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Supplementation with eicosapentaenoic acid and docosahexaenoic acid reduces high levels of circulating proinflammatory cytokines in aging adults: A randomized, controlled study

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ABSTRACT

Background: High levels of circulating proinflammatory cytokines are characteristic of inflammaging, a term coined to describe age-related chronic systemic inflammation involved in the etiology of many age-related disorders including nonhealing wounds. Some studies have shown that supplementing diets with n-3 polyunsaturated fatty acids (eicosapentaenoic acid [EPA] and docosahexaenoic acid [DHA]) lowers systemic levels of key proinflammatory cytokines associated with inflammaging. However, findings from the few studies that have focused exclusively on older adults are inconclusive. As such, the objective of this randomized controlled study was to test the effects of EPA + DHA therapy on circulating levels of proinflammatory cytokines in adults in middle to late adulthood.

Methods: Plasma levels of fatty acids and interleukin (IL)-6, IL-1β and tumor necrosis factor-α (TNF-α) were measured in 35 participants with chronic venous leg ulcers (mean age: 60.6 years) randomnly assigned to 8 weeks of EPA + DHA therapy (2.5 g/d) or placebo therapy.

Results: EPA + DHA therapy had a significant lowering effect on levels of IL-6, IL-1β and TNF-α after 4 weeks of therapy and an even greater lowering effect after 8 weeks of therapy. Further, after adjusting for baseline difference, the treatment group had significantly lower levels of IL-6 ($p = 0.008$), IL-1β ($p < 0.001$), and TNF-α ($p < 0.001$) at Week 4 and at Week 8 [IL-6 ($p = 0.007$), IL-1β ($p < 0.001$), and TNF-α ($p < 0.001$)] compared to the control group.

Conclusion: Adults in middle to late adulthood receiving EPA + DHA therapy demonstrated significantly greater reductions in circulating levels of proinflammatory cytokines compared with those receiving placebo therapy. EPA + DHA therapy may be an effective low-risk dietary intervention for assuaging the harmful effects of inflammaging.

1. Introduction

"Inflammaging" is a term used to describe the strong relationship between physiological aging and chronic systemic inflammation; a contributing factor in multiple age-related pathologies, including atherosclerosis, arthritis, nonhealing wounds and even frailty [1,2]. Studies suggest that inflammaging is likely a manifestation of immunosenescence, a remodeling of the immune system over time because of long-term exposure to internal and external injurious elements [3]. Eventually the overstimulated immune system becomes inefficient in regulating inflammation, leading to the chronic systemic inflammation involved in the etiology of many conditions causing significant morbidity and mortality for aging adults. Because of the rapidly increasing aging population, inflammaging is a global public health concern [2].

Inflammaging is typified by elevated levels of circulating proinflammatory cytokines such as interleukin (IL)-6, IL-1β and tumor necrosis factor-α (TNF-α) [2,4]. Inflammatory cytokines are small signaling proteins synthesized by a range of cells (e.g., monocytes, neutrophils) activated during inflammatory responses, regulating inflammation through a complex network of interactions. In aging, an increase in levels of circulating proinflammatory cytokines versus anti-inflammatory cytokines promotes chronic systemic inflammation and age-related diseases with inflammatory components [4]. Moreover, circulating proinflammatory cytokines can travel to the brain with the assistance of specific transporters because of increased vulnerability of

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the blood-brain barrier to prolonged peripheral inflammation [5]. A potential outcome of this proinflammmaory cytokine cross-over is neuroinflammation, which has been implicated in several age-related neurological and psychiatric illnesses such as depression and Alzheimer's disease [6]. There is also mounting evidence that chronically elevated levels of circulating proinflammatory cytokines increase risk for age-related non-Alzheimer's cognitive decline [7], a phenomenon rising in prevalence worldwide. The collective adverse effects of elevated circulating levels of pro-inflammatory cytokines and their link to inflammaging suggest that targeted interventions to reduce high levels may help prevent or reduce severity of chronic inflammatory diseases in aging. Dietary supplementation with marine-derived n-3 polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) is one relatively inexpensive therapy showing significant promise for this purpose.

