043 (8.5%)	729 (8.5%)	314 (8.3%)	
Energy (kcal/d)	2,035 (892)	2,083 (890)	1,877 (879)	**<0.001**
Fat intake (g/d)	79 (44)	81 (44)	73 (44)	**<0.001**

[1]Mean ± SD for continuous; n (%) for categorical; [2]chi-squared test with Rao & Scott's second-order correction. ω-3: omega-3 fatty acids; ω-6: omega-6 fatty acids.

predictive capability (Figures 2B,C). However, no significant association was found between the ω-6: ω-3 ratio and the reduction in frailty risk. Even when using a reference ratio of 4, the OR values in all models remained greater than 1, suggesting that increasing the ratio between the two is beneficial for reducing frailty development, though not reaching statistical significance. Further interaction analysis was performed on the covariates that showed significant differences between the healthy and frail groups in Table 1. Although there were differences between some subgroups, particularly in males, individuals with high BMI, non-smokers, low-fat intake diet, those without hypertension, diabetes, and those with cardiovascular diseases, these covariates showed no interaction with omega intake regarding frailty risk ($p > 0.05$). This suggests that the influence of covariates on the results of this study was minimal (Table 3). The decision curve analysis (DCA) showed that the net benefit probability for both ω-3 and ω-6 models in Model 3 ranged from 0 to 90%. The net benefit probability consistently remained higher than the group that received no intervention (depicted by the black solid line "None" in the graphs). This indicates that implementing the conditions in Model 3 (higher ω-3 intake and moderate ω-6 intake) is beneficial in reducing frailty risk without causing any side effects (Figures 3A,B). Furthermore, we used the RCS model to fit the relationship between ω-3 and ω-6 and frailty. After adjusting for covariates, a non-linear relationship was observed ($p = 0.0085$ for ω-3, $p = 0.0006$ for ω-6) (Figures 3C,D). To further assess the impact of drugs affecting lipid metabolism (primarily some lipid-lowering drugs) on this study, we conducted a sensitivity analysis by further grouping the population using these drugs. The results showed that in this study, there were 4,437 respondents with records of using these drugs in the past month. They still maintained a lower intake of ω-3 in the frail population but had a higher intake of ω-6 (Supplementary Table S6). After adjusting for multiple models, we still maintained the conclusion that a high level of ω-3 and moderate intake of ω-6 are associated with a reduced risk of frailty in middle-aged and elderly individuals (Supplementary Table S7).

Discussion

As far as we know, this is the first large-scale study examining the levels of ω-3 and ω-6 intake and their ratio in relation to frailty. The findings of this study align with our expectations, indicating that high levels of ω-3 (>2.05 g/d) and moderate ω-6 intake (>11.423, ≤19.160 g/d) are associated with a reduced risk of frailty in middle-aged and older individuals. Furthermore, we observed for the first time that this association exhibits a nonlinear relationship.

With advancing age, the challenge of ensuring adequate nutrition for middle-aged and older individuals becomes more pronounced due to factors such as decreased appetite (age-related anorexia), physiological changes in the gastrointestinal system, oral health issues, swallowing difficulties, and medication use. Oxidative stress and inflammation are recognized as significant factors in the aging process (23). PUFAs (polyunsaturated fatty acids) can modulate antioxidant signaling pathways and regulate inflammatory processes (24), which potentially makes ω-3 intake beneficial for reducing frailty. Many pro-inflammatory cytokines are produced by fat cells and resident macrophages in adipose tissue, contributing to the pro-inflammatory state that underlies age-related diseases and frailty. Age-related muscle loss, which is a common part of the aging process, is linked to chronic low-grade inflammation, and a decrease in muscle mass is a key phenotype of frailty (25). Normal muscle mass starts to decline after the age of 40, with muscle function declining rapidly, up to 3% per year after the age of 60 (26). This reduction in muscle mass can lead to inconveniences in daily life for middle-aged and older individuals and contributes to additional risks such as falls, fractures, and heart failure (27, 28). In the context of the growing trend of Westernized diets, ω-3 appears to play a critical role in regulating inflammation and immune modulation (29, 30), as well as in maintaining muscle mass and function (31), all

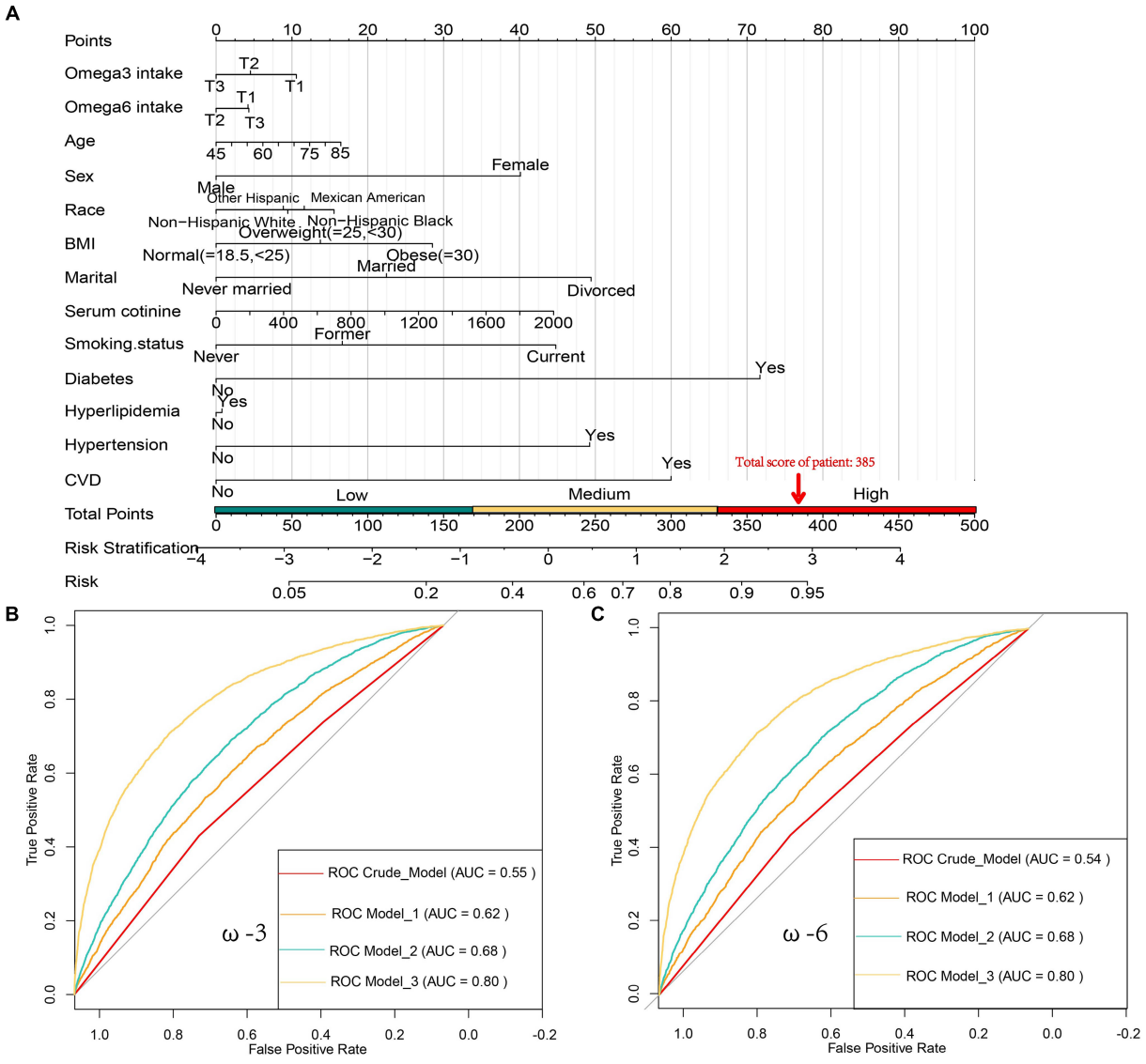

FIGURE 2
(A) Nomogram for Predicting Frailty Risk, used to assess the risk of frailty based on factors such as Age, Sex, Race, Marital Status, BMI, serum cotinine levels, Smoking status, Hypertension, Hyperlipidemia, Diabetes, daily intake of ω-3 [T1 (≤1.175 g/d), T2 (1.175<, ≤2.050 g/d), T3 (>2.050 g/d)], and daily intake of ω-6 [T1 (≤11.423 g/d), T2 (11.423<, ≤19.160 g/d), T3 (>19.160 g/d)]. Each predictor has a score, and the total score represents the likelihood of frailty. For example, an 85-year-old male, Non-Hispanic Black, obese, divorced, with a serum cotinine level of 1800, currently smoking, and having diabetes but not high cholesterol, high blood pressure, or coronary artery disease (CVD), with daily intake of 1 g ω-3 and 15 g ω-6, would have a frailty score of 385 (16 + 40 + 15 + 28 + 49 + 40 + 45 + 0 + 0 + 72 + 10 + 0 = 315), indicating a frailty risk of over 90%; **(B)** Receiver Operating Characteristic (ROC) curves showing the area under the curve (AUC) for models related to daily ω-3 intake, including the Crude model, Model 1, Model 2, and Model 3, with AUC values of 0.62, 0.55, 0.68, and 0.80, respectively; **(C)** ROC curves showing the AUC for models related to daily ω-6 intake, including the Crude model, Model 1, Model 2, and Model 3, with AUC values of 0.62, 0.54, 0.68, and 0.80, respectively.

of which are important considerations in the context of frailty in middle-aged and older individuals. Research by León-Muñoz (13) suggests that daily supplementation of 2.4 grams of EPA and DHA, the primary components of ω-3, can improve physical functioning in frail individuals. Similarly, studies have shown that supplementing 1,500 mg/day of DHA and 1860 mg/day of EPA in healthy older men and women significantly increased thigh muscle volume by 3.6% and grip strength by 2.3 kg (32), while supplementing 720 mg/day of EPA and 40 mg/day of DHA had a positive impact on walking speed in older individuals (14). León-Muñoz also suggests that the addition of dietary antioxidants may synergize with ω-3 to improve physical functioning, an area that warrants further exploration. It's important to note that some studies have reported different findings, such as the study by Orkaby (16), which found that taking 1 g/day of ω-3 did not affect frailty levels. However, this study only analyzed a single dose and did not include a control group with varying doses, while in our study, the ω-3 intake in the lowest range (T1) was less than 1.175 g/day, covering the range of Orkaby's research. Similarly, studies by Krzymińska (33) and Rolland (34) examined the impact of lower doses of DHA and EPA on muscle strength and grip strength, which may explain the difference in results. This further supports the urgency of increasing daily ω-3 intake above 2.05 g.

TABLE 2 Weighted multivariate adjusted logistic regression analysis of frailty risk with different omega intake levels in NHANES from 2005 to 2014.

Regression model	Crude model OR (95% CI)	Model 1 OR (95% CI)	Model 2 OR (95% CI)	Model3 OR (95% CI)
ω-3 (g/day)				
T1 (≤1.175)	Reference	Reference	Reference	Reference
T2 (>1.175, ≤2.050)	0.74 (0.65, 0.85) ***	0.80 (0.70, 0.92)**	0.83 (0.72, 0.95) **	0.87 (0.75, 1.02)
T3 (>2.050)	0.64 (0.55, 0.74)***	0.75 (0.64, 0.88)***	0.78 (0.66, 0.92)**	0.83 (0.70, 0.99)*
ω-6 (g /day)				
T1 (≤11.423)	Reference	Reference	Reference	Reference
T2 (>11.423, ≤19.160)	0.72 (0.64, 0.80) ***	0.81 (0.72, 0.91)***	0.83 (0.73, 0.94) **	0.86 (0.75, 1.00)*
T3 (>19.160)	0.65 (0.58, 0.73)***	0.81 (0.71, 0.92)**	0.82 (0.72, 0.93)**	0.87 (0.76, 1.00)
ω-6: ω-3 ratio				
Recommended (≤4)	Reference	Reference	Reference	Reference
Mildly high (>4, ≤10)	1.27 (0.83, 1.94)	1.19 (0.77, 1.83)	1.20 (0.76, 1.91)	1.37 (0.89, 2.09)
High (>10, ≤15)	1.35 (0.86, 2.11)	1.37 (0.85, 2.18)	1.31 (0.84, 2.04)	1.26 (0.79, 2.01)
Very high (>15)	1.29 (0.78, 2.12)	1.15 (0.73, 1.82)	1.11 (0.69, 1.80)	1.05 (0.62, 1.78)

*$P < 0.05$; **$p < 0.01$; ***$p < 0.001$.
Multiple logistic regression model: Model 1: adjusted for age, sex, race; Model 2: adjusted for age, sex, race, marital, serum cotinine, BMI, smoking status; Model 3: adjusted for age, sex, race, marital, serum cotinine, BMI, smoking status, hypertension, hyperlipidemia, diabetes, CVD. ω-3: omega-3 fatty acids; ω-6: omega-6 fatty acids.

Dietary ω-3, by itself, may potentially regulate muscle protein synthesis to maintain overall physical fitness (11). Additionally, since pro-inflammatory cytokines are associated with muscle atrophy (35), the anti-inflammatory effects of ω-3 make it a beneficial factor in preventing frailty. Another possibility is the close relationship between inflammation and apoptosis, the latter of which may be a biological pathway leading to muscle loss. For instance, tumor necrosis factor-alpha (TNF-α) induces muscle cell apoptosis (36), while ω-3 can inhibit TNF-α, interleukin-6 (IL-6), and C-reactive protein synthesis (CRP) (37, 38). LA in ω-6 plays a specific and unique role in maintaining the structural integrity and barrier function of human skin (39). The skin is the body's natural first line of defense against various non-specific pathogens. Using LA as a partial substitute for saturated fatty acids is advantageous in reducing total cholesterol and low density lipoprotein cholesterol concentrations in the blood, which is likely to lower the risk of various underlying diseases (40, 41). AA, found in ω-6, is involved in mediating and regulating inflammatory responses (42), which are fundamental to inflammation. However, early-stage inflammatory responses are also related to the production of eicosatrienoic acid and subsequently to the induction of inflammation. Daily intake of 600 mg of AA can improve physical fitness (43), and Roberts (44) suggests that a daily intake of 1,000 mg of AA actually reduces inflammation. These complexities make our understanding of the role of ω-6 more nuanced. Moderate intake of ω-6 and an increase in ω-3 intake seem to be central axes in modulating the immune system and inflammatory responses for preventing frailty risk. This underscores the importance of maintaining nutritional balance and immune function in our bodies.

However, it's worth noting that daily intake of over 2 g of ω-3 is challenging, and in most regions globally, this level of intake is not being achieved. Western diets tend to have an excess of ω-6 polyunsaturated fatty acids, often surpassing the recommended target range in this study (>11.423, ≤19.160 g/d) (8, 45), while ω-3 intake is often very low. This exposes individuals to a higher risk of frailty (46). Additionally, the high ω-3 levels in this study are also associated with high ω-6 intake, with only a small percentage of participants having a 1:1 ratio. This lack of a 1:1 ratio may have contributed to the inability to establish a direct association between ω-6/ω-3 levels and frailty, but the results indicate the importance of reducing the ratio between the two. In the future, further experimental research is needed to determine whether the anti-inflammatory and antioxidant mechanisms of ω-3 can serve as preventive mechanisms against frailty, as well as to explore the interaction between ω-3 and ω-6 in the frail process.

This work has certain limitations. First, frailty is a dynamic condition that may change over time, and there are complex and variable factors at play. Second, the intake of ω-3 and ω-6 fatty acids is assessed through questionnaire surveys, and their components are defined based on food composition lists, which may not accurately reflect the precision of each component and individuals' long-term intake levels. However, the large sample size in this study provides a reasonable representation of the average intake levels in the U.S. population. Additionally, as a cross-sectional study with the inability to track respondent information in the NHANES database, this research cannot evaluate the subsequent frailty risk in robust individuals with low ω-3 intake. Long-term tracking of frailty risk in such populations is necessary and warrants further investigation. Lastly, the lack of standardized frailty criteria makes the generalization of the study's conclusions a more cautious endeavor.

Conclusion

Our research findings support that daily intake of ω-3 exceeding 2.05 grams and ω-6 intake ranging from greater than 11.423 grams to less than or equal to 19.160 grams is beneficial in reducing frailty risk among middle-aged and elderly individuals. Based on this, we encourage individuals in this demographic in the United States to increase their intake of ω-3 while moderately reducing their intake of

TABLE 3 Subgroup analysis of the risk of frailty occurrence associated with Omega intake considering various confounding factors.

Subgroup	ω-3 Interaction *P*-Value	ω-3 T2 (>1.175, ≤2.050) OR (95%CI)	ω-3 T3 (>2.050) OR (95%CI)	ω-6 Interaction *P*-Value	ω-6 T2 (>11.423, ≤19.160) OR (95%CI)	ω-6 T3 (>19.160) OR (95%CI)
Age	$p = 0.92$			$p = 0.65$		
45–60		0.95 (0.74, 1.21)	0.84 (0.63, 1.11)		0.96 (0.75, 1.21)	0.90 (0.71, 1.14)
61–75		0.82 (0.67, 1.01)	0.79 (0.61, 1.02)		0.77 (0.61, 0.96)*	0.82 (0.64, 1.04)
>75		0.85 (0.64, 1.13)	0.87 (0.68, 1.11)		0.82 (0.62, 1.08)	0.87 (0.68, 1.12)
Sex	$p = 0.54$			$p = 0.16$		
Female		0.88 (0.74, 1.03)	0.90 (0.72, 1.12)		0.86 (0.71, 1.04)	0.98 (0.82, 1.19)
Male		0.86 (0.66, 1.12)	0.77 (0.61, 0.97)*		0.84 (0.66, 1.08)	0.74 (0.60, 0.93)**
Race	$p = 0.62$			$p = 0.46$		
Non-Hispanic White		0.78 (0.56, 1.08)	0.93 (0.68, 1.26)		0.89 (0.67, 1.17)	0.9 (0.65, 1.25)
Non-Hispanic Black		0.79 (0.63, 0.99)*	0.75 (0.60, 0.95)*		0.99 (0.79, 1.23)	0.97 (0.77, 1.23)
Mexican American		0.90 (0.74, 1.09)	0.83 (0.66, 1.05)		0.83 (0.69, 1.00)*	0.86 (0.72, 1.03)
Other Hispanic		0.99 (0.63, 1.57)	0.68 (0.41, 1.13)		0.76 (0.50, 1.16)	0.63 (0.37, 1.07)
Other Race - Including Multi-Racial		0.77 (0.43, 1.39)	1.11 (0.60, 2.06)		1.19 (0.65, 2.16)	0.90 (0.47, 1.73)
Marital	$p = 0.53$			$P = 0.65$		
Divorced		0.86 (0.70, 1.05)	0.89 (0.71, 1.12)		0.90 (0.74, 1.09)	0.90 (0.76, 1.07)
Married		0.90 (0.73, 1.10)	0.74 (0.57, 0.95)*		0.81 (0.65, 1.01)	0.80 (0.62, 1.03)
Never married		1.02 (0.48, 2.19)	1.13 (0.59, 2.19)		0.93 (0.42, 2.07)	1.59 (0.83, 3.03)
BMI (Kg/m^2)	$p = 0.72$			$p = 0.21$		
Normal (<25)		0.91 (0.73, 1.15)	0.83 (0.62, 1.10)		1.05 (0.83, 1.31)	0.92 (0.73, 1.16)
Obese (≥30)		0.83 (0.66, 1.04)	0.92 (0.69, 1.22)		0.73 (0.57, 0.92)*	0.85 (0.63, 1.15)
Overweight (≥25,<30)		0.82 (0.56, 1.20)	0.62 (0.45, 0.86)**		0.72 (0.55, 0.94)*	0.68 (0.46, 0.98)*
Smoking status	$p = 0.051$			$p = 0.35$		
Current		1.15 (0.79, 1.68)	0.8 (0.60, 1.08)		0.89 (0.67, 1.18)	0.83 (0.63, 1.09)
Former		0.94 (0.74, 1.19)	0.95 (0.76, 1.19)		0.98 (0.76, 1.27)	0.91 (0.70, 1.18)
Never		0.74 (0.62, 0.89)**	0.8 (0.63, 1.01)		0.78 (0.64, 0.96)*	0.92 (0.76, 1.11)
Hypertension	$p = 0.07$			$p = 0.51$		
Yes		0.9 (0.76, 1.06)	0.94 (0.76, 1.17)		0.88 (0.75, 1.03)	0.92 (0.76, 1.12)
No		0.82 (0.61, 1.10)	0.63 (0.47, 0.85)**		0.81 (0.62, 1.06)	0.76 (0.58, 1.00)
Hyperlipidemia	$P = 0.53$			$P = 0.53$		
Yes		0.9 (0.77, 1.05)	0.84 (0.71, 1.01)		0.88 (0.74, 1.03)	0.90 (0.77, 1.04)
No		0.73 (0.51, 1.05)	0.76 (0.52, 1.12)		0.82 (0.56, 1.20)	0.74 (0.49, 1.11)
CVD	$P = 0.51$			$p = 0.67$		
Yes		0.84 (0.70, 1.02)	0.79 (0.64, 0.96)*		0.86 (0.72, 1.02)	0.84 (0.72, 0.99)*
No		0.98 (0.74, 1.30)	1.01 (0.74, 1.36)		0.87 (0.62, 1.22)	0.99 (0.72, 1.36)
Diabetes	$p = 0.32$			$p = 0.37$		
Yes		0.96 (0.73, 1.25)	0.93 (0.72, 1.22)		0.93 (0.71, 1.21)	0.94 (0.74, 1.19)
No		0.84 (0.71, 1.01)	0.78 (0.63, 0.96)*		0.83 (0.72, 0.97)*	0.83 (0.70, 0.99)*
Fat intake level	$p = 0.10$			$p = 0.78$		
Low		0.87 (0.69, 1.10)	0.57 (0.38, 0.85)**		0.95 (0.75, 1.21)	0.82 (0.52, 1.29)
Non-low group		0.90 (0.73, 1.11)	0.88 (0.71, 1.08)		0.84 (0.68, 1.04)	0.75 (0.62, 1.06)

Subgroup analysis adjustment factors: age; sex; race; education; marital; PIR; BMI; serum cotinine; smoking status; hyperlipidemia; hypertension; diabetes; CVD, excluding sub-group variables, and the reference object in the sub-group is ω-3-(omega-3 fatty acids)-T1 (≤1.175) and ω-6 (omega-6 fatty acids)-T1 (≤11.423). *$P < 0.05$; **$p < 0.01$.