Typical Western diets contain low levels of n-3 PUFAs relative to n-6 PUFAs, largely due to changes in food production and processing over time [8,9]. This is believed to be problematic in terms of proinflammatory cytokine levels because the n-6 PUFA arachidonic acid (AA) is metabolized to eicosanoids (e.g., prostaglandin E2 [PGE2]) that induce proinflammatory cytokine production [10]. Conversely, increasing intake of n-3 EPA and DHA has been found to reduce the amount of AA available for eicosanoid synthesis because the n-6 and n-3 PUFA families are competetively metabolized [8] (Supplemental Figure 1). Further, after ingestion, EPA and DHA enter the bloodstream relatively quickly, are incorporated into cell membrane phospholipids, and then inhibit activation of key transcription factors such as nuclear factor κ B (NFκB) that increase expression of proinflammatory cytokine genes by cells involved in the inflammation process [1]. Some human studies have reported that EPA + DHA dietary supplementation is effective in lowering levels of circulating proinflammatory cytokines [11–14]. However, other EPA + DHA intervention studies in people of variable age ranges have not found reliable cytokine variations [13,15–19]. The disparities noted between studies may be due to methodological variations. For example, experimental studies focusing on older adults have varied in terms of low versus high EPA + DHA doses, placebos containing oils that are bioactive versus placebos containing oils that are chemically intert, *ex vivo* versus in vivo experiments, quantifying inflammatory factors in tissue versus circulation, and using samples of healthy people with low levels of baseline inflammation versus people with high levels of baseline inflammation [11,20–22]. As such, it is critical to clarify the extent to which EPA + DHA therapy alters circulating proinflammatory cytokine levels in aging adults. The data generated from additional well-designed randomized controlled studies will improve our understanding of the dose and time effects of EPA + DHA therapy and its potential use for preventing or treating inflammaging.

The current study used a sample of aging adults (N = 35) having at least one known chronic inflammatory non-autoimmune condition (chronic venous leg ulcers) to determine the effect of EPA + DHA supplementation (2.5 g/d) versus a placebo containing mineral oil to reduce blood plasma levels of proinflammatory cytokines IL-6, IL-1β, and TNF-α after 4 and 8 weeks of therapy.

2. Patients and methods

2.1. Study design

This randomized, double-blind, placebo-controlled, repeated measures study examined adults in middle to late adulthood (N = 35; mean age = 61 years) with chronic venous leg ulcers (CVLUs) at Week 0 (baseline), and 4 and 8 weeks after dietary supplementation with EPA + DHA or placebo. Chronic systemic and local inflammation are involved in the pathobiology of CVLUs [23,24]. Randomization of participants to one of the two treatment groups was performed by a person not associated with the study using a computer-generated randomization scheme. At Week 0, self-reported sociodemographic and comorbidity data were collected and body measurements were recorded (height, weight) for body mass index (BMI) calculations. Fasting blood samples were collected at Week 0, 4 and 8 to quantify levels of fatty acids and proinflammatory cytokines. Participants were instructed to maintain their usual diets, but to exclude fish, seafood, algae, kelp and nutritional supplements until study completion. Participants received US$250 after completing the study.

2.2. Study population

Participants were men and women 50 to 85 years of age diagnosed with a CVLU for ≥3 months, recruited over a period of 24 months from a large medical center's two out-patient wound clinics in the Midwest. The institutional review board (IRB) approved the study which was conducted at the university Clinical Research Center (CRC) associated with the medical center between August 2012 and July 2015 in compliance with ethical rules for human experimentation as stated in the 1975 Declaration of Helsinki. Participants were excluded from the study if they were allergic to fish or seafood, were prescribed warfarin therapy, had autoimmune conditions or any chronic inflammatory skin diseases (e.g. pyoderma gangrenosum), required non-steroidal anti-inflammatory drugs (NSAIDS) >2x a week, or regularly used nutritional supplements (e.g., fish oil) or corticosteroids. All participants signed an informed consent document approved by the local IRB at the beginning of the study and the study protocol was registered December 12, 2012 at ClinicalTrials.gov (NCT01754506).

As a pilot study, 40 participants were recruited during the first 24 months of the study with an almost equal allocation to the two groups (n = 21 for EPA + DHA and n = 19 for Control). After accounting for attrition, the final sample included 35 people (16 for EPA + DHA and 19 for Control). This sample size provided 80% power to detect an effect size of 0.7 for within-group comparisons and 80% power to detect an effect size of 0.9 for between-group comparisons at a two-sided significance level of 0.05. A general summary of the study design is presented in Supplementary Fig. 2 – CONSORT diagram.

2.3. EPA + DHA supplement and placebo

The softgel capsules (EPA + DHA or placebo) used in both groups were indistinguishable from each other. The manufacturer, J.R. Carlson Laboratories, Inc. (Arlington Height, IL), compounded the two types of softgels (EPA + DHA supplement and placebo) in a codified form. At Visit 1 (Week 0) EPA + DHA Group participants received instructions to take five opaque EPA + DHA softgels and one 81 mg tablet of acetylsalicylic acid (ASA) every day for the study interval. The five EPA + DHA softgels provided a total daily intake of approximately 1.5 g of EPA and 1.0 g of DHA (Table 1). This EPA/DHA ratio has been used in our previous work and was chosen because of evidence that EPA may have relatively stronger anti-inflammatory effects than DHA [25].The U.S. Federal Drug Administration (FDA) evaluated the safety of EPA and DHA and concluded that a daily intake of EPA + DHA of up to 3.0 g/d is acceptable for the general public [26]. Aspirin was administered because it has been found to enhance the specialized pro-resolving mediators from EPA and DHA (e.g., lipoxins, resolvins). Control Group participants received identical-looking placebo softgels and 81 mg ASA tablets and the same instructions for how to take the softgels as did the EPA + DHA Group participants. Five opaque placebo softgels provided a total daily intake of 5.2 g/d of light mineral oil, which is well below the therapeutic dose for constipation. Mineral oil is chemically inert and on ingestion the majority (98%) remains unabsorbed in the feces. The same daily mineral oil dose has been used as the placebo in our previous work with no adverse events being reported [27]. Participants in both groups received the appropriate number of ASA tablets and softgels for 4 weeks of treatment at Visit 1. They were instructed to bring the empty bottles to Visit 2 (4 weeks later). At Visit 2 another bottle of

Table 1
Fatty acids in the study supplement as determined by independent analysis.

Fatty acid	Common name	Mg/capsule	% of total fatty acids
C6:0	Caproic acid	0.10	0.0
C8:0	Caprylic acid	0.08	0.0
C14:0	Myristic acid	1.04	0.1
C16:0	Palmitic acid	21.00	2.0
C16:1	Palmitoleic acid	7.94	0.8
C18:0	Stearic acid	34.25	3.4
C18:1	Oleic acid	98.17	9.7
C18:2n6	Linoleic acid	30.09	3.0
C18:3n6	Gamma-linolenic acid	1.45	0.2
C18:3n3	Alpha-linolenic acid	7.27	0.7
C18:4n3	Stearidonic acid	20.46	2.1
C20:0	Arachidic acid	9.99	1.0
C20:1	Eicosenoic acid	36.80	3.7
C20:2n6	Eicosadienoic acid	2.99	0.3
C20:3n6	Dihomo-gamma-linolenic acid	2.48	0.3
C20:4n6	Arachidonic acid	20.32	2.1
C20:3n3	Eicosatrienoic acid	2.70	0.3
C20:4n3	Eicosatetraenoic acid	17.41	1.8
C20:5n3	(EPA) Eicosapentaenoic acid	324.67	33.4
C21:5n3	Heneicosapentaenoic acid	16.59	1.51
C22:0	Behenic acid	4.43	0.5
C22:1	Cetoleic acid	55.86	5.7
C22:2n6	Docosadienoic acid	1.32	0.1
C22:4n6	Adrenic acid	3.56	0.4
C22:5n6	Docosapentaenoic acid (n-3)	6.83	0.6
C22:5n3	(DHA) Docosahexaenoic acid	47.05	4.4
C22:6n3	Docosapentaenoic acid (n-6)	217.78	21.2
C24:0	Lignoceric acid	0.73	0.1
C24:1	Nervonic acid	5.45	0.6
Total fatty acids		998.81	100.0