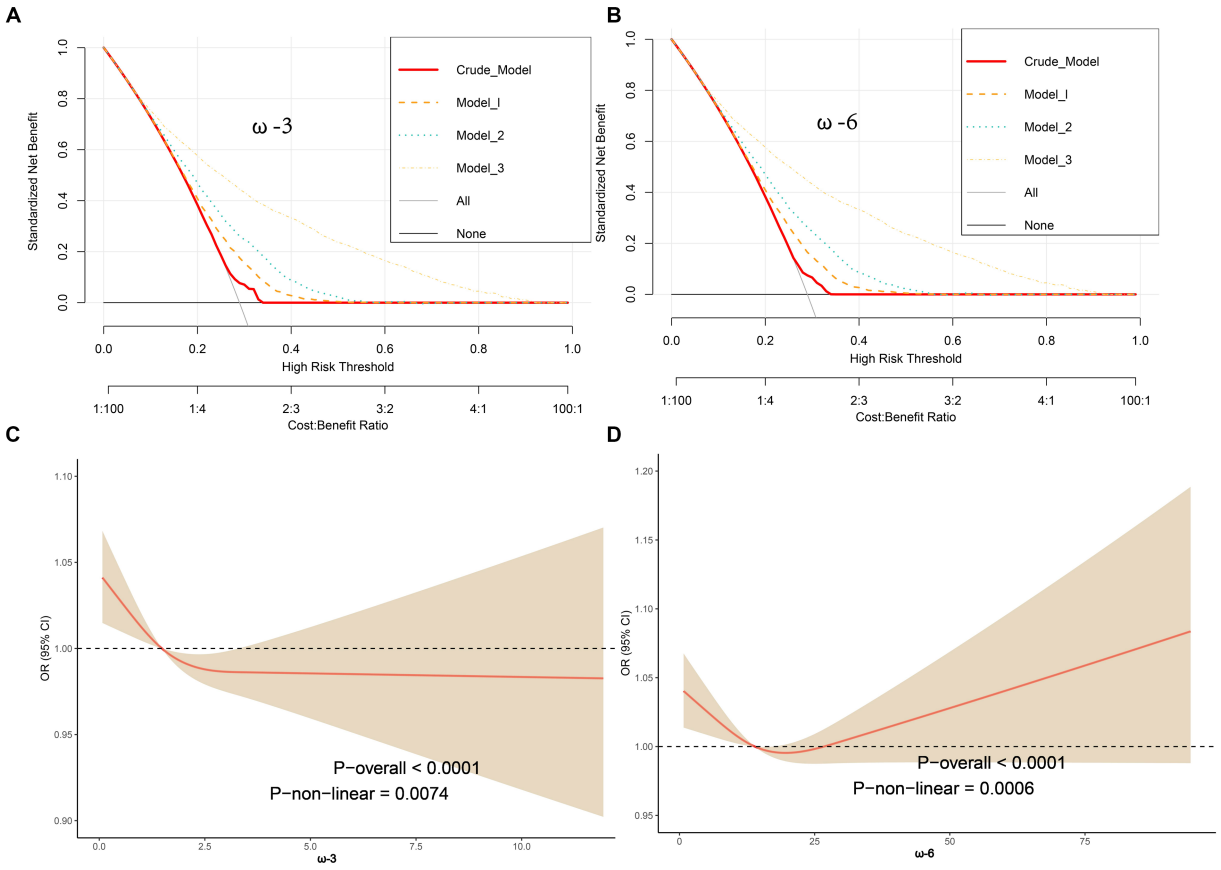

FIGURE 3
Decision Curve Analysis (DCA) showing net benefit curves for various models. The x-axis represents the threshold probability for frailty, and the y-axis represents net benefit. The red line, orange line, light blue line, and yellow line represent improved prediction nomograms for daily ω-3 and ω-6 intake, including the Crude model, Model 1, Model 2, and Model 3. The gray line represents the assumption that all patients use the nomogram model. The black line represents the assumption that no patients use the nomogram model to predict frailty risk. Based on the results from Table 2 and the DCA curves, it can be concluded that all constructed models can provide a net benefit for reducing frailty risk by increasing ω-3 intake (>2.050 g/d) **(A)** and by appropriately reducing ω-6 intake (>11.423, ≤19.160 g/d) **(B)**. The net benefit threshold is wide, and there are no side effects; adjustments were made using Restricted Cubic Spline (RCS) models for factors such as age, gender, race, BMI, marital status, serum cotinine, smoking, hypertension, hyperlipidemia, coronary artery disease, and diabetes to analyze the relationship between ω-3 and ω-6 and frailty. The solid red line represents the combined restricted cubic spline curve model, and the shaded area represents the 95% confidence interval for the combined curve. The dashed line represents the risk of frailty for different levels of ω-3 **(C)** and ω-6 **(D)** intake.

ω-6. Additionally, further investigation is warranted into the mechanisms of ω-3's anti-inflammatory and antioxidant properties in preventing frailty risk, as well as the interaction between ω-3 and ω-6.

Data availability statement

The data presented in the study are deposited in the Centers for Disease Control and Prevention. The links are as follows: 2005-2006: https://wwwn.cdc.gov/Nchs/Nhanes/2005-2006/DEMO_D.htm and https://wwwn.cdc.gov/Nchs/Nhanes/2005-2006/DR1IFF_D.htm.

Author contributions

ZY: Conceptualization, Data curation, Methodology, Software, Validation, Writing – original draft. YX: Data curation, Methodology, Validation, Writing – original draft.

KL: Data curation, Methodology, Supervision, Writing – original draft. WZ: Formal analysis, Project administration, Validation, Writing – review & editing. LL: Methodology, Supervision, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2024.1377910/full#supplementary-material

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Associations of ω-3, ω-6 polyunsaturated fatty acids intake and ω-6: ω-3 ratio with systemic immune and inflammatory biomarkers: NHANES 1999-2020

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Background: In recent years, diseases caused by abnormal immune-inflammatory responses have become increasingly severe. Dietary intervention involving omega-3 polyunsaturated fatty acids (ω-3 PUFAs) has emerged as a potential treatment. However, research investigating the relationship between ω-3, ω-6 PUFAs, and ω-6 to ω-3 ratio with inflammatory biomarkers remains controversial.

Methods: To investigate the correlation between the intake of ω-3 and ω-6 PUFAs and the ratio of ω-6: ω-3 with biomarkers of inflammation, the National Health and Nutrition Examination Survey (NHANES) data (1999 to 2020) was utilized. The systemic immune-inflammation index (SII), platelet-lymphocyte ratio (PLR), neutrophil-lymphocyte ratio (NLR), and white blood cell (WBC) were selected as study subjects. Dietary data for ω-3 and ω-6 PUFAs were collected via two 24-h dietary recall interviews. SII index and other indicators were obtained from the blood routine data. The multiple linear regression and restricted cubic spline models were utilized to evaluate the association of ω-3, ω-6 PUFAs intake, and ω-6: ω-3 ratio to SII and secondary measures.

Results: This study involved a total of 43,155 American adults. ω-3 and ω-6 PUFAs exhibited negative correlations with SII, PLR, NLR, and WBC. The correlation between ω-6: ω-3 ratio and SII, PLR, NLR, and WBC was not significant. Furthermore, the dose–response relationship showed that the relationship between the intake of ω-3 and ω-6 PUFAs and SII was an "L" pattern.

Conclusion: Intake of dietary ω-3 and ω-6 PUFAs reduces the levels of several inflammatory biomarkers in the body and exerts immunomodulatory effects.

KEYWORDS

ω-3 PUFAs, ω-6 PUFAs, ω-6: ω-3 ratio, NHANES, systemic immune-inflammation index, neutrophil-to-lymphocyte ratio, platelet-lymphocyte ratio, white blood cell

1 Introduction

An immune-inflammatory response refers to the systemic response of the immune system of the body to a particular state. This response is involved not only in acute inflammation caused by infection or injury but also in the normal homeostatic regulation of the body (1, 2). However, prolonged chronic systemic inflammation elevates the risk of various disorders, including autoimmune disease, cardiovascular disease (CVD), cancer, and diabetes (3–7).

Dietary polyunsaturated fatty acids (PUFAs), serving as vital energy sources and cell membrane components, exert a crucial impact on human health (8, 9). Clinical trials and experimental research have demonstrated that ω-3 PUFAs possess significant anti-inflammatory properties (10, 11). Although ω-6 PUFAs are often theoretically considered pro-inflammatory mediators, the findings from clinical research do not consistently support this conventional hypothesis. Arachidonic acid (AA) supplementation was found to elevate AA content in human plasma or cellular phospholipids in a randomized controlled study and crossover design study conducted in the UK and US, respectively. However, it did not exert a significant impact on pro-inflammatory cytokine production and the number of inflammatory cells (12–14). Conversely, some investigations have even proposed that ω-6 PUFAs may be linked to decreased inflammation (15–17). Additionally, research on the relationship between ω-6: ω-3 ratio and inflammatory markers has yielded conflicting results. Numerous investigations have revealed that the proportion of ω-6: ω-3 is positively correlated with inflammatory markers (18–20). While Harris (21, 22) collated and analyzed 11 case–control and two prospective cohort studies, it was considered that the clinical ω-6: ω-3 ratio could not serve as a reliable indicator for predicting disease status or providing nutritional reference.

The concept of systemic immune-inflammation index (SII) was initially introduced by Hu (23) and has been applied in several disease areas, such as CVD, respiratory diseases, autoimmune diseases, and some cancers (4, 23–27). Additionally, platelet-lymphocyte ratio (PLR), neutrophil-lymphocyte ratio (NLR), and white blood cell (WBC) count are important indicators commonly used for early detection and prediction of inflammatory diseases in clinical practice and have also been widely used in clinical studies (28–30).

Clinical interventions and experimental studies on dietary ω-3 and ω-6 PUFAs have not elucidated the relationship between the two and inflammatory mediators. Similarly, there is no consensus on the effects of the optimal ratio of ω-6 to ω:3 PUFA in humans. Therefore, this study investigated a dataset of ethnically diverse cohorts of Americans aged 20 years and older from the National Health and Nutrition Examination Survey (NHANES) data to analyze the relationship between intake of ω-3, ω-6 PUFAs and the ratio of ω-6 to ω:3 and systemic immune-inflammatory markers to provide more compelling evidence for clinical interventions and therapies.

2 Materials and methods

2.1 Study population

The National Health and Nutrition Examination Survey (NHANES), carried out by the Centers for Disease Control and Prevention (CDC), is a cross-sectional survey undertaken on a biennial basis. Its purpose is to analyze the nutritional and health status of children and adults in the US. This assessment is carried out by selecting a representative sample of the US population employing a complex multistage probability sampling design (31). NHANES contains interviews covering demographic, dietary, health-related, and socio-economic issues, alongside laboratory tests performed by highly qualified medical personnel (32). For this study, we included 116,876 participants who took part in the survey during the period 1999–2020. Our study exclusion criteria were as follows: (1) adults younger than 20 years of age ($n = 52{,}563$) (2) dietary fatty acid data were incomplete or abnormal and missing laboratory tests ($n = 10{,}328$) (3) any other covariates were missing ($n = 10{,}830$). After that, this study comprised 43,155 individuals, including 22,575 women and 20,580 men (age: ≥20 years) (Figure 1).

2.2 ω-3 and ω-6 PUFAs dietary intake

During the Mobile Examination Centre (MEC) portion of NHANES, ω-3 and ω-6 PUFAs dietary intake was obtained through two 24-h dietary recalls administered 3 to 10 days apart. The main diet interview was conducted in the MEC, and subsequent diet interview data were obtained by the Home Office via telephone. Comprehensive descriptions of the data processing procedures and diet interviews are available in the Diet Interview section of the NHANES website. α-linoleic acid (ALA, 18:3), docosapentaenoic acid (DPA, 22:5), docosahexaenoic acid (DHA, 22:6), eicosapentaenoic acid (EPA, 20:5), and stearidonic acid (SDA, 18:4) are constituents of ω-3 PUFAs. On the other hand, AA (20:4) and linoleic acid (LA, 18:2) are included in ω-6 PUFAs. Dietary intake of ω-3, ω-6 PUFAs, and ω-6: ω-3 ratios were categorized into tertiles for subsequent analyses.

2.3 SII and secondary test results

The primary outcome was SII calculated as platelet count × neutrophil count/lymphocyte count (33). NLR = Neutrophil count/lymphocyte count. PLR = platelet count/lymphocyte count. WBC count was obtained directly from a blood routine. NHANES implemented standardized protocols for the measurement of these biomarkers, and all investigators obtained written informed consent from participants.

2.4 Selection of covariates

Alongside the investigation of ω-3 and ω-6 PUFAs dietary intake, several potential confounders were examined, including age (20–40, 41–60, and ≥ 60 years), body mass index (BMI) (normal: <25 kg/m^2; overweight: 25–30 kg/m^2; obesity: ≥30 kg/m^2), educational level (below high school, high school, or above), marital status (married/cohabiting with partner or divorced/widowed/never married/separated), poverty income ratio (PIR) (below poverty line: ≤0.99, above poverty line: ≥1), race (Mexican Americans, non-Hispanic whites, non-Hispanic blacks, other Hispanics, and other races), smoking status (never, before, and now), and sex (men and women).

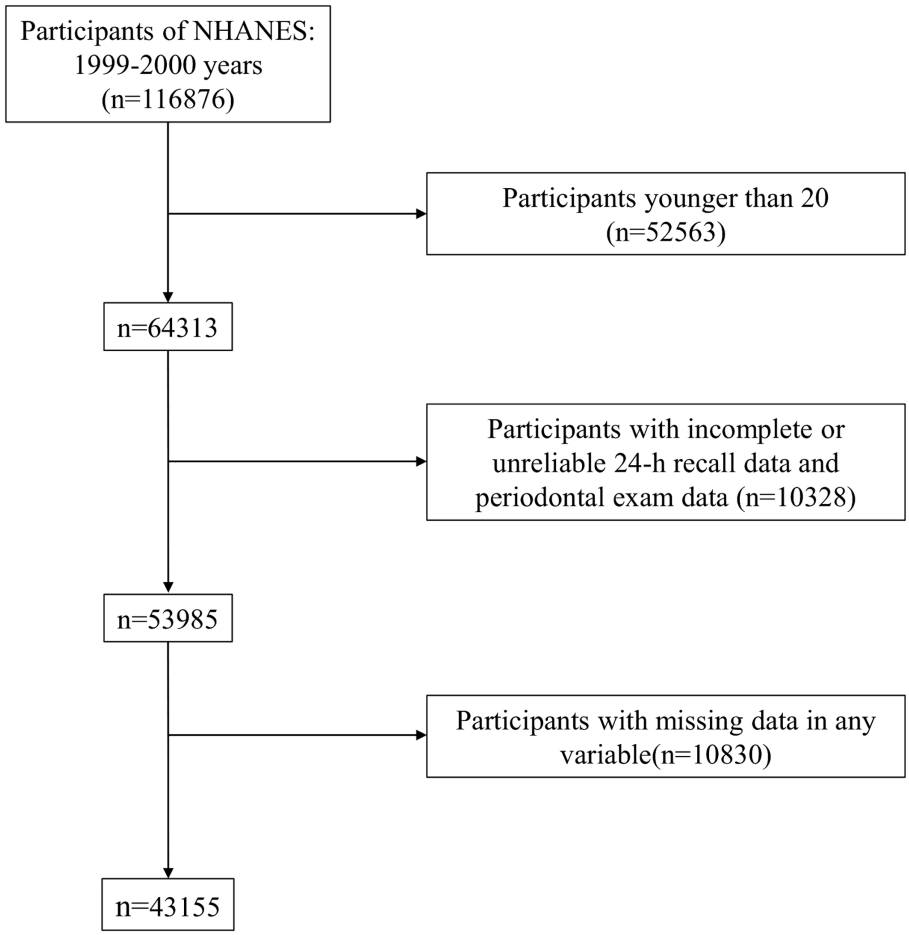

FIGURE 1
Flowchart of the screening process for the selection of eligible participants.

2.5 Statistical analyses

Due to the complex sampling design employed by NHANES, all experiments were adjusted for weighted variables and survey design to ensure that the included population was nationally representative. The Kolmogorov–Smirnov normality test was utilized to examine the normality of continuous variables, which are expressed as mean ± standard error. Variables that were not normally distributed were presented utilizing the median (interquartile range). Adjusted dietary intake of ω-3 and ω-6 PUFAs was categorized into three groups based on tertiles, with the lowest tertile serving as the reference value. Multivariate weighted linear regression models were utilized to determine the correlation between dietary ω-3, ω-6 PUFAs, and ω-6: ω-3 ratios and SII as well as other secondary outcomes. For each regression analysis, a total of three statistical models were developed. Model 1 remained unadjusted, while Model 2 was adjusted for age and sex only. Model 3 encompassed adjustments for all covariates, including age, BMI, education, monthly household poverty level index, marital status, race, sex, and smoking status. Fully adjusted models considered demographic, dietary, lifestyle, and metabolic factors. To further investigate the dose–response correlation between dietary ω-3 and ω-6 PUFAs, as well as the ω-6: ω-3 ratios with the primary measure SII, the restricted cubic splines were applied. These splines included three nodes at the 5th, 50th, and 95th percentiles of the exposure distribution in multivariate-adjusted model 3. All statistical analyses were performed using R software for data analysis. All statistical tests were two-sided, and $p < 0.05$ was deemed to reflect statistical significance.

3 Results

3.1 Baseline attributes

Tables 1–3 present the baseline attributes of the study population, categorized by dietary intake of ω-3, ω-6 PUFAs, and ω-6: ω-3 ratios in triple-digit groups, respectively. The study comprised 43,155 participants in total. The tertile intervals for ω-3 fatty acid intake were: low intake (≤ 1.167 g/day, $n = 14{,}401$), medium intake (1.167–1.928 g/day, $n = 14{,}364$), and high intake (>1.928 g/day, $n = 14{,}390$). Tertile intervals for ω-6 fatty acid intake were defined as low dose intake (≤ 10.929 g/day, $n = 14{,}387$), medium dose intake (10.929–17.63 g/day, $n = 14{,}384$), and high dose intake (>17.63 g/day, $n = 14{,}384$). The tertile interval for the ω-6: ω-3 proportion of fatty acid intake was: low (≤ 8.19, $n = 14{,}383$), medium (8.19–10.13, $n = 14{,}383$), and high (≥10.13,

TABLE 1 Weighted characteristics of the study population based on dietary ω-3 fatty acids intake.

Variable	Total ω-3 fatty acids intake (g)			*p*-value
	<=1.167 (*n* = 14,401)	1.167–1.928 (*n* = 14,364)	>1.928 (*n* = 14,390)	
Age group (%)				**< 0.0001**
20–40	4,713 (38.0)	5,147 (40.4)	5,328 (39.4)	
41–60	4,440 (36.0)	4,760 (35.6)	5,065 (38.5)	
> = 60	5,248 (26.0)	4,457 (24.0)	3,997 (22.0)	
Sex (%)				**< 0.0001**
Female	8,946 (63.6)	7,592 (53.6)	6,037 (40.9)	
Male	5,455 (36.4)	6,772 (46.4)	8,353 (59.1)	
Marital status (%)				**< 0.0001**
Married/Living with partner	8,494 (60.9)	8,900 (64.3)	9,162 (66.7)	
Windowed/Divorced/ Separated/Never married	5,907 (39.1)	5,464 (35.7)	5,228 (33.3)	
Race (%)				**0.01**
Mexican American	2,723 (8.6)	2,277 (8.0)	2037 (7.5)	
Non-Hispanic White	6,492 (68.4)	6,743 (69.6)	6,761 (70.4)	
Non-Hispanic Black	2,899 (10.9)	2,891 (10.4)	3,086 (10.0)	
Other Hispanic	1,186 (5.7)	1,177 (5.2)	1,039 (5.0)	
Other race	1,101 (6.3)	1,276 (6.8)	1,467 (7.1)	
Education level (%)				**< 0.0001**
Below high school	2048 (6.8)	1,287 (4.4)	900 (3.3)	
High school	5,742 (39.0)	5,346 (34.0)	4,802 (30.4)	
Above high school	6,611 (54.1)	7,731 (61.7)	8,688 (66.3)	
Smoking status (%)				**< 0.0001**
never	7,842 (54.0)	7,948 (55.6)	7,764 (54.5)	
former	3,528 (22.6)	3,628 (24.9)	3,920 (27.7)	
current	3,031 (23.4)	2,788 (19.5)	2,706 (17.8)	
Body mass index (%)				**0.003**
<25	4,115 (31.7)	4,046 (30.6)	4,041 (28.5)	
25–30	4,810 (32.1)	4,931 (33.3)	4,824 (34.5)	
>30	5,476 (36.2)	5,387 (36.1)	5,525 (37.0)	
Poverty income ratio (%)				**< 0.0001**
<=0.99	3,241 (16.8)	2,670 (12.9)	2,304 (10.8)	
> = 1	11,160 (83.2)	11,694 (87.1)	12,086 (89.2)	
SII	578.4 (4.3)	552.5 (4.5)	543.7 (4.4)	**< 0.0001**
NLR	2.2 (0.0)	2.2 (0.0)	2.2 (0.0)	0.1
PLR	131.1 (0.7)	127.4 (0.7)	127.7 (0.7)	**< 0.0001**
WBC (x109)	7.4 (0.0)	7.3 (0.0)	7.3 (0.0)	**0.002**

SII, systemic immune-inflammation index; NLR, neutrophil-to-lymphocyte ratio; PLR, platelet-lymphocyte ratio; WBC: white blood cell.
Bold values represent statistical significance.

n = 14,389). Participants with higher ω-3 and ω-6 PUFAs intake tended to be young and medium-aged, male, married or cohabiting with a partner, non-Hispanic white, higher educational level, wealthier, and non-smoking. Regarding laboratory parameters, individuals with higher ω-3 and ω-6 PUFAs intake demonstrated lower levels of SII, PLR, and WBC.

3.2 Associations between dietary ω-3, ω-6 PUFAs intake and ω-6: ω-3 ratio and SII, PLR, NLR, WBC

Table 4 shows the relationship between the dietary intake of ω-3, ω-6 PUFAs and ω-6: ω-3 ratio and SII. In all three models, there was

TABLE 2 Weighted characteristics of the study population based on dietary ω-6 fatty acids intake.

Variable	Total ω-6 fatty acids intake (g)			*p*-value
	<=10.929 (*n* = 14,387)	10.929–17.63 (*n* = 14,384)	>17.63 (*n* = 14,384)	
Age group (%)				**< 0.0001**
20–40	4,464 (35.8)	5,081 (39.9)	5,643 (41.7)	
41–60	4,358 (35.7)	4,761 (35.8)	5,146 (38.6)	
> = 60	5,565 (28.5)	4,542 (24.3)	3,595 (19.7)	
Sex (%)				**< 0.0001**
Female	9,073 (65.3)	7,750 (55.3)	5,752 (38.3)	
Male	5,314 (34.7)	6,634 (44.7)	8,632 (61.7)	
Marital status (%)				**< 0.0001**
Married/Living with partner	8,521 (61.6)	8,978 (64.6)	9,057 (65.7)	
Windowed/Divorced/ Separated/Never married	5,866 (38.4)	5,406 (35.4)	5,327 (34.3)	
Race (%)				**< 0.0001**
Mexican American	2,629 (8.4)	2,347 (8.2)	2061 (7.6)	
Non-Hispanic White	6,367 (67.4)	6,741 (70.0)	6,888 (70.9)	
Non-Hispanic Black	2,765 (10.4)	6,741 (70.1)	3,271 (10.8)	
Other Hispanic	1,380 (6.6)	6,741 (70.2)	920 (4.6)	
Other race	1,246 (7.2)	6,741 (70.3)	1,244 (6.2)	
Education level (%)				**< 0.0001**
Below high school	2,153 (7.6)	1,286 (4.2)	796 (2.9)	
High school	5,655 (38.5)	5,320 (33.5)	4,915 (31.4)	
Above high school	6,579 (53.9)	7,778 (62.3)	8,673 (65.7)	
Smoking status (%)				**< 0.0001**
never	8,008 (54.0)	7,971 (56.6)	7,575 (53.6)	
former	3,517 (23.5)	3,663 (24.1)	3,896 (27.6)	
current	2,862 (22.5)	2,750 (19.3)	2,913 (18.8)	
Body mass index (%)				**< 0.001**
<25	4,133 (31.7)	4,146 (31.0)	3,923 (28.2)	
25–30	4,921 (32.5)	4,876 (33.7)	4,768 (33.8)	
>30	5,333 (35.9)	5,362 (35.3)	5,693 (38.0)	
Poverty income ratio (%)				**< 0.0001**
<=0.99	3,260 (16.8)	2,607 (12.5)	2,348 (11.2)	
> = 1	11,127 (83.2)	11,777 (87.5)	12,036 (88.8)	
SII	576.6 (4.5)	552.9 (4.2)	545.4 (4.2)	**< 0.0001**
NLR	2.2 (0.0)	2.2 (0.0)	2.2 (0.0)	0.1
PLR	131.0 (0.7)	127.9 (0.7)	127.4 (0.7)	**< 0.001**
WBC (x109)	7.4 (0.0)	7.4 (0.0)	7.3 (0.0)	0.01

SII, systemic immune-inflammation index; NLR, neutrophil-to-lymphocyte ratio; PLR, platelet-lymphocyte ratio; WBC: white blood cell.
Bold values represent statistical significance.

a clear negative correlation between ω-3 and ω-6 PUFAs intake and SII. In model 1, the effect size (β) and 95% confidence intervals (CI) for SII were −34.662 (−46.069, −23.256) and − 31.157 (−41.912, −20.402) for the high-dose intake group of ω-3 and ω-6 PUFAs, respectively. In model 2, there was a negative relationship between the high-dose intake group of ω-3 and ω-6 PUFAs and SII, with *β* and 95% CI of −25.004 (−36.653, −13.354) and −18.021 (−29.131, −6.911), respectively. In model 3, a negative relationship was found between the high-dose intake group of ω-3 and ω-6 PUFAs and SII, with β and 95% CI of −21.309 (−33.098, −9.520) and −15.557 (−26.681, −4.434), respectively. The *p*-values for trend were statistically significant for ω-3 and ω-6 PUFAs intake (*p* trend <0.001). However, the correlation between the proportion of ω-6: ω-3 fatty acid intake and SII was not statistically significant. In Model 2, there was a positive correlation between the medium scale group of ω-6: ω-3 ratios and SII, with *β* and 95% CI of 12.163 (0.127, 24.199).