SD = standard deviation; BMI = body mass index
[$]There was no statistical significant difference between the two group (EPA + DHA vs. Control) in the characteristics.

ASA and another bottle of softgels were administered for the subsequent 4 weeks of treatment. Participants were instructed to store the softgels in a refrigerator and to take five softgels and one ASA tablet daily with their evening meal to reduce the incidence of “fish burps” and to encourage compliance.

2.4. Plasma fatty acid measurements

Levels of fatty acids in plasma were quantified at the three study time points by the well-established gas chromatography/mass spectrometry (GC/MS) method. Lipids were extracted from plasma samples with 2:1 (v/v) chloroform:methanol and 0.24 ml 0.88% KCL [28]. Fatty acid methyl esters were prepared using tetramethylguanidine at 100 °C. Fatty acid methyl esters were analyzed by gas chromatography using a 30-m Omegawax™ 320 (Sigma-Aldrich, St. Louis, MO) capillary column. Oven temperature was started at 175 °C and increased at a rate of 3 °C/min until reaching 220 °C. Flow rate of the carrier gas helium was 30 mL/min. Retention times were compared to standards for fatty acid methyl esters (Sigma-Aldrich, St. Louis, MO, and Matreya, LLC, Pleasant Gap, PA). The resultant values were expressed as % composition or ratios. This is routinely done to express fatty acid values and widely accepted in the field [28].

2.5. Plasma pro-inflammatory cytokine measurements

Levels of IL-1β, IL-6 and TNFα in plasma were quantified using Invitrogen (1600 Faraday Avenue Carlsbad, CA, 92008) Human ELISAs for IL-1β (cat# KHC0014), IL-6 (cat# KHC0061) and TNFα (cat# KHC3011). Assay sensitivity and range for IL-6: < 2 pg/mL and 7.8–500 pg/mL, respectively; for IL-1β: < 0.06 pg/mL and 0.31–20 pg/mL, respectively; for TNF-α: < 1.7 pg/mL, standard curve range of 15.6–1,000 pg/mL. Aliquots of blood plasma were collected and stored at −80 °C until analyzed using the Biotek Powerwave plate reader (Highland Park, PO Box 998, Winooski, VT 05404) at the OSU, College of Nursing Stress Science Laboratory.

2.6. Statistical analyses

Descriptive statistics were used to summarize sample characteristics, stratified by treatment group. Categorical variables are reported as frequencies and percentages, and continuous variables as mean and standard deviation (SD). The balance between EPA + DHA and control groups in baseline measures was tested using Chi-square statistics for categorical variables (e.g., sex) and two-sample *t*-tests for continuous variables (e.g., age and BMI). The effects of EPA + DHA on biomarkers (plasma PUFAs and cytokines) were estimated using mixed effect modeling to adjust for within-subject clustering from repeated measures. In each model, dependent variable was the level of the biomarker and the independent variables included treatment group (EPA + DHA or control), time (0, 4, 8 weeks), and their interaction. From the mixed effect modeling, we estimated the between-group comparisons at each time point and within-group comparisons of the levels of cytokines at 0, 4 and 8 weeks. If a significant baseline between-group difference was found, we adjusted for the baseline differences when conducting between-group comparisons at 4 and 8 weeks using mixed effect modeling. IBM's Statistical Package for the Social Sciences version 21.0 for Windows was used for the data analysis (SPSS, Chicago, IL). All tests were two-sided with significance level at $\alpha = 0.05$.

3. Results

3.1. Participants' characteristics

Descriptive characteristics of the study participants are shown in Table 2. The two experimental groups of older adults were similar in age, sex, race, years of education, BMI, smoking history, wound characteristics and number of comorbidities.

3.2. Plasma levels of polyunsaturated fatty acid

Fig. 1 illustrates the means for select plasma PUFA levels by

Table 2
Sample characteristics at baseline by group.

Characteristics[$]	EPA + DHA (*n* = 16)	Control (*n* = 19)
Age, mean years (SD)	60.3 (12.6)	60.9 (11.8)
Gender, *n* (%)		
Male	10 (62)	11 (58)
Female	6 (38)	14 (75)
Race, *n* (%)		
White	12 (75)	14 (75)
African American	4 (25)	5 (26)
BMI, mean (SD)	40.4 (8.2)	42.7 (13.8)
Wound size in cm^2, mean (SD)	15.6 (34.4)	19.7 (23.2)
Estimated wound age, *n* (%)		
< 6 months	8 (50)	7 (36.8)
≥ 6 months	8 (50)	12 (63.2)
Comorbidities		
Cardiovascular disease, *n* (%)	13 (81)	14 (74)
Diabetes, *n* (%)	8 (50)	7 (34)
Arthritis (rheumatoid, osteo), *n* (%)	10 (63)	8 (42)
Depression, *n* (%)	2 (13)	6 (32)
Smoking history, *n* (%)		
Current smoker	5 (31)	2 (11)
Past smoker	7 (44)	8 (42)
Never	4 (25)	9 (47)

SD = standard deviation; BMI = body mass index
[$] There was no statistical significant difference between the two groups in the characteristics.