TABLE 3 Weighted characteristics of the study population based on dietary ω-6: ω-3 ratio.

Variable	ω-6: ω-3 ratio			*p*-value
	<=8.19 (*n* = 14,383)	8.19–10.13 (*n* = 14,383)	>10.13 (*n* = 14,389)	
Age group (%)				**< 0.0001**
20–40	4,495 (35.6)	5,196 (40.0)	5,497 (42.1)	
41–60	4,688 (36.8)	4,708 (36.0)	4,869 (37.5)	
> = 60	5,200 (27.5)	4,479 (23.9)	4,023 (20.5)	
Sex (%)				**< 0.0001**
Female	7,848 (55.7)	7,514 (51.1)	7,213 (50.0)	
Male	6,535 (44.3)	6,869 (48.9)	7,176 (50.0)	
Marital status (%)				0.2
Married/Living with partner	8,981 (64.6)	8,789 (64.4)	8,786 (63.3)	
Windowed/Divorced/ Separated/Never married	5,402 (35.4)	5,594 (35.6)	5,603 (36.7)	
Race (%)				**< 0.0001**
Mexican American	2,116 (7.4)	2,449 (8.4)	2,472 (8.2)	
Non-Hispanic White	6,395 (67.9)	6,833 (70.2)	6,768 (70.4)	
Non-Hispanic Black	2,688 (9.3)	2,869 (10.0)	3,319 (11.8)	
Other Hispanic	1,492 (6.7)	1,128 (5.5)	782 (3.8)	
Other race	1,692 (8.7)	1,104 (5.9)	1,048 (5.8)	
Education level (%)				**< 0.0001**
Below high school	1,562 (5.6)	1,359 (4.5)	1,314 (4.2)	
High school	4,939 (31.2)	5,420 (35.6)	5,531 (35.8)	
Above high school	7,882 (63.2)	7,604 (59.9)	7,544 (59.9)	
Smoking status (%)				**< 0.0001**
never	8,168 (55.9)	7,863 (55.2)	7,523 (53.2)	
former	3,729 (26.0)	3,615 (24.3)	3,732 (25.2)	
current	2,486 (18.1)	2,905 (20.5)	3,134 (21.5)	
Body mass index (%)				**< 0.0001**
<25	4,308 (31.8)	3,872 (28.3)	4,022 (30.5)	
25–30	5,021 (35.0)	4,736 (32.5)	4,808 (32.6)	
>30	5,054 (33.2)	5,775 (39.1)	5,559 (36.9)	
Poverty income ratio (%)				0.5
<=0.99	2,714 (13.2)	2,699 (13.1)	2,802 (13.7)	
> = 1	11,669 (86.8)	11,684 (86.9)	11,587 (86.3)	
SII	555.5 (4.6)	564.2 (4.9)	552.5 (3.9)	0.1
NLR	2.2 (0.0)	2.2 (0.0)	2.2 (0.0)	**0.01**
PLR	128.6 (0.7)	128.8 (0.7)	128.5 (0.7)	1
WBC (x109)	7.3 (0.0)	7.4 (0.0)	7.3 (0.0)	0.2

SII, systemic immune-inflammation index; NLR, neutrophil-to-lymphocyte ratio; PLR, platelet-lymphocyte ratio; WBC: white blood cell.
Bold values represent statistical significance.

Table 5 shows the dietary intake of ω-3, ω-6 PUFAs, and the relationship between ω-6: ω-3 ratio and PLR. In model 1, the β and 95% CI for PLR were −3.369 (−5.145, −1.593) and −3.606 (−5.428, −1.784) for the high-dose intake group of ω-3 and ω-6 PUFAs, respectively. In model 2, there was an inverse relationship between the high-dose intake group of ω-3 and ω-6 PUFAs and PLR, with β and 95% CI of −2.777 (−4.595, −0.959) and −1.958 (−3.608, −0.308), respectively. In model 3, a negative relationship was found between the high-dose intake group of ω-3 and ω-6 PUFAs and PLR, with β and 95% CI of −2.555 (−4.374, −0.735) and −1.867 (−3.702, −0.033), respectively. The *p*-values for trend for ω-3 and ω-6 PUFAs intake were statistically significant ($p < 0.001$). The correlation between ω-6: ω-3 ratio and PLR did not exhibit statistical significance.

TABLE 4 Survey-weighted multivariate regression analyses of associations between dietary ω-3 and ω-6 PUFAs intake and ω-6:ω-3 ratio and SII.

SII	Model 1 *β* (95%CI) *p*-value	Model 2 *β* (95%CI) *p*-value	Model 3 *β* (95%CI) *p*-value
Total ω-3 PUFAs intake (g)			
<=1.167	ref	ref	ref
1.167–1.928	−25.9 (−36.554, −15.246)**	−21.198 (−32.102, −10.295)**	−18.628 (−29.451, −7.806)**
>1.928	−34.662 (−46.069, −23.256)**	−25.004 (−36.653, −13.354)**	−21.309 (−33.098, −9.520)**
p for trend	<0.0001	<0.0001	<0.001
Total ω-6 PUFAs intake (g)			
<=10.929	ref	ref	ref
10.929–17.63	−23.686 (−34.169, −13.203)**	−18.418 (−28.841, −7.996)**	−16.115 (−26.451, −5.780)**
>17.63	−31.157 (−41.912, −20.402)**	−18.021 (−29.131, −6.911)**	−15.557 (−26.681, −4.434)**
p for trend	<0.0001	<0.0001	<0.001
Total ω-6:ω-3 ratio			
<=8.19	ref	ref	ref
8.19–10.13	8.65 (−3.452, 20.752)	12.163 (0.127, 24.199)*	8.198 (−3.488, 19.884)
>10.13	−3.061 (−13.079, 6.956)	1.943 (−8.029, 11.914)	−0.583 (−10.800, 9.633)
p for trend	0.524	0.732	0.867

SII, systemic immune-inflammation index; *β*, standardized coefficients; CI, confidence interval. Non-adjusted model: no covariates were adjusted. Minimally-adjusted model: age and gender were adjusted. Fully-adjusted model: age, gender, race, marital status, education level, smoking status, body mass index, and family monthly poverty level index were adjusted. *$p < 0.05$; **$p < 0.01$.

TABLE 5 Survey-weighted multivariate regression analyses of associations between dietary ω-3 and ω-6 PUFAs intake and ω-6:ω-3 ratio and PLR.

PLR	Model 1 *β* (95%CI) *p*-value	Model 2 *β* (95%CI) *p*-value	Model 3 *β* (95%CI) *p*-value
Total ω−3 PUFAs intake (g)			
<=1.167	ref	ref	ref
1.167–1.928	−3.731 (−5.511, −1.951)**	−2.777 (−4.595, −0.959)**	−3.499 (−5.292, −1.706)**
>1.928	−3.369 (−5.145, −1.593)**	−1.576 (−3.365, 0.213)	−2.555 (−4.374, −0.735)**
p for trend	<0.001	0.1	<0.01
Total ω−6 PUFAs intake (g)			
<=10.929	ref	ref	ref
10.929–17.63	−3.128 (−4.802, −1.455)**	−1.958 (−3.608, −0.308)**	−2.782 (−4.395, −1.169)**
>17.63	−3.606 (−5.428, −1.784)**	−0.882 (−2.711, 0.947)	−1.867 (−3.702, −0.033)*
p for trend	<0.001	0.383	0.059
Total ω−6:ω−3 ratio			
<=8.19	ref	ref	ref
8.19–10.13	0.161 (−1.679, 2.002)	1.053 (−0.765, 2.872)	1.494 (−0.190, 3.177)
>10.13	−0.117 (−1.891, 1.656)	1.233 (−0.512, 2.979)	1.574 (−0.203, 3.352)
p for trend	0.893	0.168	0.086

PLR, platelet-lymphocyte ratio; *β*, standardized coefficients; CI, confidence interval. Non-adjusted model: no covariates were adjusted. Minimally-adjusted model: age and gender were adjusted. Fully-adjusted model: age, gender, race, marital status, education level, smoking status, body mass index, and family monthly poverty level index were adjusted. *$p < 0.05$; **$p < 0.01$.

Table 6 depicts the dietary intake of ω-3, ω-6 PUFAs, and the relationship between ω-6: ω-3 ratio and NLR. In model 1, the *β* and 95% CI for NLR were −0.041 (−0.076, −0.006) and −0.04 (−0.077, −0.003) for the medium-dose intake group of ω-3 and ω-6 PUFAs, respectively. In model 2, there was an inverse relationship between the high-dose intake group of ω-3 and ω-6 PUFAs and NLR, with *β* and 95% CI of −0.045 (−0.084, −0.005) and −0.038 (−0.075， −0.001), respectively. In model 3, a negative relationship was found between the medium-dose intake group of ω-3 PUFAs and NLR, with *β* and 95% CI of −0.037 (−0.071, −0.002). The *p*-values for trend for ω-3 and ω-6 PUFAs intake were statistically significant ($p < 0.05$). In Model 1, there was a negative correlation between the high-scale group of ω-6: ω-3 ratios and NLR, with *β* and 95% CI of −0.037 (−0.073, −0.002).

Table 7 shows the ω-3 and ω-6 PUFAs dietary intake and the relationship between ω-6: ω-3 ratio and WBC. In model 1, the *β* and 95% CI for WBC were −0.169 (−0.264, −0.073) and − 0.128 (−0.214,

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TABLE 6 Survey-weighted multivariate regression analyses of associations between dietary ω-3 and ω-6 PUFAs intake and ω-6:ω-3 ratio and NLR.

NLR	Model 1 *β* (95%CI) *p*-value	Model 2 *β* (95%CI) *p*-value	Model 3 *β* (95%CI) *p*-value
Total ω−3 PUFAs intake (g)			
<=1.167	ref	ref	ref
1.167–1.928	−0.041 (−0.076, −0.006)*	−0.042 (−0.077, −0.007)*	−0.037 (−0.071, −0.002)*
>1.928	−0.032 (−0.072, 0.008)	−0.045 (−0.084, −0.005)*	−0.036 (−0.076, 0.004)
p for trend	0.188	<0.05	0.082
Total ω−6 PUFAs intake (g)			
<=10.929	ref	ref	ref
10.929–17.63	−0.04 (−0.077, −0.003)*	−0.034 (−0.070, 0.002)	−0.031 (−0.067, 0.005)
>17.63	−0.039 (−0.077, −0.001)*	−0.038 (−0.075, −0.001)*	−0.033 (−0.070, 0.004)
p for trend	<0.05	<0.05	0.086
Total ω−6:ω−3 ratio			
<=8.19	ref	ref	ref
8.19–10.13	0.02 (−0.022, 0.063)	0.033 (−0.009, 0.075)	0.024 (−0.017, 0.065)
>10.13	−0.037 (−0.073, −0.002)*	−0.014 (−0.050, 0.021)	−0.02 (−0.055, 0.016)
p for trend	<0.05	0.402	0.263

NLR, neutrophil-to-lymphocyte ratio; β, standardized coefficients; CI, confidence interval. Non-adjusted model: no covariates were adjusted. Minimally-adjusted model: age and gender were adjusted. Fully-adjusted model: age, gender, race, marital status, education level, smoking status, body mass index, and family monthly poverty level index were adjusted. *$p < 0.05$; **$p < 0.01$.

TABLE 7 Survey-weighted multivariate regression analyses of associations between dietary ω-3 and ω-6 PUFAs intake and ω-6:ω-3 ratio and WBC.

WBC	Model 1 *β* (95%CI) *p*-value	Model 2 *β* (95%CI) *p*-value	Model 3 *β* (95%CI) *p*-value
Total ω−3 PUFAs intake (g)			
<=1.167	ref	ref	ref
1.167–1.928	−0.098 (−0.175, −0.021)*	−0.091 (−0.169, −0.014)*	−0.023 (−0.098, −0.051)
>1.928	−0.169 (−0.264, −0.073)**	−0.142 (−0.246, −0.039)**	−0.049 (−0.148, 0.050)
p for trend	<0.01	<0.01	0.334
Total ω−6 PUFAs intake (g)			
<=10.929	ref	ref	ref
10.929–17.63	−0.043 (−0.139, 0.054)	−0.042 (−0.142, 0.058)	0.028 (−0.067, 0.123)
>17.63	−0.128 (−0.214, −0.041)**	−0.113 (−0.206, −0.021)*	−0.037 (−0.121, 0.048)
p for trend	<0.05	0.015	0.354
Total ω−6:ω−3 ratio			
<=8.19	ref	ref	ref
8.19–10.13	0.06 (−0.031, 0.151)	0.051 (−0.038, 0.140)	−0.024 (−0.109, 0.060)
>10.13	−0.006 (−0.108, 0.097)	−0.024 (−0.124, 0.076)	−0.074 (−0.171, 0.023)
p for trend	0.899	0.625	0.132

WBC, white blood cell; *β*, standardized coefficients; CI, confidence interval. Non-adjusted model: no covariates were adjusted. Minimally-adjusted model: age and gender were adjusted. Fully-adjusted model: age, gender, race, marital status, education level, smoking status, body mass index, and family monthly poverty level index were adjusted. *$p < 0.05$; **$p < 0.01$.

−0.041) for the high-dose intake group of ω-3 and ω-6 PUFAs, respectively. The *p*-values trend for ω-3 and ω-6 PUFAs intake were less than 0.01 and 0.05, respectively, and were statistically significant. In model 2, there was a negative relationship between the high-dose intake group of ω-3 and ω-6 PUFAs and WBC, with *β* and 95% CI of −0.142 (−0.246, −0.039) and-0.113 (−0.206, −0.021), respectively. The *p*-value for trend for ω-3 PUFAs intake was statistically significant. ($p < 0.01$). No notable relationship was depicted between ω-6: ω-3 ratio and WBC.

3.3 Stratified analyses of the associations between dietary ω-3, ω-6 PUFAs intake and SII

The study participants were divided into groups based on sex, age, smoking status, race, BMI, and education (Supplementary Tables S1, S2). The analysis showed that the relationship between dietary intake of ω-3 and ω-6 fatty acids and the SII index remained consistent across

all the groups and did not exhibit significant variations. However, it is important to note that the association between ω-3 and ω-6 fatty acid intake and SII was found to be stronger in the obese population (BMI > 30).

3.4 Nonlinear associations between dietary ω-3, ω-6 PUFAs intake and SII

Figures 2, 3 illustrate the findings of the dose–response relationship between ω-3, ω-6 PUFAs and SII index. As shown in the figure, an L-type correlation was observed between ω-3 and ω-6 PUFAs intake and SII (p for nonlinearity <0.05). The dose–response relationship between intake of ω-3 and ω-6 PUFAs and SII showed an overall trend of first decrease and then increase, with inflection points of 2.35 g/day and 19.79 g/day, respectively. However, the non-linear relationship between the ω-3: ω-6 ratio and SII was not significant (p for nonlinearity >0.05).

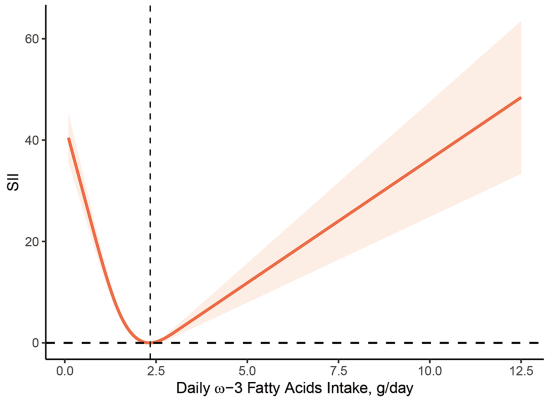

FIGURE 2
Dose–response relationship between ω-3 PUFAs intake and SII. The association was adjusted for age, gender, race, marital status, education level, smoking status, body mass index, family monthly poverty level index.

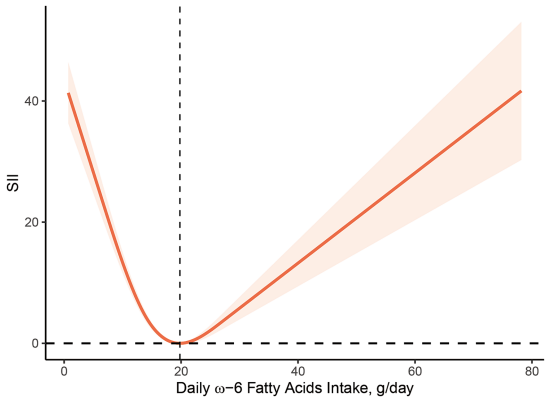

FIGURE 3
Dose–response relationship between ω-6 PUFAs intake and SII. The association was adjusted for age, gender, race, marital status, education level, smoking status, body mass index, family monthly poverty level index.

4 Discussion

A comprehensive cross-sectional survey was carried out in this research to assess the relationship between dietary intake of ω-3, ω-6 PUFAs, and ω-6 to ω-3 ratio with systemic immune and inflammatory biomarkers. Data from the NHANES survey for 1999 to 2020, representing the US population, were utilized. In this study, we found a significant negative correlation between dietary intake of ω-3 and ω-6 PUFAs and SII, NLR, PLR, and WBC, which supports the contention that ω-3 PUFAs exert an anti-inflammatory effect, and that ω-6 PUFAs have a similar anti-inflammatory effect. In addition, the dose–response relationship suggests that ω-3 and ω-6 PUFAs intakes are associated with SII in a non-linear L-form, whereas ω-6: ω-3 ratios are not substantially related to these inflammatory biomarkers.

Theoretical studies suggest that ω-3 and ω-6 PUFAs compete with each other at cyclooxygenase (COX) and lipoxygenase (LOX) sites to generate different types of eicosanoids, such as prostaglandins (PGs) and leukotrienes (LTs), etc. (34). In response to inflammatory stimuli, AA within the ω-6 PUFA is released from membrane phospholipids. Subsequently, it undergoes conversion into PGE2 and LTB4 in a COX- and LOX-dependent manner. This process exerts a strong pro-inflammatory effect, contributing to platelet aggregation and vasoconstriction. In contrast, the metabolism of EPA and DHA in ω-3 PUFA produces PGE3 and LTB5, which exhibit anti-inflammatory and antiplatelet aggregation effects (35).

However, the relationship between fatty acid elongation and desaturase action is rather complex, and clinical outcome is not easily predicted based on biochemical pathways alone. The association between dietary PUFAs and inflammatory markers has not yet been fully elucidated (36). The anti-inflammatory impacts of ω-3 fatty acids have been extensively documented in multiple clinical and experimental studies. Two extensive studies by Derosa et al. (10, 11) revealed substantially reduced levels of serum of high-sensitivity C-reactive protein (hs-CRP), matrix metalloproteinase (MMP)-2, and MMP-9 in patients with dyslipidemia after 6 months of EPA + DHA intake at 2.6 g/day compared with placebo. ω-3 PUFAs also protect from CVD by addressing arrhythmias, lowering blood pressure, plasma homocysteine and serum triglycerides, prolonging clotting time, and suppressing platelet aggregation (37). This is consistent with the findings of this research. However, it is crucial to highlight that there is no substantial correlation between consuming low doses of ω-3 fatty acids over short courses and inflammation biomarkers (38).

At the same time, it was found that ω-6 PUFAs also had some anti-inflammatory effects. Although ω-6 PUFAs are theoretically and widely recognized as pro-inflammatory mediators, multiple clinical studies have failed to substantiate this hypothesis (12–14). In a study examining the impact of dietary AA supplementation on peripheral blood mononuclear cells (PBMCs), participants who received 0.7 g/day of AA-rich single-cell oil (ARASCO) for 12 weeks exhibited an elevation in the proportion of AA in PBMC phospholipids of total fatty acids (12). In another study, ARASCO

supplementation for 7 weeks significantly increased the secretion of PGE2 and LTB4 from lipopolysaccharide-stimulated cultured PBMCs. However, this supplementation did not lead to the increased secretion of interleukin-6 (IL-6), IL-1β, or tumor necrosis factor-α (TNF-α), nor did it affect the number of circulating lymphocytes labeled with specific subsets (13). A randomized controlled study of healthy elderly adults (55 to 70 years old) in Japan discovered that the plasma phospholipid content of AA increased dose-dependently following 4 weeks of AA supplementation at 0.24 g/day or 0.73 g/day. However, this supplementation did not influence the levels of AA metabolites and the plasma concentrations of CRP, IL-6, and TNF-α (14). The provided evidence indicates that increasing AA intake results in elevated AA content in plasma or cellular phospholipids. However, it does not exert a notable impact on pro-inflammatory cytokine production and the number of inflammatory cells. In parallel, studies focusing on LA, a synthetic substrate of AA, revealed that increasing LA intake did not elevate AA concentrations in plasma or PBMC. Moreover, it was not significantly associated with multiple inflammatory markers (39–41). This observation could be attributed to the saturation of the pathway for synthesizing AA from LA. Conversely, some studies even suggest that AA and LA may be associated with reduced inflammation (15–17). A cross-sectional study of 364 patients with CVD secondary prevention showed that augmented dietary consumption of ω-3 and ω-6 PUFAs was inversely correlated with levels of CRP, IL-1β, IL-10, and IL-12 (16). Another cross-sectional study showed that ω-3 and ω-6 PUFAs intake was linked to a reduced risk of developing CVD in comparison with intake of either fatty acid alone (17). These findings align with our observation that ω-6 PUFA intake is inversely associated with inflammatory markers. The inhibitory effect of ω-6 PUFAs on inflammatory responses may be achieved through eicosanoid-independent pathways as well as the production of precursors related to the inflammatory abrogation mediators. Nonetheless, additional research is required to explore the specific mechanisms involved (42).

In addition, this study revealed no noteworthy correlation between ω-6: ω-3 ratio and SII, along with other secondary measures. It is noteworthy that research investigating the relationship between the ω-6: ω-3 ratio and inflammatory markers has yielded conflicting results (18–22). Kalogeropoulos et al. (18) demonstrated a strong relationship between the proportion of ω-6: ω-3 and hs-CRP, IL-6, TNF-α, fibrinogen, and homocysteine in serum from 374 healthy people in the ATTICA's study database. This suggests that the inflammatory balance of the body may be regulated by the relative amounts of ω-6 and ω-3 fatty acids. Another study involving 1,123 healthy individuals discovered an inverse relationship between the proportion of ω-6: ω-3 in fasting plasma and the anti-inflammatory marker IL-10 (20). Zhang et al. (19) utilized a population cohort from the UK Biobank to identify an elevated risk of all-cause, cancer, and CVD mortality with an elevated ω-6: ω-3 ratio in the population. Harris (21, 22) conducted an analysis of 11 case–control and two prospective cohort studies, revealing that the ω-6: ω-3 ratio was not effective in distinguishing coronary artery disease cases from healthy subjects. Therefore, it is suggested that more evidence is necessary to substantiate the utility of the ω-6: ω-3 ratio as a biomarker for predicting disease status or serving as a nutritional reference. This is supported by our findings. The ω-6: ω-3 ratio may not allow for the efficacy of each fatty acid to be assessed individually. Therefore, no recommendation can be given for a more accurate assessment.

The specific mechanism for the "L" shaped dose–response relationship between dietary ω-3 and ω-6 fatty acid intake and SII index is unclear, but there are several possibilities. First, dietary fatty acid intake is strongly associated with age, BMI, and individual metabolism. The results of stratified analyses show that there are some differences in BMI among the included study populations. One study found that a high dietary ω-6: ω-3 PUFA ratio was positively associated with excessive obesity and worsened metabolic status (43). And BMI is a better predictor of response to dietary supplements than simple body weight (44). Therefore, BMI differences in the study population may contribute to the over-activation of the inflammatory state, resulting in an "L" shaped dose–response relationship between PUFAs intake and inflammatory biomarkers in the body. Secondly, the effect of a single dietary component on homeostasis is limited, as the structure and function of cell membranes are regulated by other dietary factors, such as antioxidants and polyphenols, in addition to PUFAs. Several studies have shown that PUFAs is highly susceptible to oxidation, and their peroxidation produces lipid peroxides, which can harm the organism. The dietary intake of PUFA is accompanied by the intake of certain antioxidants, such as LA and vitamin E which are also obtained through vegetable oils. An epidemiological study investigating the relationship between PUFA intake and CRP concentrations found that the negative correlation between dietary ω-3 and ω-6 fatty acid intake and elevated CRP was only significant in individuals with low vitamin E intake, demonstrating some interaction between vitamin E and PUFAs (45). Thus with the gradual increase in ω-3 and ω-6 fatty acid intake, antioxidants in the body are unable to antagonize the higher levels of lipid peroxides and an increase follows a decrease in the level of inflammation. In addition to this, PUFA is also closely associated with the platelet-activating factor (PAF), the synthesis and catabolism of which involves the participation of a series of enzymes, among which lipoprotein-associated phospholipase A2 (Lp-PLA2) is considered to be a marker of vascular inflammatory response in the body. A cross-sectional study including 2,246 participants found that AA, EPA, and DHA plasma levels were negatively correlated with Lp-PLA2 mass and activity (46). In addition, a negative correlation between EPA and DHA and Lp-PLA2 concentrations was also observed in adipose tissue (47). Further studies revealed that this may affect Lp-PLA2 expression through activation of p38 mitogen-activated protein kinase and phosphatidylinositol 3-kinase (48–50). This may be related to the "L" shaped relationship between ω-3 and ω-6 fatty acid intake and SII index. However, the association between ω-3 and ω-6 PUFAs and PAF has not yet been established, and further studies are needed (51, 52).

On the one hand, this research exhibits multiple notable strengths. Firstly, it examined the correlation between dietary ω-3, ω-6 PUFAs intake and ω-6: ω-3 ratio and systemic immune-inflammatory status, utilizing multiple inflammatory indicators. Secondly, it explored the dose–response relationship between the main indicator SII and the dietary intake of ω-3 and ω-6 PUFAs. Finally, it employed a large, nationally representative sample,

which enhances statistical power and augments the accuracy and reliability of the findings. On the other hand, this research also has certain limitations. Firstly, due to its cross-sectional design, establishing causality is challenging. Secondly, dietary data were collected via a 24-h dietary recall interview, which may lead to inaccuracy.

5 Conclusion

This research proposes that there is an inverse relationship between ω-3, ω-6 PUFAs intake and systemic inflammatory biomarkers in humans. The intake ω-3 and ω-6 PUFAs in the dose–response relationship exhibited an "L"-type association with the SII, indicating an initial decrease followed by an increase. However, no noticeable association was observed between ω-6: ω-3 ratio and these inflammatory markers. Further investigation is warranted to elucidate the mechanisms underlying this observation.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by National Center for Health Statistics. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

YL: Data curation, Formal analysis, Methodology, Software, Validation, Writing – original draft, Writing – review & editing. HT: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft. XY: Conceptualization, Methodology, Visualization, Writing – original draft. LilM: Formal analysis, Software, Visualization, Writing – review & editing. HZ: Data curation, Supervision, Visualization, Writing – review & editing. GZ: Data curation, Formal analysis, Methodology, Software, Writing – review & editing. XC: Conceptualization, Data curation, Formal analysis, Writing – original draft. LijM: Data curation, Methodology, Writing – original draft. JG: Formal analysis, Software, Validation, Writing – review & editing. WJ: Conceptualization, Methodology, Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2024.1410154/full#supplementary-material

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Effect of FADS1 SNPs rs174546, rs174547 and rs174550 on blood fatty acid profiles and plasma free oxylipins

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Introduction: Previous studies have indicated that activity of fatty acid desaturase 1 (FADS1), is involved in cardiometabolic risk. Recent experimental data have shown that FADS1 knockdown can promote lipid accumulation and lipid droplet formation in liver cells. In this study, we aimed to characterize whether different FADS1 genotypes affect liver fat content, essential fatty acid content and free oxylipin mediators in the blood.

Methods: We analyzed the impact of FADS1 single-nucleotide polymorphisms (SNPs) rs174546, rs174547, and rs174550 on blood fatty acids and free oxylipins in a cohort of 85 patients from an academic metabolic medicine outpatient center. Patients were grouped based on their genotype into the homozygous major (derived) allele group, the heterozygous allele group, and the homozygous minor (ancestral) allele group. Omega-3 polyunsaturated fatty acids (n-3 PUFA) and omega-6 polyunsaturated fatty acids (n-6 PUFA) in the blood cell and plasma samples were analyzed by gas chromatography. Free Oxylipins in plasma samples were analyzed using HPLC–MS/MS. Liver fat content and fibrosis were evaluated using Fibroscan technology.

Results: Patients with the homozygous ancestral (minor) FADS1 genotype exhibited significantly lower blood levels of the n-6 PUFA arachidonic acid (AA), but no significant differences in the n-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). There were no significant differences in liver fat content or arachidonic acid-derived lipid mediators, such as thromboxane B2 (TXB2), although there was a trend toward lower levels in the homozygous ancestral genotype group.

Discussion: Our findings suggest that FADS1 genotypes influence the blood levels of n-6 PUFAs, while not significantly affecting the n-3 PUFAs EPA and DHA. The lack of significant differences in liver fat content and arachidonic acid-derived lipid mediators suggests that the genotype-related variations in fatty acid levels may not directly translate to differences in liver fat or inflammatory lipid mediators in this cohort. However, the trend towards lower levels of certain lipid mediators in the homozygous ancestral genotype group warrants

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further investigation to elucidate the underlying mechanisms of different FADS1 genotypes and potential implications for cardiometabolic risk.

KEYWORDS

steatosis hepatis, MAFLD, FADS1, Oxylipins, PUFA

1 Introduction

Metabolic-dysfunction associated fatty liver disease (MAFLD, steatosis hepatis) is a common disease with an estimated prevalence of 20–30% (1, 2). It is the most common liver disease in the Western world, with a rising trend. MAFLD can be considered as hepatic manifestation of the metabolic syndrome (MetS) (3, 4). MAFLD patients are at risk to develop persistent inflammation (Nonalcoholic steatohepatitis, NASH), which can lead to liver fibrosis, cirrhosis and is associated with an increased risk of developing hepatocellular carcinoma (5, 6).

Genes involved in the elongation and desaturation of long-chain (lc) PUFAs from short-chain precursors have been linked to diet-dependent risks of cardiovascular disease (CVD) and metabolic syndrome MetS) (7–9).

One of the rate-limiting steps in the synthesis of lc-PUFAs from sc-PUFAs is catalyzed by the fatty acid (FA) delta-5-desaturase (D5D; Figure 1) (10). D5D or fatty acid desaturase 1 (FADS1) is a membrane-bound desaturase that catalyzes the synthesis of lc omega-3 (n-3) and omega-6 (n-6) PUFAs from dietary linoleic acid (LA, 18:2 n-6) and α-linolenic acid (18:3 n-3) (11). The human D5D gene is located on chromosome 11 in the *FADS* region, where several polymorphisms are located. These *FADS* gene polymorphisms affect PUFA synthesis in addition to dietary regulation of FA supply and composition (10). Two alleles of the SNPs rs174546, rs 174547 and rs174550 are distinguished at the *FADS1* locus. The major or derived allele corresponds to the following bases at these three SNPs: C at rs174546, T at rs 174547 and T at rs174550. The minor or ancestral allele corresponds to the following bases at the three SNPs analyzed here: T at rs174546, C at rs174547 and C at rs174550. The minor alleles of these SNPs in the FADS1 gene were found to be associated with lower blood concentrations of lc n–3 and n–6 PUFAs (12–14). Some data suggest that the major alleles are associated with an increased risk of atherosclerosis in the context of a diet high in animal fats (15), whereas other studies found that the minor alleles might confer an increased cardiovascular risk that could be alleviated by dietary measures (16).

It has been shown that the *FADS1* minor ancestral allele has been the only relevant allele in human populations in Europe until approximately 8.500 years ago, when the derived allele was introduced (17).

The minor ancestral allele, which is characterized by lower *FADS1* expression, has been implicated to confer increased metabolic risk and increased liver steatosis (18–21).

In this pilot study, we aimed to characterize liver fat content and lipidomics characteristics from patients enrolled in a routine setting of an outpatient metabolic medicine center for diagnosis, monitoring or treatment of MetS and MAFLD. We wanted to determine whether the homozygous minor (ancestral) FADS1 genotype is associated with higher liver fat in unselected patients presenting to our center, and whether different FADS1 genotypes affect essential fatty acid content and free oxylipin mediators in the blood.

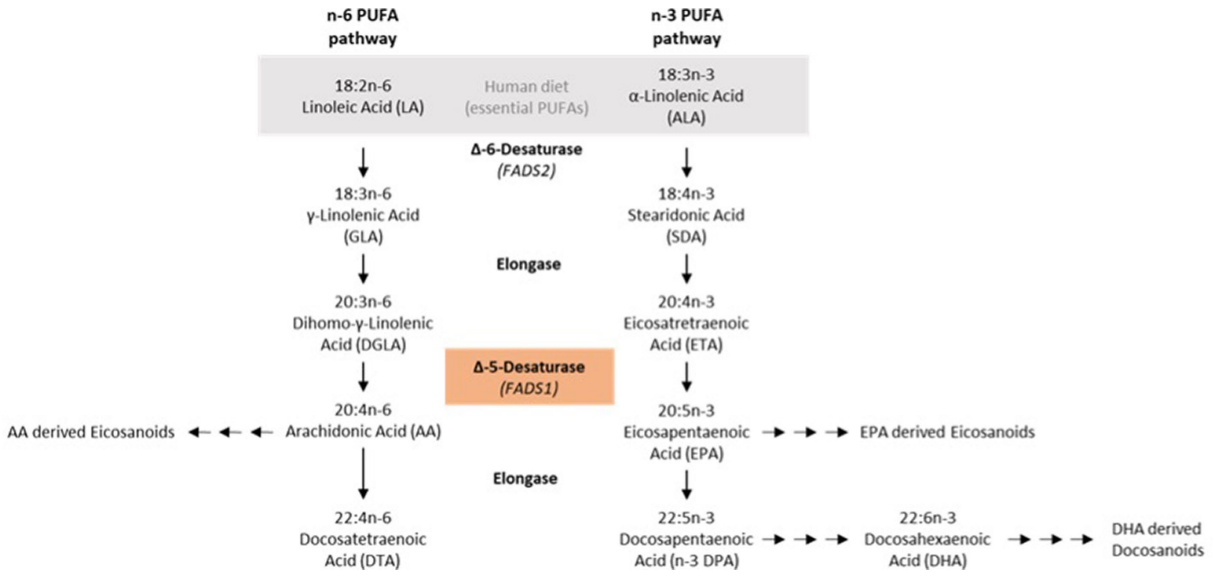

FIGURE 1
N-6/n-3 PUFA synthesis pathways.

2 Methods

Patients presenting to our metabolic disease clinic were recruited, containing patients with risk for, or manifest MAFLD, MetS, type 2 diabetes mellitus (T2DM), and/or dyslipidemia (elevation of triglycerides, total cholesterol, or LDL cholesterol or lowering of HDL cholesterol). Patients gave their informed consent for this research project investigating essential n-6 and n-3 fatty acids in the context of metabolic disease (approved by the institutional ethics committee, Nr. Z02-20170508). Only patients with signed informed consent were included in the study.

To assess PUFA levels in blood cells and plasma, blood samples were collected after at least 6 h of fasting from patients ($n = 85$) enrolled in a routine setting. All samples were centrifuged at 3500 rpm for 10 min at 4°C, separated blood cell and plasma samples were stored at −80°C until FA analysis. Extraction and quantification were carried out according to established protocols. According to a previous study, we expected a prevalence of patients homozygous for the minor FADS1 allele (that has lower D5D activity) of 11% (16), and aimed for 9–10 patients with this genotype to allow for meaningful statistical analysis of the FA and oxylipins.

In brief, FA from 50 µL erythrocytes and 75 µL plasma per sample were analyzed. Boron trifluoride (BF_3) derivatization was applied for the blood cell fraction (22) and a combined BF_3 + NaOH method for derivatization was used for the plasma samples (23, 24). FA values from blood cells are presented as percentage [%] of total FA content, FA concentration from plasma are presented in absolute amounts [µg/ml]. Free Oxylipins in plasma samples [ng/ml] were analyzed as described previously using LC–MS/MS (Lipidomix, Berlin) (25).

Sonographic evaluation was performed using FibroScan (Echosens, Paris), a standardized and reproducible point-of-care technique for quantification of steatosis and fibrosis as described previously (25). This is a non-invasive measurement method for quantifying a fatty liver, in which acoustically generated transient ultrasound waves cause the liver to vibrate intermittently. This method is based on shear wave elastography (SWE) (26).The cutoff value reported in the literature for the detection of hepatic steatosis ranges from 222 decibels per meter (dB/m) in a cohort of patients with chronic hepatitis C to 294 dB/m in a meta-analysis of patients with nonalcoholic fatty liver disease (NAFLD) (27). The examination was performed in all patients in a fasting state (the last meal should have been at least 6 h before the measurement). The examination was performed in the supine position with maximal abduction of the right arm and positioning of the right leg over the left to gain sufficient intercostal access. The probe was placed at the intersection of the xiphoid process and the midaxillary line. Patients weighing <100 kg were examined with the M probe (standard probe—transducer frequency 3.5 MHz) and patients weighing >100 kg were examined with the XL probe (transducer frequency 2.5 MHz). Reliable measurements were defined as: Median of 13 valid LS measurements with an interquartile range ≤ 30% (IQR/med = the difference between the 75 and 25th percentiles, i.e., the range of the middle 50% of the data relative to the median).

The *FADS1* SNPs rs174546, rs174547 and rs174550 were characterized in venous blood collected from the patients in EDTA tubes using an Illumina Platform (Life and Brain, Bonn, Germany).

For statistical analysis FA or oxylipin values were tested for normal distribution using the Shapiro–Wilk test. For normally distributed values, a one-way ANOVA between the three groups and Tukey's Honest Significance Difference (HSD) test as follow-up to assess significances between subsets of two groups was performed. For non-normally distributed values, testing for significant differences between the three groups was performed using the Kruskal-Wallis test and followed up with Dunn's testing for subsets of the possible pairwise comparisons. Categorical values (age) were compared using Pearson Chi Square testing. Statistical analyses were done using GraphPad prism or Excel software. $p \leq 0.05$ was considered as significant.

3 Results

We aimed to include at least 10 patients homozygous for the minor (ancestral) *FADS1* allele. This objective was achieved after having screened 85 patients for whom we performed liver fat determination, FA analyses and, of a subset, lipidomics analyses. Out of these 85 patients, 37 were homozygous for the major (derived) *FADS1* alleles (rs174546 CC, rs174547 TT and rs174550 TT), 37 heterozygous (rs174546 CT, rs174547 CT and rs174550 CT), and 11 homozygous for the minor (ancestral) *FADS1* allele (rs174546 TT, rs174547 CC and rs174550 CC). As shown in Table 1 there were no significant differences between the groups regarding age and sex. Furthermore, there were no significant differences between groups regarding steatosis and fibrosis, as assessed by Fibroscan.

In order to assess FADS1 activity the delta-5-desaturase index (D5D index) was calculated as the following ratio of arachidonic acid (AA) and its precursor dihomo-gamma-linolenic acid (DGLA) in FA and plasma (10, 28):

$$D5D\,index = \frac{arachidonic\ acid\ (AA; C20:4n-6)}{dihomo-gamma-linoleinic\ acid\ (DGLA; C20:3n-6)}$$

As expected from previously published data we found highly significant D5D index differences between genotypes containing the major allele (rs174546 CC and CT,) and the homozygous minor

TABLE 1 Liver fat and fibrosis parameters of included patients depending on rs174546 genotype.

	Total	CC	CT	TT
	$n = 85$	$n = 37$	$n = 37$	$n = 11$
N (Female/Male)	36/49	11/26	19/18	6/5
Age (years)	52.8 ± 1.7	53.2 ± 2.6	54.2 ± 2.6	46.6 ± 4.9
CAP (dB/m)	283.0 ± 7.1	290.4 ± 10.5	281.6 ± 10.8	267.3 ± 20.6
LSM (kPa)	10.4 ± 1.6	11.0 ± 2.6	8.8 ± 2.0	13.5 ± 5.1

Data are given as data as mean ± SEM, CAP, common attenuation parameter in dB/m (decibels per meter), LSM, liver stiffness measurement in kPa (kilopascal). All differences between groups were not significant assuming a p-value of < 0.05 for significance.

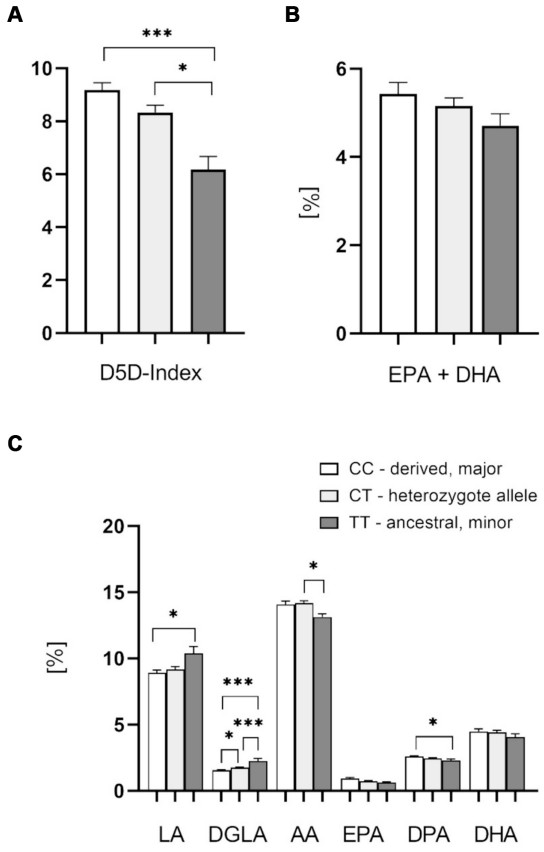

FIGURE 2
Effect of the FADS1 rs174546 genotype on PUFAs in the blood cell fraction. **(A)** D5D index is significantly higher with the C allele carriers (CC > CT > TT). **(B)** Slightly lower EPA + DHA content (determined in analogy to the Omega-3-Index) in the blood cell fraction from patients with the homozygous ancestral FADS1 genotype (TT). **(C)** Significantly higher levels of n-6 PUFAs linoleic acid (LA) and dihomo-gamma-linolenic acid (DGLA), and lower levels of arachidonic acid (AA) and n-3 PUFA docosapentaenoic acid (DPA) with the minor TT genotype. $n = 37$ for the homozygous derived genotype CC, $n = 37$ for the heterozygous genotype CT, $n = 11$ for the homozygous ancestral genotype TT, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, one-way ANOVA with subsequent Tukey's HSD testing.

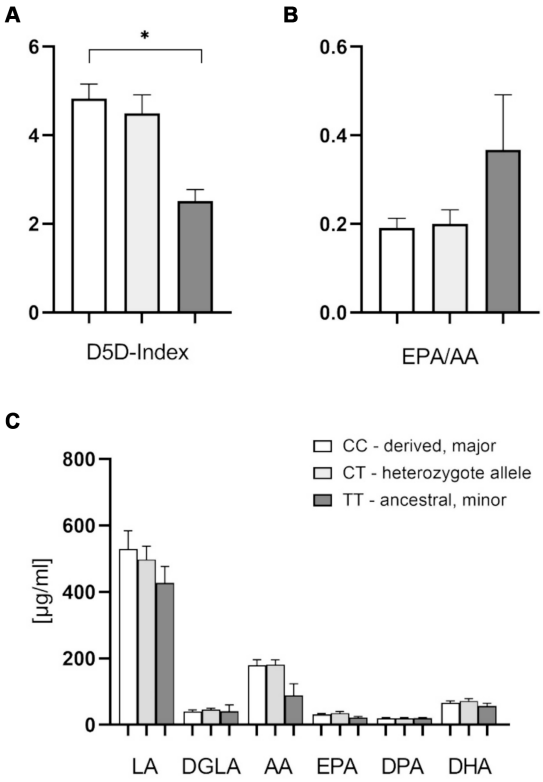

FIGURE 3
Effect of the FADS1 rs174546 genotype on plasma PUFAs content. **(A)** The D5D index is significantly higher with the CC alleles in comparison to TT alleles. **(B)** Slightly higher EPA/AA plasma ratio with the homozygous ancestral FADS1 genotype (ns, $p = 0.067$). **(C)** Slightly lower AA (20:4 n-6) levels with the homozygous ancestral FADS1 genotype. $n = 19$ for the homozygous derived genotype, $n = 19$ for the heterozygous genotype, $n = 4$ for the homozygous ancestral genotype, $^{*}p < 0.05$, Kruskal-Wallis and subsequent Dunn's testing.

allele (rs174546 TT), supporting higher FADS1 gene activity with the major derived C allele, leading to higher levels of AA and lower levels of DGLA (Figures 2A,C). Indeed, there were significant differences in blood cell FA content for LA (18:2 n-6), DGLA (20:3 n-6), AA (20:4 n-6), and n-3 docosapentaenoic acid (DPA, 22:5 n-3). No significant differences were found for eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3; Figure 2C). The content of EPA plus DHA, determined in analogy to the well-established Omega-3-Index (29), was slightly lower in the homozygous minor allele group (Figure 2B).

In a subset of patients, we also analyzed plasma fatty acids. These data confirmed the significantly higher D5D index, as well as higher levels of AA (20:4 n-6) in the derived genotype carriers (Figures 3A,C). There was a significant difference of D5D indices between the homozygous CC and TT allele carriers. In this subset we did not find significant differences for other plasma FAs. Interestingly, there was a higher plasma EPA/AA-ratio in patients with the homozygous ancestral genotype, although this finding did not reach statistical significance (Figure 3B). This parameter, with a cutoff at 0.4 and higher levels being beneficial, has been implicated as a risk stratification marker for cardiovascular protection (30, 31).

There were no significant differences of plasma free oxylipin content between different FADS1 genotypes, with slightly higher, but not significantly different levels of metabolites in the homozygous ancestral genotype as compared to the other genotypes (Figures 4A–E). There were indications, however, that platelet-related mediators such as thromboxane B2 (TXB2) and 12-HETE tended to be higher with the derived genotype (Figures 4A,B). Potentially hepatoprotective epoxy metabolites also showing a trend to higher levels as compared to the minor homozygotes (Figure 4E).

4 Discussion

The development of MetS is affected not only by lifestyle but also by genetics. In a retrospective study, the difference of dietary patterns was not enough to cause the change of MetS outcomes (32). Since genetic variants affect lipid metabolism which is closely associated with MetS (33), attention focused on the association between MetS

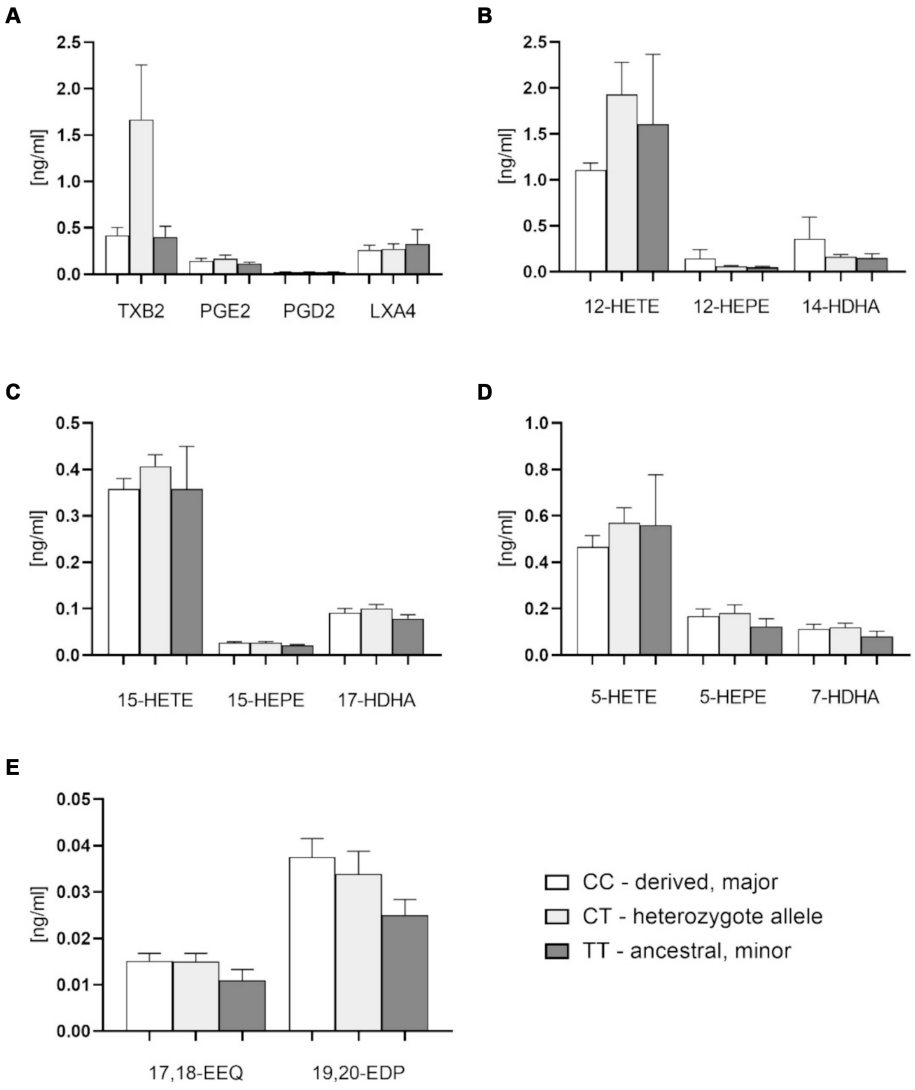

FIGURE 4
Plasma free oxylipins in groups with FADS1 rs174546 genotypes. **(A)** Thromboxane B2 (TXB2), prostaglandin D2 (PGD2), prostaglandin E2 (PGE2) and lipoxin A4 (LXA4) contents in plasma of different FADS1 genotypes. **(B)** 12-Lipoxygenase-derived oxylipin contents from AA (12-hydroxyeicosatetraenoic acid, 12-HETE), EPA (12-hydroxyeicosapentaenoic acid, 12-HEPE) and DHA (14-hydroxydocosahexaenoic acid, 14-HDHA) in plasma of different FADS1 genotypes. **(C)** 15-Lipoxygenase-derived oxylipin contents from AA (15-HETE), EPA (15-HEPE) and DHA (17-HDHA). **(D)** 5-Lipoxygenase-derived oxylipins from AA (5-HETE), EPA (5-HEPE) and DHA (7-HDHA) in plasma of different FADS1 genotypes. **(E)** Cytochrome P450-derived epoxy metabolites from EPA (17,18-epoxyeicosatetraenoic acid 17,18-EEQ) and DHA (19,20-epoxydocosapentaenoic acid, 19,20-EDP) in plasma of different FADS1 genotypes. $n = 36$ for the homozygous derived genotype, $n = 36$ for the heteozygous genotype, $n = 10$ for the homozygous ancestral genotype. There were no significant differences between groups from Kruskal-Wallis testing.

and genetic factors (34–36), and FADS genotype has been found to contribute to biochemical and metabolic variations in the MetS. In our pilot study in a routine clinical setting, we chose the *FADS1* SNP rs174546 which has been reported to be in linkage disequilibrium (LD) with other *FADS1* variants as a representative variant for differences in the FADS1 genotype (12, 36) and confirmed this with the SNPs rs174547 and rs174550.

Carriers of the major allele are known to have higher FADS1 enzyme activities than homozygous carriers of the ancestral minor allele. This leads to higher substrate precursor concentrations (LA, 18:2 n-6), higher substrate concentrations (DGLA, 20:3 n-6) and lower product (AA, 20:4 n-6) concentrations in patients homozygous for the minor alleles (12, 16, 37).

We confirmed the previously described significantly lower D5D index and activity for patients with the homozygous minor (ancestral) *FADS1* allele as compared to those homo- or heterozygous for the major (derived) allele in the blood cell fraction as well as in plasma for homozygous minor allele carriers in comparison with homozygous major allele carriers. Furthermore, the amounts of LA (18:2 n-6) and DGLA (20:3 n-6) were significantly higher in blood cells with the homozygous ancestral FADS1 genotype, whereas amounts of AA (20:4 n-6) and DPA (22:5 n-3) were significantly lower.

The lc n-3 PUFA concentrations in plasma and blood cells also tended to show higher concentrations in the derived allele carriers. This also indicates increased D5D activities in the derived allele carriers. We could not find any significance here except for n-3 DPA

contents in blood cells, with higher values in the derived allele carriers. The observed reduction in D5D product concentrations of n-3 LC PUFAs, compared to the significant differences in n-6 LC PUFAs among derived allele carriers, may result from an excess of available linoleic acid (LA).

Our findings regarding D5D index and n-6 PUFAs are in agreement with earlier studies. EPIC, a multicenter prospective cohort study analyzing 2,653 patients, the TT genotype at SNP rs174546 was inversely related to D5D activity (39). Another large study also observed that D5D activity was significantly lower among carriers of the rs174546 minor T allele. In addition, this study also indicated higher LA and DGLA concentrations in patients with the TT genotype, while lower concentrations were observed for AA (40). In the PREOBE cohort, women who carried the T allele at rs174546 had higher DGLA and lower AA levels (41). Furthermore, higher serum phospholipid concentrations of LA were observed in people with the T allele of rs174546, while the lower serum phospholipid levels were observed for AA (42).

However, contrary to earlier studies (15, 43–45), we did not observe that FADS1 gene variants were associated with significant differences in blood levels of n-3 PUFAs EPA or DHA. Interestingly, other data also indicate that genetic differences at the *FADS1* and *FADS2* loci probably have stronger effects on n-6 PUFAs than n-3 PUFAs (46). Having said that, these differences could also be due to differences in dietary consumption of n-3 PUFA, for which there is more variation than for n-6 PUFA in western diet between individuals with high or low fish consumption, which we did not monitor in our study.

Liver steatosis and fibrosis assessed by Fibroscan (47) were not associated with FADS1 allele variations, which is in contrast to a pediatric study indicating that the minor *FADS1* variant was associated with a higher degree of liver steatosis (19).

In order to expand knowledge and analyses regarding FADS1 genotype variations we also performed oxylipin analyses in plasma. While we did not observe significant differences between the groups, we found a trend toward higher thrombocyte AA-mediator formation in the derived group—which might indicate higher vascular risk and could fit with earlier observations that the derived FADS1 genotype is associated with increased cardiovascular risk (48, 49). Furthermore, we found a trend toward a lower plasma EPA/AA ratio in the derived group. At the same time n-3 PUFA derived epoxy metabolites that were identified as steatosis-protective factors in mouse experiments (50, 51), were lower in the ancestral group. This could be a mechanism involved in increased steatosis and metabolic risk that has been described for patients with the ancestral genotype (19, 21). Our data are not sufficient to prove these effects, however, they form the basis for hypotheses that now need to be tested in other studies.

Limiting factors of this observational study are mainly the heterogeneous group composed of a random selection of patients. In addition, the group of homozygous ancestral allele carriers is relatively small. In future studies it will be important to collect additional clinical data from patients, such as routine lipid parameters, blood pressure measurements and BMI, to further stratify risk constellation for steatosis hepatis and cardiovascular disease. Looking at the FADS1 gene alone is also a limitation. For example, Shetty et al. described an association of FADS2 gene polymorphisms with increased insulin resistance and type 2 diabetes (52). In the current cohort, diabetes mellitus status was not considered and thus the inclusion of this criterion is needed to better assess the risk constellation between diabetes mellitus and cardiovascular risk in MAFLD.

To further assess the effects of the minor, ancestral allele on PUFA and oxylipin concentrations, an increase in the study population is desirable.

5 Conclusion

In this pilot study in a routine clinical setting, we were able to confirm previous observations of significantly different blood fatty acid profiles depending on FADS1 genotype, with a lower D5D index in patients homozygous for the ancestral (minor) allele. Carriers of the derived genotype had a higher D5D index and higher AA concentrations and also showed a trend toward higher AA-derived thromboxane B2 and 12-HETE in blood plasma.

Data availability statement

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving humans were approved by Ethics committee of Brandenburg Medical School. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

MRa: Data curation, Methodology, Writing – review & editing, Investigation. ZW: Data curation, Visualization, Writing – original draft, Writing – review & editing. CL: Data curation, Writing – review & editing. JE: Data curation, Writing – review & editing, Conceptualization, Methodology, Supervision. MRo: Methodology, Writing – review & editing, Formal analysis, Investigation. AJ: Methodology, Writing – review & editing, Data curation. CS: Writing – review & editing. UE: Writing – review & editing. KW: Writing – review & editing, Conceptualization, Data curation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft. AP: Writing – review & editing, Investigation, Methodology, Project administration, Supervision.

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Conflict of interest

MRo is owner of Lipidomix GmbH.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The differential effects of eicosapentaenoic acid and docosahexaenoic acid on cardiovascular risk factors: an updated systematic review of randomized controlled trials

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Cardiovascular disease remains a major global health concern. The combination of the omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) has been shown to beneficially modify a range of cardiovascular risk factors. However, whether EPA and DHA have differential effects or potencies is currently unclear. A systematic review of randomized controlled trials (RCTs) that compared ≥2 g/day of near pure EPA and DHA was conducted. A total of 24 publications from nine unique RCTs were included. EPA and DHA both lower triglyceride levels, with DHA most likely having a slightly greater effect. Furthermore, both EPA and DHA increase high density lipoprotein (HDL) 2 cholesterol, which is cardioprotective, with the increase being greater with DHA. DHA appears to increase low density lipoprotein (LDL) cholesterol; however, DHA also increases LDL particle size, which would render LDL less atherogenic. DHA seems more effective than EPA in decreasing heart rate and blood pressure. Both EPA and DHA alter platelet function decreasing thrombogenicity, although they may have different actions on platelets. Both EPA and DHA decrease F2-isoprostanes, interpreted as a reduction in oxidative stress. They both decrease inflammatory gene expression and promote an anti-inflammatory oxylipin profile. These are all favorable effects with regard to cardiovascular disease risk. Effects of EPA and DHA on blood glucose are inconsistent. This review is constrained by the small number of high quality RCTs that directly compare EPA to DHA and report on outcomes other than blood lipids. There is a need for additional high-quality research to assess the independent effects of EPA and DHA on cardiovascular risk factors (e.g., inflammation, blood pressure, vascular function, platelet function) in larger and more diverse study populations.

KEYWORDS

blood lipids, triglycerides, cholesterol, blood pressure, inflammation

Introduction

Cardiovascular disease (CVD) remains the leading cause of mortality worldwide, with around 18.56 million deaths globally in 2019 (1). Of particular concern are the modifiable risk factors that contribute to CVD, which include (but are not limited to) elevated blood cholesterol and triglycerides, inflammation, hypertension, and diabetes. The omega-3 (n-3) polyunsaturated

fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have emerged as potential interventions to control these risk factors (2–4) resulting in cardioprotective properties (3–5). Although fish is the main dietary source of EPA and DHA, the study of these fatty acids has been helped by the ready availability of supplemental forms and their effects have most often been studied as the combination of EPA and DHA. This combination has been shown to lower triglycerides (6), blood pressure (7), inflammation (8–10) and heart rate (11, 12), to raise high-density lipoprotein (HDL) cholesterol (2), to improve vascular reactivity (13–15), and to reduce platelet reactivity and thrombosis (16). They may also raise low density lipoprotein (LDL) cholesterol (2) and fasting blood glucose (17). However, whether EPA and DHA have differential effects or potencies on risk factors for CVD is uncertain, in part because most trials have focussed on the effects of EPA and DHA when used in combination. It is of interest to know which is the more effective, EPA or DHA, and this information would be useful for regulators, industry and consumers. A small number of trials have been performed that directly compare the effects of pure EPA with pure DHA; these trials were subject to a systematic review published in 2018 (18) that focused on the differential effects of EPA and DHA using the findings from six randomized controlled trials (RCTs) reported in 18 publications (19–36). Knowing that there have been several more trials and publications on this topic published since then, this new systematic review aims to update the previous one and provide a comprehensive and up-to-date analysis of the current literature that specifically compares the effects of EPA and DHA on risk factors for CVD. While there is not a universally agreed-upon recommended dosage for EPA and DHA, this systematic review employed a strict inclusion criterion of ≥2 g per day due to evidence suggesting that this threshold shows favorable impacts on several relevant risk factors (37–39). Furthermore, trials included in this review needed to have used ≥90% of n-3 polyunsaturated fatty acids as either EPA or DHA in order to avoid, as best as possible, the biological effects of the "other" n-3 polyunsaturated fatty acid. These inclusion criteria are consistent with those of the previous systematic review (18).

Materials and methods

Literature search

This systematic review was conducted according to the principles of Preferred Reporting for Systematic Reviews and Meta-Analyses (PRISMA) (40). Searches were conducted in October 2023 in PubMed (2017 to October 2023), EMBASE (2017 to October 2023) and CINAHL (2017 to October 2023) databases. Earlier years were not searched because eligible publications up until 2017 had been identified in the previous systematic review (18). Search terms used included: "EPA," "DHA," "eicosapentaenoic acid," "docosahexaenoic acid" together with "blood lipid," "lipid," "triglyceride," "cholesterol," "LDL," "HDL," "lipoprotein," "blood pressure," "inflamm*," "interleukin-6," "IL-6," "C-reactive protein," "CRP," "vascular," "heart rate," "cardiovascular," "cardiometabolic." These search terms are consistent with those used in the previous systematic review (18). The full search strategies for the three databases are shown in Supplementary Table 1. This systematic review was not registered because it was conducted for educational purposes.

Publication selection

Publications had to meet the following criteria to be included in the qualitative synthesis: study must be in humans; study must compare pure or near pure EPA and DHA; study design must be a RCT; dose of EPA and DHA used must be ≥2 g per day; study has to include outcomes of interest (predefined risk factors for CVD); study must be published in the English language; study has to be available as full text. Publications from a previous systematic review (18) on the comparative effects of EPA and DHA on risk factors for CVD that included literature published up to 2017 were also included for qualitative synthesis as shown on the PRISMA flow chart (Figure 1). Publications which met the following criteria were excluded: animal studies; *in vitro* studies; dose of EPA and DHA used did not meet ≥2 g per day; outcomes of interest not stated or included; study was not an RCT; not published in the English language; study compared other interventions alongside EPA and DHA.

Data extraction

The data extracted for each trial included the study design, study population, sample size, dosage levels of EPA, DHA and placebo, study duration and relevant outcomes measured.

Quality assessment

The "parent" publication for each trial was assessed for methodological quality and validity using the Jadad scale (41). Risk of bias was assessed for each individual publication using the Cochrane Risk of Bias tool for RCTs (42).

Results

Identification of included publications

From the electronic literature searches, 1,566 publications of potential relevance were identified. Of these, 249 were removed due to being duplicates and 1,317 were assessed for eligibility based on title and abstract. From these, 1,290 were excluded due to not meeting the eligibility criteria. Based on the abstracts of the remaining 27 publications, 20 were excluded due to the following reasons: trials described in 14 publications administered EPA and DHA together, 1 trial utilized <2 g/day of EPA and DHA, trials reported in three publications administered EPA only, and two publications had already been recorded in the previous systematic review (35, 36). One of these latter publications included in the previous review (35) did not report outcomes of relevance and so was excluded. Hence, seven newly-identified publications were included for qualitative synthesis (43–49). Two of these newly-identified publications (43, 44) report additional results from a trial with publications included in the previous review. Five of the newly-identified publications (45–49) report results from three trials not included in the previous review. From the previous systematic review, 17 publications were also included (19–34, 36). Therefore, a total of 24 publications were included in this systematic review (Figure 1). A number of these publications were from the same

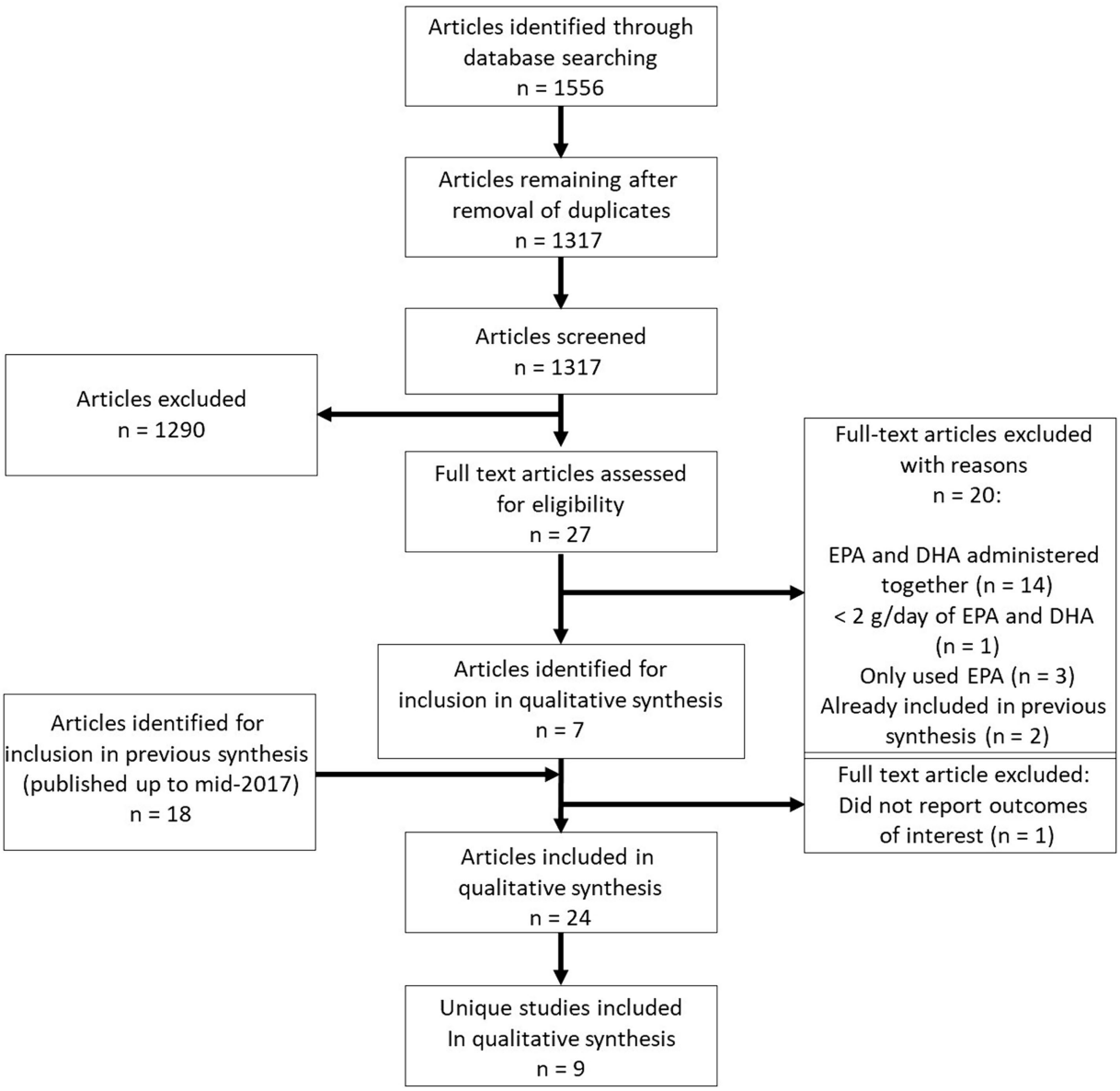

FIGURE 1
PRISMA flow chart showing selection of publications for inclusion in the systematic review.

trials; hence a total of nine unique RCTs were identified for inclusion; results from six unique RCTs were included in the previous review.

Characteristics of included studies

Table 1 summarizes the characteristics of the nine unique trials including design, population, sample size, dose of EPA, DHA and placebo used, duration, outcomes reported and Jadad score (based on the "parent" publication).

The sample size of the included trials varied between 21 and 224, with a total of 763 participants studied overall. Four trials included healthy participants and one, participants described as healthy with abdominal obesity and chronic inflammation. Other trials included those with overweight and hyperlipidaemia, with dyslipidaemia, with chronic inflammation or with diabetes and being treated for hypertension. The dosage of EPA and DHA given ranged from 2.8 to 4 g/day. The trial durations varied between 4 and 12 weeks (mean 7 weeks). All trials provided EPA, DHA, and placebo in capsules. Placebos used included olive oil ($n = 4$ trials), corn oil ($n = 2$), high oleic sunflower oil ($n = 1$), mixed oils ($n = 1$) and safflower oil ($n = 1$). Trial locations varied and included Australia ($n = 4$ trials), Canada ($n = 2$), USA ($n = 2$) and Norway ($n = 1$). Jadad scores, based on the "parent" publication, varied from 3 to 5, with 4 studies receiving the maximum score of 5. Reasons for not achieving the maximum score were lack of information on method of randomization and/or on method of blinding.

A summary of risk of bias, according to the Cochrane criteria for RCTs, is shown in Table 2. This analysis was completed for each individual publication because the details of different publications from the same trial (e.g., participant numbers) sometimes varied. Most publications, including all publications from 6 of the trials, had

TABLE 1 Summary of characteristics of the nine included studies.

Study	Country	Study design and population	Sample size	Dose of EPA/DHA/Placebo (g/day)	Duration (Weeks)	Outcomes	Jaded score based on the "parent" publication
Grimsgaard et al. (19, 20)	Norway	Double-blind, randomized controlled trial. Healthy men.	*n* = 75 (EPA) *n* = 72 (DHA) *n* = 77 (corn oil) *n* = 224 (total)	3.8 (EPA) 3.6 (DHA) 4.0 (corn oil)	7	Serum lipids (total cholesterol, LDL cholesterol, HDL cholesterol, ApoA1, ApoB, triglycerides) Haemodynamics (blood pressure, heart rate, left ventricular function)	5 (19)
Mori et al. (21–24), Mas et al. (25)	Australia	Double-blind, randomized controlled trial. Overweight mildly hyperlipidaemic men.	*n* = 19 (EPA) *n* = 17 (DHA) *n* = 20 (olive oil) *n* = 56 (total)	3.8 (EPA) 3.7 (DHA) 3.0 (olive oil)	6	Serum lipids (total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, LDL particle size) Oxidative stress markers (urinary and plasma F_2-isoprostanes) Glycaemic control (serum glucose, serum insulin) Haemodynamics (blood pressure, heart rate, vascular reactivity)	3 (21)
Nestel et al. (30)	Australia	Double-blind, randomized controlled trial. Dyslipidaemic subjects.	*n* = 12 (EPA) *n* = 12 (DHA) *n* = 14 (olive oil) *n* = 38 (total)	3.0 (EPA) 2.8 (DHA) 2.8 (olive oil)	7	Plasma lipids (total cholesterol, LDL cholesterol, HDL cholesterol, VLDL triglycerides, total triglycerides) Haemodynamics (blood pressure, pulse pressure, heart rate, total vascular resistance, systemic arterial compliance)	4 (30)
Woodman et al. (26–28), Mori et al. (29), Mas et al. (25)	Australia	Double-blind, randomized controlled trial. People with type-2 diabetes and treated for hypertension.	*n* = 17 (EPA) *n* = 18 (DHA) *n* = 16 (olive oil) *n* = 51 (total)	3.8 (EPA) 3.7 (DHA) 3.0 (olive oil)	6	Serum lipids (total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, LDL particle size) Haemodynamics (blood pressure) Glycaemic control (serum glucose, insulin, C-peptide, insulin sensitivity, insulin secretion) Plasma inflammatory markers (CRP, TNF-α, IL-6) Platelet, fibrinolytic and vascular function (collagen and PAF-stimulated platelet aggregation, collagen-stimulated thromboxane release, plasma tPA and PAI-1 antigen, von Willebrand factor, P-selectin, brachial artery dilatation) Oxidative stress markers (urinary and plasma F_2-isoprostanes)	3 (26)
Park and Harris (31, 32), Park et al. (33)	USA	Double-blind, randomized controlled trial. Healthy participants.	*n* = 11 (EPA) *n* = 11 (DHA) *n* = 11 (safflower oil) *n* = 33 (total)	3.8 (EPA) 3.8 (DHA) 4.0 (safflower oil)	4	Plasma lipids (total cholesterol, HDL cholesterol, LDL cholesterol, VLDL cholesterol, triglycerides, chylomicron size, ApoB48, ApoB100, chylomicron clearance) Plasma LPL and hepatic lipase activities Platelet count and platelet volume	3 (31)

(Continued)

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TABLE 1 (Continued)

Study	Country	Study design and population	Sample size	Dose of EPA/DHA/Placebo (g/day)	Duration (Weeks)	Outcomes	Jaded score based on the "parent" publication
Allaire et al. (34, 43, 44), Vors et al. (36)	Canada	Double-blind randomized controlled crossover study with 9-week washout. Healthy subjects with abdominal obesity and subclinical inflammation.	*n* = 121 (EPA) *n* = 123 (DHA) *n* = 125 (corn oil) *n* = 125 (total)	2.7 (EPA) 2.7 (DHA) 3.0 (corn oil)	10	Plasma lipids (total cholesterol, LDLcholesterol, HDL cholesterol, ApoB, triglycerides) Plasma triglyceride-lowering responders LDL particle size, % small-dense LDL PCSK9 *In vivo* kinetics of ApoB100-containing lipoproteins (in a subset of 19) Whole blood expression of lipid metabolism genes (HMGCoA reductase, LDLR, SREBP1c, SREBP2) (in a subset of 44) Plasma inflammatory markers (CRP, IL-6, IL-18, TNF-α, adiponectin) Whole blood expression of inflammatory genes (PPARA, TNFA, CD14, TRAF3, CCL2, IL10, IL1B, IL1RN, NFKB, TNFRSF1A) (in a subset of 44)	5 (34)
Klingel et al. (45), Lee et al. (46)	Canada	Double-blind, randomized controlled trial. Healthy men and women.	*n* = 29 (EPA) *n* = 30 (DHA) *n* = 30 (olive oil)	3.0 (EPA) 3.0 (DHA) 3.0 (olive oil)	12	Serum lipids (total cholesterol, HDL cholesterol, triglycerides) Serum glucose Serum LPL activity Marker of *de novo* lipogenesis Haemodynamics (blood pressure, heart rate, cardiac function)	5 (46)
So et al. (47, 48)	USA	Double-blind randomized controlled crossover study with 10-week washout. Men and postmenopausal women (age 50–75 years) with chronic inflammation.	*n* = 21 (EPA) *n* = 21 (DHA) *n* = 21 (high-oleic sunflower oil)	3.0 (EPA) 3.0 (DHA) 3.0 (high-oleic sunflower oil)	10	Plasma lipids (total cholesterol, LDL cholesterol, HDL cholesterol, ApoB, ApoA1, triglycerides) Plasma activities of LPL, CETP and LCAT Plasma inflammatory markers (CRP, TNF-α, IL-6, MCP-1, IL-10) Cytokine gene expression by LPS-stimulated blood monocytes (TNFA, IL6, MCP1, IL10) Plasma oxylipin profile	4 (47)
Pisaniello et al. (49)	Australia	Double-blind randomized controlled trial. Healthy participants.	*n* = 10 (EPA) *n* = 10 (DHA) *n* = 10 (fish oil concentrate) *n* = 10 (palm oil, sunflower oil, rapeseed oil, and fish oil)	4.0 (EPA) 4.0 (DHA) 4.0 (fish oil or mixed oils)	4	Serum lipids (total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides) Serum inflammatory marker (CRP) Effect of serum on TNF-stimulated inflammatory gene expression in endothelial cells (ICAM, VCAM, CCL2, NFKB subunit) Haemodynamics (blood pressure, heart rate)	5 (49)

Apo, apolipoprotein; CCL, chemokine (C-C motif) ligand; CETP, cholesteryl ester transfer protein; CRP, C-reactive protein; HDL, high density lipoprotein; HMGCoA, hydroxymethylglutaryl coenzyme A; ICAM, intercellular adhesion molecule; IL, interleukin; LCAT, lecithin cholesterol acyl transferase; LDL, low density lipoprotein; LDLR, low density lipoprotein receptor; LPL, lipoprotein lipase; LPS, lipopolysaccharide; MCP, monocyte chemoattractant protein; NFKB, nuclear factor kappa-light-chain-enhancer of activated B cells; PAF, platelet activating factor; PAI, plasminogen activator inhibitor; PCSK9, proprotein convertase subtilisin/kexin type 9; PPAR, peroxisome proliferator activated receptor; SREBP, sterol receptor element binding protein; TNF, tumor necrosis factor; TNFRSF, tumor necrosis family receptor superfamily; tPA, tissue plasminogen activator; TRAF, tumor necrosis factor receptor associated factor; VCAM, vascular cell adhesion molecule; VLDL, very low density lipoprotein.

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TABLE 2 Risk of bias assessment for each individual publication.

Study	Bias arising from the randomization process	Bias due to deviations from intended intervention	Bias due to missing outcome data	Bias in measurement of the outcome	Bias in selection of the reported result	Overall bias	Comments
Grimsgaard et al. (19)	🟢	🟢	🟢	🟢	🟢	🟢	
Grimsgaard et al. (20)	🟢	🟢	🟢	🟢	🟢	🟢	
Mori et al. (21)	🟢	🟢	🟢	🟢	🟢	🟢	
Mori et al. (22)	🟢	🟢	🟢	🟢	🟢	🟢	40 participants from the 59 randomized and 56 completers agreed to the experimental procedure
Mori et al. (23)	🟢	🟢	🟢	🟢	🟢	🟢	
Mori et al. (24)	🟢	🟢	🟡	🟢	🟡	🟡	Baseline values for the outcome are not provided. It is not stated how many participants' data are reported (i.e., sample size is not specified). It is not stated whether this outcome was pre-specified.
Mas et al. (25)	🟢	🟢	🟡	🟢	🟡	🟡	It is not stated how many participants' data are reported (i.e., sample size is not specified). It is not stated whether this outcome was pre-specified.
Woodman et al. (26)	🟢	🟢	🟢	🟢	🟢	🟢	
Woodman et al. (27)	🟢	🟢	🟢	🟢	🟢	🟢	
Woodman et al. (28)	🟢	🟢	🟡	🟢	🟢	🟡	It is not stated how many participants' data are reported (i.e., sample size is not specified).
Mori et al. (29)	🟢	🟢	🟢	🟢	🟢	🟢	
Nestel et al. (30)	🟢	🟢	🟢	🟢	🟢	🟢	
Park and Harris (31)	🟢	🟢	🟢	🟢	🟢	🟢	
Park and Harris (32)	🟢	🟢	🟢	🟢	🟢	🟢	
Park et al. (33)	🟢	🟢	🟢	🟢	🟢	🟢	

(Continued)

TABLE 2 (Continued)

Study	Bias arising from the randomization process	Bias due to deviations from intended intervention	Bias due to missing outcome data	Bias in measurement of the outcome	Bias in selection of the reported result	Overall bias	Comments
Allaire et al. (34)	●	●	●	●	●	●	
Allaire et al. (43)	●	●	●	●	●	●	Completed in a subset of the first 20 participants
Allaire et al. (44)	●	●	●	●	●	●	It is not stated how the sub-set of 44 participants for gene expression analysis was selected
Vors et al. (36)	●	●	●	●	●	●	It is not stated how the sub-set of 44 participants was selected
Lee et al. (46)	●	●	●	●	●	●	
Klingel et al. (45)	●	●	●	●	●	●	
So et al. (47)	●	●	●	●	●	●	
So et al. (48)	●	●	●	●	●	●	
Pisanello et al. (49)	●	●	●	●	●	●	

Green circle = low risk of bias; Amber circle = some concerns.

a low risk of bias. Reasons for concern are listed, where relevant, in Table 2.

Effects of EPA and DHA on the concentration of EPA and DHA in blood pools

Table 3 summarizes the publications that report on the EPA and DHA concentration in different blood pools. Most publications report fatty acids as % of total fatty acids in the pool, although one reports absolute concentration (19). Of the 12 publications listed, six report fatty acids in plasma or plasma or serum phospholipids (19, 21, 26, 30, 34, 48), while three report fatty acids in platelets (23, 29, 31), two - both from the same trial - in erythrocytes (45, 46) and one in whole blood (49). Supplementing with near pure EPA increases the EPA content of all pools reported on, while supplementing with near pure DHA increases the DHA content of all pools reported on (Table 3). Furthermore, most studies report that supplementing near-pure DHA increases the EPA content of the pools reported on. However, effects of near-pure EPA on DHA concentration are inconsistently observed, with some studies observing a decrease in DHA (19, 23, 26, 29), others little or no change (21, 30, 31, 34, 45, 46, 48, 49). No studies report a significant increase in DHA when EPA is supplemented.

Comparative effects of EPA and DHA on cardiovascular risk factors

Effect of EPA vs. DHA on blood lipids and lipoproteins

All nine trials (13 publications) (19, 23, 26, 28, 30, 32–34, 43–45, 48, 49) included outcomes related to the effect of EPA and DHA on blood lipids (Table 4).

The study of Grimsgaard et al. (19) in healthy men, found that both EPA (3.8 g/day) and DHA (3.6 g/day) for 7 weeks led to significant reductions in triglycerides (21 and 26%, respectively) compared to corn oil. Moreover, EPA demonstrated additional benefits by lowering total cholesterol, ApoA1 and ApoB, while DHA increased HDL cholesterol. Both EPA and DHA decreased the ratio of total to HDL cholesterol. Compared to EPA, DHA increased HDL cholesterol and showed a greater decrease in triglycerides, although the latter effect was not statistically significant.

The Mori et al. (23) study involving overweight mildly hyperlipidaemic men using 3.8 g/day of EPA or 3.7 g/day of DHA for 6 weeks demonstrated a decrease in triglycerides for both EPA and DHA (−18% and −20%, respectively) compared to olive oil. EPA also decreased the HDL3 cholesterol subfraction by 7%, while DHA increased LDL cholesterol by 8%, HDL2 cholesterol by 29% and LDL particle size. EPA did not affect total, LDL, HDL or HDL2 cholesterol

TABLE 3 Summary of the effects of EPA and DHA supplementation on blood and cell levels of these fatty acids.

Study	Study population	Dose of EPA or DHA (g/day)	Duration (Weeks)	Where fatty acids measured	Units	Group	EPA before supplementation	EPA after supplementation	DHA before supplementation	DHA after supplementation
Grimsgaard et al. (19)	Healthy men	3.8 (EPA) 3.6 (DHA)	7	Serum phospholipids	μmol/L	EPA	61.4	+182.1	184.0	−28.0
						DHA	59.8	+17.6	185.0	+128.0
Mori et al. (21)	Overweight mildly hyperlipidaemic men	3.8 (EPA) 3.7 (DHA)	6	Plasma phospholipids	%	EPA	1.7	9.8	4.1	4.0
						DHA	1.5	2.3	4.0	10.9
Mori et al. (23)	Overweight mildly hyperlipidaemic men	3.8 (EPA) 3.7 (DHA)	6	Platelets	%	EPA	~0.1	~3.6	~0.2	~ − 0.7
						DHA	~0.1	~0.6	~0.2	~4.3
Nestel et al. (30)	Dyslipidaemic subjects	3.0 (EPA) 2.8 (DHA)	7	Plasma	%	EPA	1.6	9.0	NR	NR but no change
						DHA	1.1	2.7	2.2	7.2
Woodman et al. (26)	People with type-2 diabetes and treated for hypertension	3.8 (EPA) 3.7 (DHA)	6	Plasma phospholipids	%	EPA	1.6	~10.2 (+540%)	4.3	~4.0 (−7%)
						DHA	1.7	~2.8 (+64%)	4.3	~11.0 (+156%)
Mori et al. (29)	People with type-2 diabetes and treated for hypertension	3.8 (EPA) 3.7 (DHA)	6	Platelets	%	EPA	1.0	4.8	2.3	1.7
						DHA	1.0	1.5	2.4	5.2
Park and Harris (31)	Healthy participants	3.8 (EPA) 3.8 (DHA)	4	Platelets	%	EPA	0.2	3.3	1.5	1.6
						DHA	0.4	0.4	1.4	4.1
Allaire et al. (34)	Healthy subjects with abdominal obesity and subclinical inflammation	2.7 (EPA) 2.7 (DHA)	10	Plasma phospholipids	%	EPA	NR but 1.1 in control group	6.0	NR but 3.3 in control group	3.3
						DHA	NR but 1.1 in control group	2.1	NR but 3.3 in control group	8.1
Klingel et al. (45), Lee et al. (46)	Healthy men and women	3.0 (EPA) 3.0 (DHA)	12	Erythrocytes	%	EPA	0.5	3.9	3.0	2.5
						DHA	0.5	1.2	2.9	7.2
So et al. (48)	Men and postmenopausal women (age 50–75 years) with chronic inflammation	3.0 (EPA) 3.0 (DHA)	10	Plasma phospholipids	%	EPA	0.7	5.3	2.8	3.1
						DHA	0.7	1.6	2.8	7.7
Pisaniello et al. (49)	Healthy participants	4.0 (EPA) 4.0 (DHA)	4	Whole blood	%	EPA	0.8	~2.8 (+253%)	1.9	NR but no change
						DHA	0.5	NR but no change	1.7	~4.2 (+145%)

NR, not reported.

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TABLE 4 Summary of findings related to effects of EPA and DHA on blood lipids and lipoproteins.

Study	Population	Control	Effect of EPA vs. control on blood lipids and lipoproteins	Effect of DHA vs. control on blood lipids and lipoproteins	Effect of EPA vs. DHA on blood lipids and lipoproteins
Grimsgaard et al. (19)	Healthy men	Corn oil	↓ Triglycerides (−21%, $p = 0.0001$) ↓ Total cholesterol (−0.15 ± 0.55 mmol/L, $p < 0.05$) ↓ ApoA1 (−0.04 ± 0.10 g/L, $p < 0.001$) ↓ ApoB (−0.03 ± 0.11 g/L, $p < 0.05$) ↑ HDL:ApoA1 (+0.04 ± 0.08, $p = 0.0001$) ↓ Total:HDL cholesterol (−0.13 ± 0.47, $p = 0.007$)	↓ Triglycerides (−26%, $p = 0.0001$) ↑ HDL cholesterol (+0.06 ± 0.13 mmol/L, $p < 0.001$) ↑ HDL:ApoA1 (+0.04 ± 0.07, $p < 0.001$) ↓ Total/HDL cholesterol (−0.19 ± 0.52, $p < 0.01$)	EPA ↓ total cholesterol and ApoA1 more than DHA DHA ↑ HDL cholesterol more than EPA ($p = 0.009$)
Mori et al. (23)	Overweight mildly hyperlipidaemic men	Olive Oil	↓ Triglycerides (−18%, $p = 0.012$) ↓ HDL_3 cholesterol (−7%, $p = 0.032$) No effect on total, HDL, HDL_2 or LDL cholesterol No effect on LDL particle size	↓ Triglycerides (−20%, $p = 0.003$) ↑ LDL cholesterol (+8%, $p = 0.019$) ↑ LDL particle size (+0.25 ± 0.08 nm, $p = 0.002$) ↑ HDL_2 cholesterol (+29%, $p = 0.004$) No effect on total, HDL or HDL_3 cholesterol	N/A
Woodman et al. (26, 28)	People with type-2 diabetes treated for hypertension	Olive oil	↓ Triglycerides (−19%, $p = 0.022$) ↑ HDL_2 cholesterol (+16%, $p = 0.026$) ↓ HDL_3 cholesterol (−11%, $p = 0.026$) No effect on total, LDL- or HDL cholesterol No effect on LDL particle size	↓ Triglycerides (−15%, $p = 0.022$) ↑ HDL_2 cholesterol (+12%, $p = 0.05$) No effect on total, LDL, HDL or HDL_3 cholesterol ↑ LDL particle size (+0.26 ± 0.10 nm, $p = 0.02$)	N/A
Nestel et al. (30)	Dyslipidaemic subjects	Olive oil	↓ Total triglycerides (−23%, $p = 0.026$) ↓ VLDL triglycerides ($p = 0.006$) No effect on total, HDL or LDL cholesterol	↓ Total triglycerides (−32%, $p = 0.026$) ↓ VLDL triglycerides ($p = 0.006$) No significant difference in total, HDL or LDL cholesterol	No significant difference between EPA and DHA
Park and Harris (32), Park et al. (33)	Healthy subjects	Safflower oil	No effect on plasma lipids (total, LDL-, HDL- or VLDL cholesterol, triglycerides) ↓ ApoB48 after an oral fat challenge ($p < 0.001$) ↓ ApoB100 after an oral fat challenge ($p < 0.01$) ↓ Chylomicron triglyceride half-life (fed state) ($p < 0.05$) ↓ Chylomicron particle size (−53%, $p < 0.01$) ↑ Pre-heparin LPL activity (47%, $p < 0.05$); no effect on post-heparin LPL activity or hepatic lipase activity ↑ Margination volume in the fasted state ($p < 0.001$)	No effect on plasma lipids (total, LDL-, HDL- or VLDL cholesterol, triglycerides) ↓ ApoB48 after an oral fat challenge (−28%, $p < 0.001$) ↓ ApoB100 after an oral fat challenge (−24%, $p < 0.01$) ↓ Chylomicron triglyceride half-life (fed state) ($p < 0.05$) ↓ Chylomicron particle size (−24%, $p < 0.01$) ↑ Pre-heparin LPL activity (73%, $p < 0.05$); no effect on post-heparin LPL activity or hepatic lipase activity ↑ Margination volume in the fasted state ($p < 0.001$) ↑ Margination volume in the fed state ($p < 0.05$)	No difference between EPA and DHA

(Continued)

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TABLE 4 (Continued)

Study	Population	Control	Effect of EPA vs. control on blood lipids and lipoproteins	Effect of DHA vs. control on blood lipids and lipoproteins	Effect of EPA vs. DHA on blood lipids and lipoproteins
Allaire et al. (34, 43, 44)	Healthy subjects with abdominal obesity and subclinical inflammation	Corn Oil	↓ Triglycerides (−12%, $p < 0.0001$) ↑ LDL cholesterol (+2%, $p = 0.046$) ↓ mean LDL particle size ↑ Proportion of small dense LDL ↓ PCSK9 concentrations ↑ VLDL ApoB100 fractional catabolism rate ↓ LDL ApoB100 fractional catabolism rate	↓ Triglycerides (−13%, $p < 0.0001$) ↑ Total cholesterol (+4%, $p = 0.001$) ↑ LDL cholesterol (+7%, $p < 0.0001$) ↑ HDL cholesterol (+8%, $p < 0.0001$) ↓ Cholesterol/HDL cholesterol ratio (−3%, $p < 0.001$) ↑ ApoB (+5%, $p = 0.02$) ↑ mean LDL particle size ↓ Proportion of small dense LDL ↓ PCSK9 concentrations ↑ VLDL ApoB100 fractional catabolism rate	Compared to EPA, DHA ↓ Plasma triglycerides ($p = 0.005$) ↑ Plasma total cholesterol ($p < 0.001$) ↑ Plasma LDL cholesterol ($p = 0.04$), more so in men than women ($p = 0.046$) ↑ Plasma HDL cholesterol ($p < 0.0001$) ↓ Plasma cholesterol/HDL cholesterol ratio ($p = 0.006$) ↑ LDL particle size (+0.7 Å; $p < 0.001$) ↓ The proportion of small dense LDL (−3.2%; $p < 0.01$) ↑ LDL ApoB100 fractional catabolic rate (+11.4%; $p = 0.008$) and the production rate (+9.4%; $p = 0.03$). ↑ Proportion of responders where plasma triglyceride concentrations reduced by >0·25 mmoL/L (45 vs. 32%, $p < 0·001$).
Klingel et al. (45)	Healthy men and women	Olive oil	No effect on serum triglycerides, total and HDL cholesterol ↑ Lipogenic index and *de novo* lipogenesis Trend for ↑ serum LPL activity	↓ Serum triglycerides No effect on serum total and HDL cholesterol No effect on lipogenic index or *de novo* lipogenesis ↑ Serum LPL activity	N/A
Pisaniello et al. (49)	Healthy adults	Mixed oils	No effect on serum total, HDL or LDL cholesterol or triglycerides	No effect on serum total, HDL or LDL cholesterol ↓ Serum triglycerides by an average of 0.31 mmoL/L (−27%, $p = 0.02$)	No differences
So et al. (48)	Older men and postmenopausal women with chronic inflammation	Sunflower oil	↓ Plasma triglycerides No effect on plasma total, HDL, LDL and non-HDL cholesterol, ApoA1 and ApoB	↓ Plasma triglycerides ↑ Plasma LDL No effect on plasma total, HDL and non-HDL cholesterol, ApoA1 and ApoB	No differences

Apo, apolipoprotein; HDL, high density lipoprotein; LDL, low density lipoprotein; LPL, lipoprotein lipase; PCSK9, proprotein convertase subtilisin/kexin type 9; VLDL, very low density lipoprotein. N/A indicates that a statistical comparison between the effects of EPA and DHA was not made.

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or LDL particle size, while DHA did not affect total, HDL or HDL3 cholesterol.

In the Woodman et al. (26, 28) study on hypertensive diabetics, EPA (3.8 g/day) or DHA (3.7 g/day) for 6 weeks had no impact on total, LDL or HDL cholesterol. However, there was a significant decrease in triglycerides with both EPA and DHA (−19% and −15%, respectively) and there was an elevation in HDL2 (+16% and +12%, respectively). EPA decreased HDL3 (−11%) but DHA had no effect. DHA but not EPA increased LDL particle size.

In the Nestel et al. (30) study involving dyslipidaemic patients that lasted 7 weeks, there was no observed change in total, LDL or HDL cholesterol levels with either EPA or DHA (3.0 and 2.8 g/day, respectively) compared to olive oil. However, both EPA and DHA were found to lower triglycerides (−23% and −32%, respectively) and VLDL triglycerides, although there was no significant difference between the effects of the two fatty acids.

Surprisingly, in the Park and Harris study (32, 33), neither EPA nor DHA, at a dosage of 3.8 g/day for 4 weeks, had an impact on triglycerides or on total, LDL, HDL or VLDL cholesterol. This may be because of the short duration of this trial. Nevertheless, there was a significant reduction in ApoB48 and ApoB100 after a high fat challenge, suggesting more efficient handling of dietary fat. In agreement with this, both EPA and DHA increased chylomicron clearance (shorter half-life) and decreased chylomicron particle size when compared to safflower oil. Furthermore, lipoprotein lipase (LPL) activity increased following supplementation of either EPA or DHA, perhaps explaining the faster clearance of chylomicrons. The margination volume, which indicates the extent to which triglyceride-rich lipoproteins adhere to endothelial LPL, was increased with both EPA and DHA (+64% and +53%, respectively) in the fasted state and with DHA in the fed state, consistent with more rapid triglyceride clearance.

The Allaire et al. (34, 43, 44) study conducted in healthy subjects with subclinical inflammation and abdominal obesity, showed that both EPA and DHA at 2.7 g/day for 10 weeks lowered triglycerides (−12% and −13%, respectively), and increased LDL cholesterol (+2% and +7%, respectively), compared to corn oil. The proportion of responders where there was a reduction in plasma triglyceride concentrations by >0.25 mmoL/L was greater with DHA than EPA (45 and 32%, respectively), although the average magnitude of triglyceride reduction was similar between DHA and EPA (0.59 and 0.57 mmol/L, respectively). Compared to control, DHA increased total cholesterol by 4%, increased HDL cholesterol by 8%, lowered the cholesterol/HDL ratio by 3% and increased ApoB by 5%. EPA did not have those effects. Compared to EPA, DHA resulted in a decrease in triglycerides, an increase in total cholesterol and LDL cholesterol (this was more prominent in men than women), an increase in HDL cholesterol and a decrease in the cholesterol/HDL cholesterol ratio. EPA decreased mean LDL particle size, while DHA increased it. Compared to EPA, DHA increased mean LDL particle size and decreased the proportion of small dense LDL by 3.2%. EPA and DHA both decreased PCSK9 concentrations (18.2 and 25% respectively). Furthermore, compared to EPA, DHA increased both the LDL ApoB100 production rate and the fractional catabolic rate.

Klingel et al. (45), found that DHA (3 g/day for 12 weeks) decreased serum triglycerides (from 0.85 ± 0.04 mmol/L to 0.65 ± 0.03 mmol/L) in healthy adults but EPA at the same dose did not. Neither EPA nor DHA significantly affected total or HDL cholesterol in this trial. Both EPA and DHA led to similar increases in serum LPL activity (from 44.1 to 49.1 mU/ml and from 42.9 to 51.5 mU/ml). In another trial involving healthy adults (49), DHA (4 g/day) was found to decrease triglycerides by an average of 27%, but there was no effect on total, LDL or HDL cholesterol or effect of EPA on blood lipids. Overall, there was no difference in effects of EPA and DHA on blood lipids including triglycerides. The duration of this study was only 4 weeks, which is shorter than other trials that show greater effects.

So et al. (48) found that both EPA and DHA (3.0 g/day for 10 weeks) decreased plasma triglyceride concentrations (−20% and −22%, respectively) in older adults with inflammation without changes in ApoB concentrations, resulting in a significant reduction in triglyceride/ApoB ratio with both EPA and DHA. EPA did not affect total, HDL, LDL or non-HDL cholesterol, while DHA raised LDL cholesterol. The LDL-C/ApoB and HDL-C/ApoA1 ratios were increased with both EPA and DHA but the increase of the HDL-C/ApoA1 ratio was greater with DHA. This study also reported the effects of EPA and DHA on the activities of LPL, cholesteryl ester transfer protein (CETP) and lecithin cholesterol acyl transferase (LCAT). These were all affected by both EPA and DHA but in a sex-specific way. DHA increased LPL activity and decreased LCAT activity with the latter only seen in women. EPA decreased CETP and LCAT activity with the decrease in LCAT again seen only in women.

Effect of EPA vs. DHA on inflammatory markers

Four trials (five publications) (29, 34, 36, 47, 49) included outcomes related to the effect of EPA and DHA on inflammatory markers (Table 5). The Allaire et al. (34)/Vors et al. (36) study observed a significant reduction in plasma levels of CRP, IL-6, IL-18 and TNF-α (−8, −12, −7, and −15% respectively) and an increase in adiponectin (3%) with DHA supplementation (2.7 g/day) compared to corn oil. In contrast, EPA at the same dose only decreased plasma IL-6 (−13%). Compared to EPA, DHA resulted in a greater decrease in IL-18 and a greater increase in adiponectin. Both EPA and DHA decreased CD14 gene expression and increased PPARA gene expression. EPA also increased expression of the TRAF3 gene, while DHA increased expression of the TNFA gene. In contrast to some of these findings, Mori et al. (29) saw no effect of EPA (3.8 g/day) or DHA (3.7 g/day) on plasma CRP or IL-6, while both EPA and DHA tended to decrease plasma TNF-α, with DHA having a greater effect, although this was not formally tested statistically. So et al. (47) reported no effect of EPA or DHA (3 g/day) on plasma CRP, TNF-α, IL-6, MCP-1 or IL-10 and no difference in effect of EPA and DHA on these biomarkers of inflammation. DHA decreased monocyte secretion of TNF-α, IL-6 and MCP-1 in response to lipopolysaccharide and decreased expression of the TNFA, IL6, MCP1 and IL10 genes. EPA only decreased expression of the TNFA gene. Both EPA and DHA modified the plasma oxylipin profile; both decreased several arachidonic acid (AA)-derived oxylipins. EPA increased several EPA-derived oxylipins while DHA increased several DHA- and EPA-derived oxylipins. Finally, Pisaniello et al. (49) reported no effect of EPA or DHA (4 g/day) on serum CRP in healthy adults. Serum from those supplemented with EPA decreased CCL2 gene expression in endothelial cells. Other genes were unaffected and serum from those supplemented with DHA had no effect on any genes.

Effect of EPA vs. DHA on blood pressure, haemodynamics, and vascular function

Six trials (eight publications) (20–22, 26, 27, 30, 46, 49) included outcomes related to effects of EPA and DHA on blood pressure, haemodynamics and vascular function (Table 6). Grimsgaard et al. (20)

TABLE 5 Summary of findings related to effects of EPA and DHA on inflammatory markers.

Study	Population	Control	Effect of EPA vs. control	Effect of DHA vs. control	Effect of EPA vs. DHA
Mori et al. (29)	People with type-2 diabetes and treated for hypertension	Olive oil	No effect on plasma IL-6 and CRP Trend for ↓ plasma TNF-α (−19.5%, n.s.)	No effect on plasma IL-6 and CRP Trend for ↓ plasma TNF-α (−32.8%, n.s.)	N/A
Allaire et al. (34), Vors et al. (36)	Healthy subjects with abdominal obesity and low-grade inflammation	Corn oil	No effect on plasma CRP, IL-18, TNF-α or adiponectin ↓ Plasma IL-6 (−13%, $p = 0.03$) ↓ CD14 gene expression ($p = 0.008$) ↑ PPARA ($p = 0.003$) and TRAF3 gene expression ($p = 0.002$)	↓ Plasma CRP (−8%, $p = 0.02$), TNF-α (−15%, $p = 0.01$), IL-6 (−12%, $p = 0.01$) and IL-18 (−7%, $p = 0.002$) ↑ Plasma adiponectin (+3%, $p = 0.047$) ↓ CD14 gene expression ($p = 0.02$) ↑ PPARA ($p = 0.01$) and TNFA gene expression ($p = 0.01$)	Compared to EPA, DHA ↓ IL-18 ($p = 0.01$) and ↑ adiponectin (<0.001) No difference for CRP, IL-6, and TNF-α or for gene expression
Pisaniello et al. (49)	Healthy adults	Mixed oils	No effect on serum CRP Serum from EPA supplementation ↓ CCL2 gene expression by endothelial cells (−25%, $p = 0.03$); other genes unaffected	No effect on serum CRP No effect of serum from DHA supplementation on inflammatory gene expression by endothelial cells	N/A
So et al. (47)	Older men and postmenopausal women with chronic inflammation	Sunflower oil	No effect on plasma CRP, TNF-α, IL-6, MCP-1 or IL-10 ↓ LPS-induced monocyte TNFA gene expression but no effect on IL6, MCP1 or IL10 ↓ ratio of LPS induced monocyte TNF to IL10 and MCP1 to IL10 gene expression ↑ plasma EPA-derived oxylipins	No effect on plasma CRP, TNF-α, IL-6, MCP-1 or IL-10 ↓ LPS induced monocyte secretion of TNF-α, IL-6 and MCP-1 ↓ LPS induced monocyte TNFA, IL6, MCP1 and IL10 gene expression ↑ plasma DHA-derived oxylipins	No difference for plasma CRP, TNF-α, IL-6, MCP-1 or IL-10 DHA ↓ LPS induced monocyte IL10 gene expression compared with EPA Different plasma oxylipin profiles

CCL, chemokine (C-C motif), ligand; CRP, C-reactive protein; IL, interleukin; LPS, lipopolysacharide; MCP, monocyte chemoattractant protein; PPAR, peroxisome proliferator activated receptor; TNF, tumor necrosis factor; TRAF, tumor necrosis factor receptor associated factor. N/A indicates that a statistical comparison between the effects of EPA and DHA was not made.

reported an increase in heart rate (+1.9 bpm) with EPA (3.8 g/day) but a decrease in heart rate (−2.2 bpm) with DHA (3.6 g/day) in healthy men. When directly compared with each other, DHA resulted in a decreased heart rate compared to EPA. Both EPA and DHA improved left ventricular diastolic filling but there was found to be no significant effect of either EPA or DHA on blood pressure. In the Mori et al. (21, 22) study in overweight mildly hyperlipidaemic men, DHA (3.7 g/day) decreased both systolic and diastolic blood pressure compared to placebo. However, EPA (3.8 g/day) was found to have no significant effect on blood pressure. DHA decreased heart rate by around 3.5 bpm over a 24-h period compared to placebo. Furthermore, DHA increased vasodilator responses and attenuated constrictor responses in forearm blood flow compared to placebo. In the Nestel et al. (30) study in dyslipidaemic subjects, there was no effect of EPA (3 g/day) or DHA (2.8 g/day) on heart rate or blood pressure. However, there was an increase in systemic arterial compliance with EPA (+36%) and DHA (+27%) compared to placebo. There was also a non-significant lowering of vascular resistance with both EPA and DHA. There was no significant difference between the effects of EPA and DHA for any of the parameters. In the study of anti-hypertensive-treated type 2 diabetics by Woodman et al. (26, 27), there was no significant effect of EPA (3.8 g/day) or DHA (3.7 g/day) on blood pressure or vascular function. However, there was a non-significant decrease in 24 h heart rate with both EPA and DHA. In a study of healthy young men and women, Lee et al. (46) reported an increase in heart rate (4.2 beats/min) and systolic and diastolic blood pressure with EPA compared to the olive oil placebo, but no effect of DHA (both at 3 g/day); effects of EPA were different from those of DHA. DHA, but not EPA, increased cardiac muscle sympathetic nerve activity burst frequency and incidence. Finally, Pisaniello et al. (49) reported no effect of EPA (4 g/day) on heart rate or

TABLE 6 Summary of findings related to effect of EPA and DHA on blood pressure, haemodynamics and vascular function.

Study	Population	Control	Effect of EPA vs. control	Effect of DHA vs. control	Effect of EPA vs. DHA
Grimsgaard et al. (20)	Healthy adult men	Corn oil	↑ Heart rate (increased 1.9 bpm, $p = 0.04$) No effect on systolic or diastolic blood pressure Improved left ventricular diastolic filling	↓ Heart rate (decreased 2.2 bpm, $p = 0.006$) No effect on systolic or diastolic blood pressure Improved left ventricular diastolic filling	DHA ↓ heart rate compared with EPA ($p = 0.0001$)
Mori et al. (21, 22)	Overweight mildly hyperlipidaemic men	Olive oil	No significant effect on blood pressure Small nonsignificant rise in heart rate No effect on vasodilator or constrictor responses of forearm blood flow	↓ 24 h systolic (−5.8 mmHg, p = 0.022) and diastolic (−3.3 mmHg, $p = 0.029$) blood pressure ↓ daytime systolic (−3.5 mmHg, $p = 0.041$) and diastolic (−2.0 mmHg, p = 0.046) blood pressure ↓ 24 h (−3.5 bpm, p = 0.001), daytime (−3.7 bpm, p = 0.001), night-time (−2.8 bpm, $p = 0.025$) ambulatory heart rate ↑ vasodilator responses and ↓ constrictor responses of forearm blood flow	N/A
Woodman et al. (26, 27)	People with type-2 diabetes treated for hypertension	Olive oil	No effect on systolic or diastolic blood pressure Nonsignificant decrease in 24 h heart rate No effect on flow-mediated dilatation or glyceryl-trinitrate mediated dilatation	No effect on systolic or diastolic blood pressure Nonsignificant decrease in 24 h heart rate No effect on flow-mediated dilatation or glyceryl-trinitrate mediated dilatation	N/A
Nestel et al. (30)	Dyslipidaemic subjects	Olive Oil	No effect on heart rate No effect of systolic or diastolic blood pressure No effect on pulse pressure ↑ Systemic arterial compliance (+36%, $p = 0.028$) Trend to decrease total vascular resistance	No effect on heart rate No effect of systolic or diastolic blood pressure No effect on pulse pressure ↑ Systemic arterial compliance (+27%, $p = 0.091$) Trend to decrease total vascular resistance	No differences between EPA and DHA
Lee et al. (46)	Healthy young adults	Olive Oil	↑ Heart rate (4.2 bpm, $p = 0.04$) ↑ Systolic blood pressure ($p = 0.01$) ↑ Diastolic blood pressure ($p = 0.002$) No effect on cardiac muscle sympathetic nerve activity burst frequency and burst incidence	No effect on heart rate No effect on systolic or diastolic blood pressure ↑ Cardiac muscle sympathetic nerve activity burst frequency ($p = 0.001$) and burst incidence ($p = 0.003$)	EPA ↑ heart rate compared with DHA (p = 0.05) EPA ↑ systolic blood pressure compared with DHA ($p = 0.008$) EPA ↑ diastolic blood pressure compared with DHA ($p = 0.04$) DHA ↑ cardiac muscle sympathetic nerve activity burst frequency (p = 0.02) and burst incidence ($p = 0.058$) compared to EPA
Pisaniello et al. (49)	Healthy adults	Mixed oils (palm oil, sunflower oil, rapeseed oil, and fish oil)	No effect on heart rate No effect on systolic blood pressure No effect on diastolic blood pressure	No effect on heart rate No effect on systolic blood pressure ↓ Diastolic blood pressure (−4.1 ± 1.8 mmHg, p = 0.05)	N/A

N/A indicates that a statistical comparison between the effects of EPA and DHA was not made.

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TABLE 7 Summary of findings related to effects of EPA and DHA on glycaemic control.

Study	Population	Control	Effect of EPA vs. Control	Effect of DHA vs. Control	Effect of EPA vs. DHA
Mori et al. (23)	Overweight mildly hyperlipidaemic men	Olive oil	Trend toward increased fasting glucose (+4%, $p = 0.062$) ↑ Fasting insulin (+18%, $p = 0.035$)	No effect on fasting glucose ↑ Fasting insulin (+27%, $p = 0.001$) ↓ Glucose to insulin ratio ($p = 0.018$)	N/A
Woodman et al. (26)	People with type-2 diabetes treated for hypertension	Olive oil	↑ Fasting glucose (+19%, $p = 0.002$) No effect on glycated hemoglobin, fasting insulin, fasting C-peptide, insulin sensitivity or secretion	↑ Fasting glucose (+12%, $p = 0.002$) No effect on glycated hemoglobin, fasting insulin, fasting C-peptide, insulin sensitivity or secretion	N/A
Klingel et al. (45)	Healthy participants	Olive oil	No effect on fasting glucose	No effect on fasting glucose	No effect on fasting glucose

N/A indicates that a statistical comparison between the effects of EPA and DHA was not made.

TABLE 8 Summary of findings related to effect of EPA and DHA on platelet and fibrinolytic function.

Study	Population	Control	Effect of EPA vs. Control	Effect of DHA vs. Control	Effect of EPA vs. DHA
Park and Harris (31)	Healthy subjects	Safflower oil	↓ Mean platelet volume ↑ Platelet count	No effect	EPA ↓ Mean platelet volume EPA ↑ Platelet count
Woodman et al. (28)	People with type-2 diabetes treated for hypertension	Olive oil	Platelet function: No effect on collagen-or PAF-stimulated platelet aggregation or platelet-derived TXB_2 Fibrinolytic function: No effect on PAI-1, tPA, von Willebrand factor, or P-selectin.	Platelet function: ↓ Collagen-stimulated platelet aggregation (−17%, $p = 0.054$) ↓ Platelet-derived TXB_2 (−19%, $p = 0.03$) No effect on PAF-stimulated platelet aggregation Fibrinolytic function: No effect on PAI-1, tPA, von Willebrand factor, or P-selectin	N/A

PAF, platelet activating factor; PAI, plasminogen activator inhibitor; tPA, tissue plasminogen activator; TX, thromboxane. N/A indicates that a statistical comparison between the effects of EPA and DHA was not made.

TABLE 9 Summary of findings related to effect of EPA and DHA on oxidative stress.

Study	Population	Control	Effect of EPA vs. control	Effect of DHA vs. control	Effect of EPA vs. DHA
Mori et al. (24), Mas et al. (25)	Overweight mildly hyperlipidaemic men	Olive oil	↓ Urinary F_2 isoprostanes (−27%, $p < 0.0001$) ↓ Plasma F_2 isoprostanes (−24%, $p < 0.0001$)	↓ Urinary F_2 isoprostanes (−26%, $p < 0.0001$) ↓ Plasma F_2 isoprostanes (−14%, $p = 0.009$)	N/A
Mori et al. (29); Mas et al. (25)	People with type-2 diabetes treated for hypertension	Olive oil	↓ Urinary F_2 isoprostanes (−19%, $p = 0.017$) ↓ Plasma F_2 isoprostanes (−19%, $p = 0.039$)	↓ Urinary F_2 isoprostanes (−20%, $p = 0.014$) ↓ Plasma F_2 isoprostanes (−23%, $p = 0.011$)	N/A

N/A indicates that a statistical comparison between the effects of EPA and DHA was not made.

blood pressure in heathy adults. DHA (4 g/day) also did not affect heart rate or systolic blood pressure, but decreased diastolic blood pressure.

Effect of EPA vs. DHA on glycaemic control

Three trials (23, 26, 45) included outcomes related to effects of EPA and DHA on glycaemic control (Table 7). In the Mori et al. (23) trial in overweight mildly hyperlipidaemic men, fasting insulin was found to be increased by both EPA (+18%) and DHA (+27%). With EPA there was also a trend toward increased fasting glucose (+4%) but there was no effect of DHA on fasting glucose. With DHA, there was a decrease in the glucose:insulin ratio, as a result of the effect on insulin. In type-2 diabetics treated for hypertension (26), both EPA and DHA increased fasting glucose, with a larger effect of EPA than DHA (+19 vs. +12%). There was no effect of either EPA or DHA on fasting insulin, glycated hemoglobin, fasting C-peptide, insulin sensitivity or insulin secretion compared to control. Klingel et al. (45) reported no effect of either EPA or DHA (3 g/day) on fasting glucose compared to olive oil in healthy participants.

Effect of EPA vs. DHA on platelet and fibrinolytic function

Two trials (28, 31) included outcomes related to effects of EPA and DHA on platelet and fibrinolytic function (Table 8). The Park and Harris (31) trial conducted in healthy subjects reported a decrease in mean platelet volume and an increase in platelet numbers with EPA (3.8 g/day) compared with safflower oil. However, with DHA (3.8 g/day), there was no effect on platelet volume or count. The effects of EPA were significantly different from those of DHA. The effects on platelet aggregation were not assessed in that trial. In the Woodman et al. (28) trial in anti-hypertensive-treated type 2 diabetics DHA, in contrast to EPA, led to a decrease in collagen-stimulated platelet aggregation (−17%) and platelet-derived thromboxane B2 release (−19%) when compared to olive oil. Thrombaxane B2 is derived from thromboxane A2 which promotes platelet aggregation and so these two observations with DHA may be related. In that trial, neither EPA nor DHA demonstrated any effect on markers of fibrinolytic function.

Effect of EPA vs. DHA on oxidative stress

Two trials (three publications) (24, 25, 29) included outcomes related to effects of EPA and DHA on oxidative stress as assessed by measuring F2 isoprostances in urine and plasma (Table 9). In a trial in overweight mildly hyperlipidaemic men (24, 25), both EPA and DHA significantly decreased urinary F2 isoprostanes (by 27 and 26% respectively) and plasma F2 isoprostanes (by 24 and 14% respectively) compared to olive oil. Likewise, in a trial in type 2 diabetics treated for hypertension (25, 29), both EPA and DHA significantly decreased urinary F2 isoprostanes (by 19 and 20% respectively) and plasma F2 isoprostanes (by 19 and 23% respectively) compared to olive oil. The effects of EPA and DHA were not formally compared but DHA tended to have greater effects than EPA on F2 isoprostanes as a marker of oxidative stress.

Discussion

Context of this systematic review

Higher dietary intakes and higher blood and tissue status of the omega-3 PUFAs EPA and DHA are associated with lower risk of developing CVD and mortality from CVD, including coronary heart disease (3, 4) and some studies have reported that intervention with these fatty acids decreases adverse cardiovascular outcomes in at risk patients (50, 51). EPA and DHA act through beneficial effects on multiple cardiovascular risk factors, as demonstrated in numerous trials and meta-analyses of RCTs (2–4, 6–16). An important question that is not fully resolved is whether EPA and DHA have the same or unique actions (52, 53). A systematic review of RCTs that compared the effect of ≥2 g/day of near pure EPA and DHA on cardiovascular risk factors was published in 2018 (18). It included 18 publications from 6 unique RCTs and concluded that EPA and DHA do appear to have differential effects on at least some risk factors for CVD. New information on this topic has been published since mid-2017 when the literature search for that systematic review was conducted. Therefore, the search was updated and this systematic review produced. The new search identified an additional seven publications including five publications from 3 new RCTs (45–49) and 2 from one of the previously included RCTs (43, 44). New data on plasma lipids and lipoproteins (43–45, 47, 48), inflammatory biomarkers (47, 49), blood pressure, haemodynamics and vascular function (46, 49) and glycaemic control (45) are included.

Summary of effects of EPA vs. DHA on cardiovascular risk factors

Six out of nine trials reported that EPA lowered triglycerides compared with placebo, while seven out of nine trials reported that DHA lowered triglycerides. Although some trials suggest a greater triglyceride lowering effect of DHA than EPA, the difference sometimes appears to be small, and is not apparent in some trials. Effects of EPA and DHA on cholesterol, LDL and HDL are less consistent. Most trials report no effect of EPA on LDL cholesterol, although one reported an increase (34). Although some trials report no effect of DHA on LDL cholesterol, 3 reported an increase (23, 34, 48). No included trials reported that EPA affects HDL cholesterol and, while most trials also report no effect of DHA, two did report increased HDL cholesterol with DHA (19, 34). There may be effects of EPA and DHA on HDL subfractions: two trials reported that EPA lowers HDL3 (26, 34) while one reported higher HDL2 (26). Two trials reported that DHA increases HDL2 (23, 26). Regarding LDL particle size, two trials reported no effect of EPA (23, 28), while one reported a decrease in size (43). Three trials reported that DHA increased LDL particle size (23, 28, 43). The trials of Park and Harris (32, 33) and So et al. (48) indicate that EPA and DHA can have effects on the enzymes that metabolize lipoproteins and control the transfer of lipid moieties between lipoproteins. Thus, high dose EPA and DHA lower triglycerides, with DHA possibly being more potent. EPA has little impact on either LDL or HDL cholesterol, but may lower the level of the harmful HDL3 subfraction. DHA can raise both LDL and HDL cholesterol, may raise the level of the protective HDL2 subfraction, and increases LDL particle size, perhaps rendering LDL less atherogenic.

Regarding inflammatory markers, all four included trials reported that EPA did not alter CRP levels; 3 out of the 4 trials also report no effect of DHA, but one (34) reported that DHA lowered CRP. No included trial reported that EPA altered any of the cytokines assessed (TNF-α, IL-6, IL-10, MCP-1, IL-18, adiponectin), but these were mostly only measured in one trial. One trial did report a trend to lower TNF-α with EPA (29). The trial that reported that DHA lowered

CRP also found lower TNF-α, IL-6 and IL-18 and higher adiponectin with DHA (34). Another trial (29) reported a trend to lower TNF-α with DHA. Both EPA and DHA decreased CD14 gene expression and increased PPARA gene expression in one trial (36). Both EPA and DHA decreased the inflammatory response seen with LPS stimulation of monocytes studied *ex vivo*, with a stronger effect of DHA (47). Both EPA and DHA modified the plasma oxylipin profile (47) Thus, both EPA and DHA promoted an anti-inflammatory gene expression profile and reduced the response of monocytes to an inflammatory stimulus; however circulating biomarkers of inflammation like CRP and IL-6 are little impacted by EPA, but these may be lowered by DHA suggesting that DHA has stronger anti-inflammatory actions. Both EPA and DHA foster a less inflammatory plasma profile of oxylipins.

Five out of 6 trials reported no effect of EPA on blood pressure, 4 out of 6 reported no effect on heart rate and 2 out of 3 reported no effect on vascular function. One trial reported that EPA increased systolic and diastolic blood pressure in healthy young adults compared to the olive oil placebo (46). Two trials reported that EPA increased heart rate in healthy adult men (20, 46), while another reported a trend to decreased heart rate in people with diabetes being treated for hypertension (26). One trial reported that EPA improved arterial compliance in people with dyslipidaemia (30). Four out of 6 trials reported no effect of DHA on blood pressure, 3 out of 6 reported no effect on heart rate and 1 out of 3 reported no effect on vascular function. One trial reported that DHA decreased systolic and diastolic blood pressure in overweight mildly hyperlipidaemic men (21) while another reported lower diastolic, but not systolic, blood pressure in healthy adults (49). Two trials reported that DHA lowered heart rate (20, 21), while a third trial reported a trend for this (26). Two trials reported that DHA improved vascular function (22, 30). Overall, DHA appears to have stronger and more favorable effects on blood pressure, heart rate and vascular function than EPA.

One trial reported that EPA increased fasting glucose (26); another reported a trend for this outcome (23) but a third found no effect of EPA (45). One trial reported that EPA increased fasting insulin (23) but a second trial did not see this (26). That trial found no other effects of EPA on markers of glucose homeostasis including insulin sensitivity. One trial reported that DHA increased fasting glucose (26); two others found no effect of DHA (23, 45). One trial reported that DHA increased fasting insulin (23) but a second trial did not see this (26). That trial found no other effects of DHA on markers of glucose homeostasis including insulin sensitivity. Overall, there seems to be little impact of EPA and DHA on glucose homeostasis.

One trial reported that EPA decreases mean platelet volume and increases platelet number, with DHA not having these effects (31). A second trial reported that DHA decreased collagen-stimulated platelet aggregation and thromboxane B2 generation, but EPA did not have these effects (28). There was no effect of EPA or DHA on markers of fibrinolytic function in the one trial that reported these (28).

Both EPA and DHA were found to decrease urinary and plasma F2 isoprostanes assessed as markers of oxidative stress, with little difference in potency (24, 25, 29).

Discussion of the findings

This systematic review suggests that EPA and DHA have quantitatively different effects on CVD risk factors such as blood lipids including triglycerides, blood pressure, heart rate, vascular function, platelet function, and inflammatory markers. The trials included in this review show that DHA has a more favorable impact on several of these parameters.

It is suggested that the plasma triglyceride lowering effect of both EPA and DHA is due to a number of factors, including an inhibitory effect on hepatic triglyceride synthesis and VLDL assembly and secretion and an enhanced triglyceride hydrolysis and clearance of VLDL by LPL (54). This was supported by the Klingel et al. (45) trial that showed that both EPA and DHA increased LPL activity. DHA had a greater effect on LPL activity, which might account for its (slightly) greater triglyceride lowering action. The decrease in hepatic triglyceride synthesis and VLDL secretion is thought to be due to the decrease in synthesis of ApoB100, which is required for VLDL assembly. A decrease in hepatic VLDL assembly and secretion is thought to be beneficial for lowering blood triglyceride levels, as VLDL is the main lipoprotein that transports triglycerides (54). Interestingly, Allaire et al. (44) reported an increase in the VLDL ApoB100 catabolism rate which is the rate at which VLDL is cleared from the bloodstream, which further supports the decrease in triglyceride levels observed with EPA and DHA. VLDL catabolism generates LDL, so this could be part of the mechanism for the elevated LDL-cholesterol reported in some studies of DHA.

In terms of the results with other blood lipids and blood lipid-related factors, one of the newer trials identified a decrease in PCSK9 levels with both EPA and DHA (43). PCSK9 degrades the cell surface LDL receptors responsible for clearing circulating LDL and therefore contributes to elevated LDL-cholesterol (55). An EPA- or DHA-mediated decrease in PCSK9 would be anticipated to result in better LDL clearance and so lower LDL-cholesterol. Contrary to this expectation, some trials report elevated LDL-cholesterol, especially with DHA (23, 34, 48). Apart from an increase in LDL levels, some trials reported an increase in LDL particle size with DHA supplementation (23, 28, 43). Allaire et al. (43) suggest that the reason for this could be due to the decrease in ApoCIII secretion from the liver through the regulation of transcriptions factors and binding proteins. This apparent reduction in ApoCIII production after DHA leads to increased conversion of VLDL to LDL and the formation of larger LDL particles compared to EPA, as reported in several trials. In this regard DHA also decreased the proportion of pro-atherogenic small dense LDL particles (43). The increase in HDL cholesterol that is reported with DHA could be explained by altered activity of lipid transfer proteins such as CETP (48) which results in the transfer of cholesteryl esters from HDL toward more triglyceride-rich lipoproteins.

Regarding the effects of EPA and DHA on inflammation, there is some inconsistency in the findings of the included trials. In terms of their actions on inflammation, EPA and DHA are thought to have anti-inflammatory effects through the replacement of, and competition with, AA in the cell membrane which results in decreased production of eicosanoids from AA which tend to be more inflammatory (such as prostaglandin E2 and leukotriene B4) and increased production of eicosanoids from EPA (leukotriene B5 and prostaglandin E3) which are less inflammatory (56, 57). Furthermore, EPA and DHA both give rise to lipid mediators termed specialized pro-resolving mediators (58–60). Interestingly, So et al. (47) reported a greater reduction in plasma phospholipid AA with DHA than EPA linked with a greater reduction in AA derivatives such as prostaglandin E2 and thromboxane B2. There was also an elevation in some of the

EPA- and DHA-derived oxylipins (47). EPA and DHA are also known to have a role in regulating inflammatory gene expression (56, 57), effects seen in the studies reported in Allaire et al. (34, 36) and So et al. (47). Markers of inflammation such as CRP, IL-6, IL-1, and TNF-α are linked with an increased likelihood of CVD and cardiovascular events (61, 62). Furthermore, there is a relationship between the inflammatory markers themselves, for example IL-6 triggers CRP to be synthesized in the liver. Despite the reported effects on oxylipins and gene expression in the included trials (34, 36, 47), there were few effects on circulating markers of inflammation, although Allaire et al. (34) reported a decrease in plasma CRP, TNF-α, IL-6, and IL-18 with DHA and a decrease in IL-6 with EPA.

With respect to the limited trials that show a decrease in blood pressure with EPA and DHA, it is suggested that the mechanism for this is related to a decrease in systemic vascular resistance due to the increase in nitric oxide production (63), decreased response to angiotensin II and noradrenalin (64, 65) and an increase in arterial compliance (66) leading to a reduction in systolic and diastolic pressure. The changes in heart rate observed with DHA can be explained by the beneficial impacts on cardiac muscle cell function and the likely changes in membrane fluidity, which alter the conductive properties of ion channels within those membranes (67) resulting in lowered heart rate.

Regarding glycaemic control, the results are limited, but some trials report that EPA and DHA may increase fasting glucose and fasting insulin, which may be seen as a deleterious effect. These effects may be due to an increase in hepatic glucose output and an increase in plasma glucagon concentrations. It is worth noting that the recent trial by Klingel et al. (45) reported no effect of either EPA or DHA on blood glucose.

EPA and DHA appear to have different effects on platelet function with EPA reducing mean platelet volume and DHA reducing collagen-induced platelet aggregation. The mechanism behind effects on platelet aggregation is similar to the effects on inflammation, in that EPA and DHA displace AA from the platelet membrane leading to a decrease in thromboxane A2 which is a platelet aggregator and an increase in EPA-derived prostacyclin PGI3 which is an inhibitor of platelet aggregation (68). Regarding platelet volume, an increase in platelet volume would suggest a more pro-atherogenic environment; therefore, EPA may reduce the incidence of atherogenic events via the reduction of mean platelet volume; DHA seems not to have this effect.

In the context of oxidative stress, both plasma and urinary F2-isoprostanes have been established as biomarkers indicative of *in vivo* lipid peroxidative damage (69). AA is the precursor for the synthesis of F2-isoprostanes (69). The reduction in F2-isoprostanes by EPA and DHA is interpreted to indicate reduced oxidative stress. However, since AA is the precursor to F2-isoprostanes, it may be that lower F2-isoprostanes also partly reflect the lowering of AA that is a feature of increased intake of EPA and DHA. Nevertheless, Mas et al. (25) reported that the effects of EPA and DHA on plasma F2-isoprostanes are retained when the data are adjusted for the change in plasma AA.

The broader context

Irrespective of whether there are differences in their quantitative effects, the beneficial impact of both EPA and DHA on a range of recognized and emerging risk factors for CVD suggests that they play an important role in disease prevention. This is supported by multiple epidemiological studies which evaluate the association between intake or blood or tissue levels of EPA and DHA and incident disease during a, usually long, follow-up period. Chowdhury et al. (70) aggregated such prospective studies investigating risk of coronary outcomes. Data from 16 studies, including over 422,000 subjects, showed a 13% reduction in risk for those in the top third of dietary intake of EPA + DHA than those in the lower third of intake. Data from 13 studies with over 20,000 participants showed a 22, 21, and 25% reduction in risk of coronary outcomes for those in the top third of blood levels of EPA, DHA, or EPA + DHA, respectively, compared to those in the lower third (70). Using data from 17 prospective cohort studies, Alexander et al. (71) reported an 18% lower risk for any coronary heart disease event for subjects with higher dietary intake of EPA + DHA than for those with lower intake. There were also significant reductions of 19, 23, and 47% in the risk for fatal coronary death, coronary events, and sudden cardiac death, respectively. Another study pooled data from 19 trials investigating the association between EPA or DHA concentration in a body pool, such as serum, plasma, red blood cells, or adipose tissue, and risk of future coronary heart disease in adults who were healthy at study entry (72). Both EPA and DHA were independently associated with a reduction in the risk of fatal coronary heart disease, with about a 10% reduced risk for each one standard-deviation increase in either EPA or DHA. Harris et al. (73) gathered together 10 cohort studies and found a 15% lower risk of fatal coronary heart disease for each one-standard-deviation increase in the omega-3 index (i.e., the sum of EPA + DHA in red blood cells). A *de novo* pooled analysis of 17 prospective cohort studies with 42,466 individuals confirmed the association between a lower risk for death from CVD in those with the highest vs. the lowest quintile of circulating EPA, DHA, and EPA + DHA (74). These analyses support a clear role for EPA and DHA in the primary prevention of coronary heart disease and, perhaps more widely, of CVD, as discussed elsewhere (3, 4), findings that underpin current dietary recommendations for intake of these fatty acids (75–78). The Vitamin D and Omega-3 (VITAL) trial also provides some support for these recommendations. This was an RCT conducted as a two-by-two factorial design of vitamin D3 (at a dose of 50 µg/day) and EPA + DHA (1 g/day) among 25,871 healthy participants aged over 50 years for the primary prevention of CVD and cancer (79). After a median follow-up of 5.3 years, there was no statistically significant difference between the groups receiving EPA + DHA or placebo in the primary outcome of major cardiovascular events (a composite of myocardial infarction, stroke, or death from cardiovascular causes). However, an analysis of the individual components of the composite showed a significant reduction in the EPA + DHA arm for myocardial infarction (28% reduction) and coronary heart disease (17% reduction). Correspondingly, there was also a lower risk of death from these two non-prespecified outcomes (50% for myocardial infarction and 24% for coronary heart disease), although the effect on coronary heart disease mortality was not significant. There was a significant reduction in major adverse cardiovascular events (19%) and risk of myocardial infarction (40%) for those who consumed fewer than 1.5 fish meals per week and then supplemented with EPA + DHA. Although there was no effect on the primary outcome (first serious vascular event) between EPA + DHA (840 mg/day) and placebo groups over a median follow-up on 7.4 years in A Study of Cardiovascular Events in Diabetes (ASCEND), a study of 15,480 people living with diabetes but

with no evidence of CVD, there were 19% fewer deaths from vascular events in the EPA + DHA arm as well as a trend toward reduced risk of death (21%) from coronary heart disease (80).

Despite the consistent evidence for EPA and DHA reducing risk of CVD, findings from trials using these fatty acids therapeutically in those already with high risk or with advanced disease have proven to be inconsistent (3, 4). This has led to discussion about the relative impact of EPA and DHA, since different therapeutic trials have used different formulations. In the GISSI-Prevenzione study (81) involving survivors of recent myocardial infarction (≤ 3 months since myocardial infarction), treatment with EPA + DHA (840 mg/day) significantly reduced the composite primary outcomes (−15% and −20%, respectively) and several secondary outcomes, including cardiovascular death by 30%, sudden death by 45%, and total fatal events by 20%. In the GISSI-HF trial (82), patients with chronic heart failure received EPA + DHA (840 mg/day) or placebo for approximately 4 years, and there a small (9%) but significant reduction in all-cause mortality. These trials suggest that the combination of EPA + DHA may be effective therapeutically. The randomized, open-label Japan EPA Lipid Intervention Study (JELIS) included patients with hypercholesterolemia who were assigned to receive either a statin alone or a statin along with highly purified EPA (1.8 g/day EPA) with a 5-year follow-up (83). The primary outcome was any major coronary event, including sudden cardiac death, fatal and nonfatal myocardial infarction, and other nonfatal events, including unstable angina pectoris, angioplasty, stenting, and coronary artery bypass grafting. Long-term use of EPA-ethyl ester as an addition to statin therapy had no effect over statin alone on the primary outcome in the primary prevention arm of the trial, but in the secondary prevention arm, EPA supplementation resulted in a significant 19% reduction in nonfatal coronary events vs. statin alone (83). JELIS highlights that EPA may be effective in the absence of DHA. This latter conclusion is supported by more recent trials. In the Reduction of Cardiovascular Events with Icosapent Ethyl Intervention Trial (REDUCE-IT) (50), 8,179 high risk patients received 3.6 g/day of EPA as ethyl ester or mineral oil as placebo with a median follow-up of 4.9 years. The primary outcome (a composite of cardiovascular death, nonfatal myocardial infarction, nonfatal stroke, coronary revascularization, or unstable angina) was reduced by 25% in patients who received EPA-ethyl ester compared to placebo. The key prespecified secondary outcome (a composite of cardiovascular death, nonfatal MI, or nonfatal stroke) was also significantly reduced in the EPA-ethyl ester group as were a whole range of other clinical outcomes (50). Another positive EPA-ethyl ester trial was Effect of Vascepa on Improving Coronary Atherosclerosis in People with High Triglycerides Taking Statin Therapy (EVAPORATE) (84). This study involved 80 patients with known angiographic coronary artery disease taking statins and with no history of myocardial infarction, stroke, or life-threatening arrhythmia within the prior 6 months. The same EPA-ethyl ester preparation and the same dose were used as in REDUCE-IT. EVAPORATE demonstrated that EPA might directly promote atherosclerotic plaque attenuation in hypertriglyceridemic individuals at 18 months (84). In contrast to this series of trials demonstrating significant therapeutic benefit of EPA provided in the absence of DHA (50, 83, 84), trials of the combination of EPA + DHA conducted since the two GISSI trials provide inconsistent findings. The Outcome Reduction with an Initial Glargine Intervention (ORIGIN) trial with 840 mg/day EPA + DHA in 12,536 dysglycemic patients with recent myocardial infarction or heart failure and a median follow-up of 6.2 years was null (85), as was the Risk and Prevention Study which assessed the effect of 840 mg/day EPA + DHA in 12,513 patients at high cardiovascular risk but with no myocardial infarction for a median of 5 years (86). However, in a prespecified subgroup analysis, compared with placebo, EPA + DHA resulted in an 18% lower incidence of the revised primary outcome among women (composite of the time to death from cardiovascular causes or first hospital admission for cardiovascular causes). Also, admissions for heart failure were significantly lower in the long-chain omega-3 fatty acid group. It is worth noting that the trials of EPA + DHA have used a lower dose (840 mg/day) than trials of pure EPA (1.8 or 3.6 g/day), so any difference in findings of these trials could relate to dosing.

One trial that has questioned the impact of the combination of EPA + DHA is the Long Term Outcomes Study to Assess Statin Residual Risk with Epanova in High Cardiovascular Risk Patients with Hypertriglyceridemia (STRENGTH) trial (87). In this study, patients with hypertriglyceridemia and high cardiovascular risk on statin therapy were treated with 4 g/day of an oil containing EPA and DHA (as free fatty acids); this provided about 2.2 g EPA and 0.8 g DHA daily. Corn oil was used as placebo. There was no significant difference in a composite outcome of major adverse cardiovascular events among patients who received additional omega-3 fatty acids to usual background therapies vs. control, and the trial was stopped early (87). The Omega-3 Fatty Acids in Elderly with Myocardial Infarction (OMEMI) trial randomized a total of 1,027 patients with a recent myocardial infarction (in the previous 2–8 weeks) to receive approximately 1.6 g/day of EPA + DHA (930 mg EPA and 660 mg DHA as triglycerides) or corn oil (placebo) as an addition to standard care (88). After 2 years of follow-up, there was no significant difference between the two groups in the primary composite cardiovascular outcome.

Thus, REDUCE-IT and EVAPORATE both report benefits of pure EPA while STRENGTH and OMEMI report no benefit of the combination of EPA + DHA. The reasons for this discrepancy between REDUCE-IT and STRENGTH have been discussed elsewhere (89, 90) and include choice of placebo, formulation of omega-3 fatty acids (ethyl ester vs. free fatty acids) and exact omega-3 dose (3.6 vs. 3 g/day). Another possibility is that DHA negates the benefits of EPA so that the combination of EPA + DHA is less effective than EPA alone, although the mechanisms for how this would occur are unclear. There are no long-term studies comparing the therapeutic effect of pure EPA, pure DHA and the combination of EPA + DHA on hard cardiovascular endpoints.

Conclusion

The results of this systematic review suggest that EPA and DHA have some similar and some different effects on cardiovascular risk factors. EPA and DHA both lower triglyceride levels with DHA most likely having a slightly greater effect. Furthermore, both EPA and DHA increase HDL2 cholesterol, which is cardioprotective, with the increase being greater with DHA. DHA appears to increase LDL cholesterol and LDL particle size which would render LDL less atherogenic. From the more limited study data, both EPA and DHA decreased some inflammatory markers and pro-inflammatory gene expression, with DHA having stronger effects. DHA may be more effective than EPA in decreasing heart rate and blood pressure. Both EPA and DHA alter platelet function decreasing thrombogenicity, although they have different actions on platelets. Both EPA and DHA decrease F2-isoprostanes, interpreted as a reduction in oxidative

stress. They both decrease inflammatory gene expression and promote an anti-inflammatory oxylipin profile. These are all favorable effects with regard to cardiovascular risk. Reported effects of EPA and DHA on blood glucose are inconsistent.

Although the new data on the effects of EPA and DHA on blood lipids including triglycerides may create a clearer picture of those effects, the overall data around whether the two omega-3 fatty acids have differential effects on other cardiovascular risk factors is still inconsistent, but generally speaking there is a signal that DHA has a stronger impact than EPA. However, this updated systematic review is constrained by the small number of high quality RCTs that directly compare EPA to DHA and report on outcomes other than blood lipids. Therefore, there is a need for additional high-quality research to assess the independent effects of EPA and DHA on a range of cardiovascular risk factors (e.g., inflammation, blood pressure, vascular function, platelet function) in larger and more diverse study populations.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

GC: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. PC: Conceptualization, Project administration, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2024.1423228/full#supplementary-material

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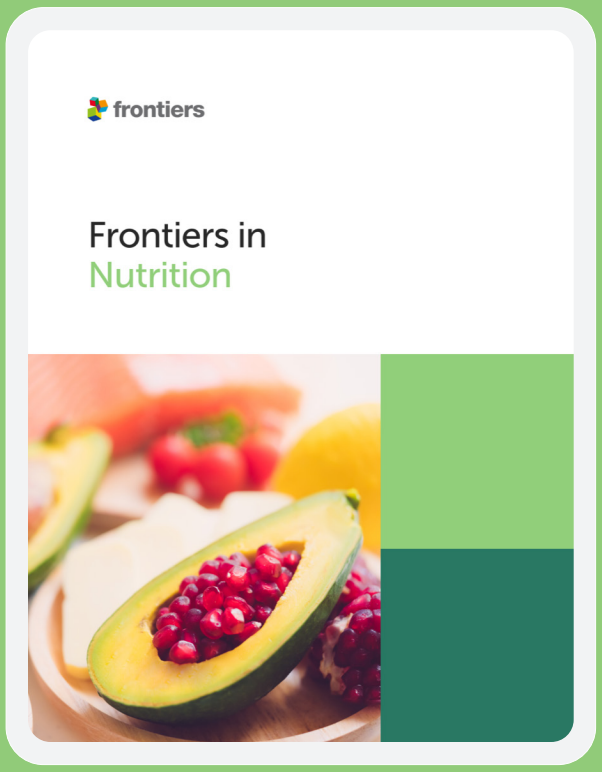

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