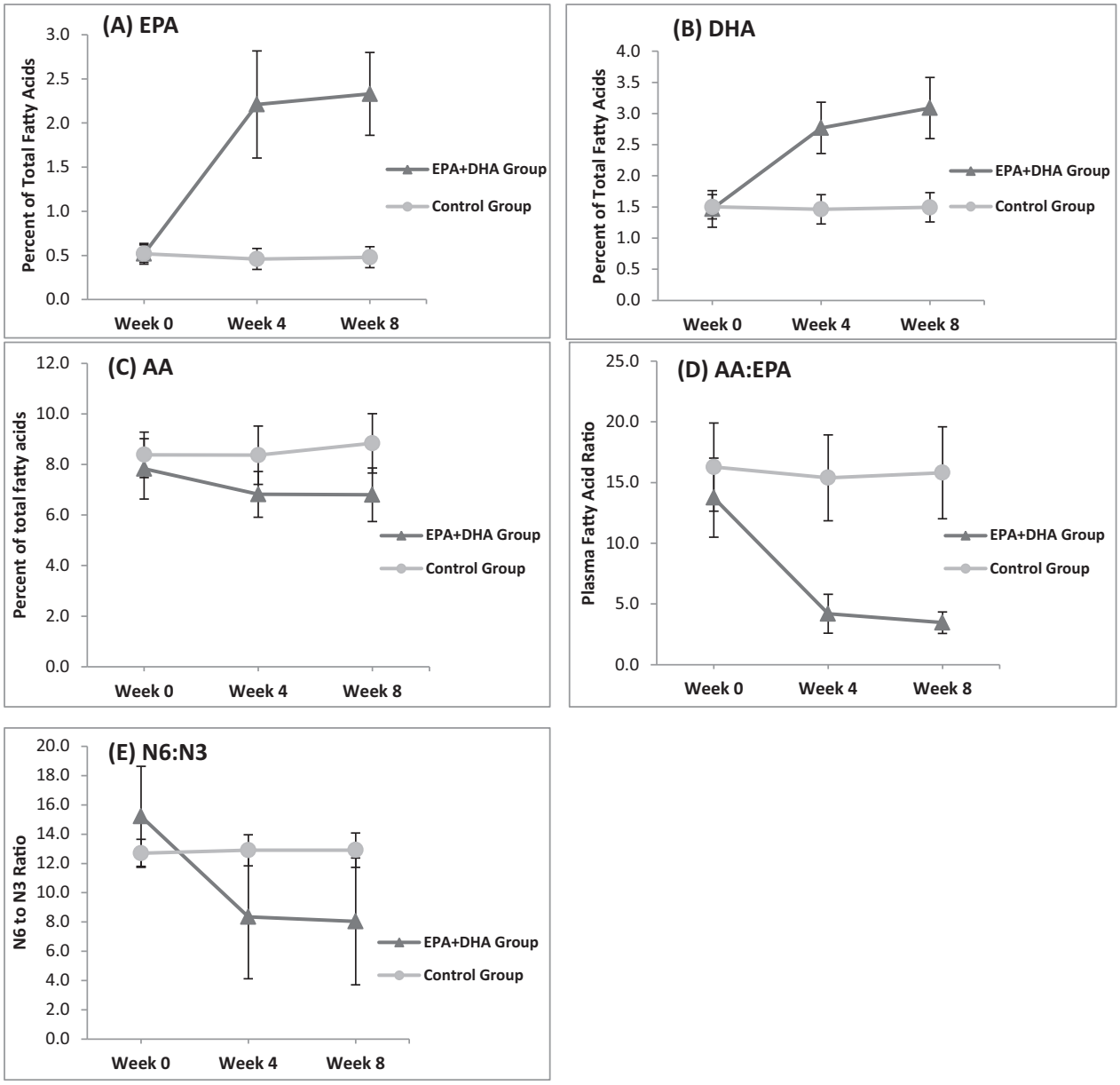

Fig. 1. Comparison of levels of (A) EPA (percent of total fatty acids in plasma), (B) DHA (percent of total fatty acids in plasma), (C) AA (percent of total fatty acids in plasma), (D) AA to EPA ratios (plasma), and (E) N6 to N3 ratios (plasma) between the three study time points in the EPA + DHA Group and the Control Group. Error bars represent the 95% confidence intervals of the group means.

treatment group and study time points. The within-group analysis of the EPA + DHA Group showed that plasma PUFA levels were significantly higher for EPA at Week 4 ($p < 0.001$) and at Week 8 ($p < 0.001$) relative to Week 0 (Fig. 1A). Similarly, DHA levels were significantly higher at Week 4 ($p < 0.001$) and Week 8 ($p < 0.001$) when compared to Week 0 (Fig. 1B). Conversely, levels of AA were significantly lower at Week 4 ($p = .003$) and Week 8 ($p = 0.003$) than at baseline, as was the AA: EPA ratio (Week 4: $p < 0.001$; Week 8: $p < 0.001$) (Fig. 1C), suggesting that the EPA study dose resulted in significantly increased proportions of EPA in the plasma that occurred partly at the expense of AA. Further, the n-6:n-3 ratio was significantly reduced over time in the EPA + DHA group (Week 4: $p < 0.001$; Week 8: $p < 0.001$). In comparison, there were no significant changes in the levels of EPA, DHA, AA, AA:EPA or n-6:n-3 across time in the Control Group.

As expected, the between-group comparisons showed that at Week 4 post enrollment, the EPA + DHA Group demonstrated significantly higher plasma levels of EPA ($p < 0.001$) and DHA ($p < 0.001$), lower levels of AA (marginally significant, $p = 0.055$), and significantly lower ratios of AA:EPA ($p < 0.001$) and n-6:n-3 ($p = 0.040$) than the Control Group. The same pattern was noted when comparing levels of EPA ($p < 0.001$), DHA ($p < 0.001$), AA ($p = 0.010$) and ratios of AA: EPA ($p < 0.001$) and n-6:n-3 ($p = 0.030$) between the two groups at Week 8 post enrollment (Fig. 1).

3.3. Plasma levels of cytokines IL-6, IL-1β and TNF-α

Table 3 provides a summary of the results for plasma levels of IL-6, IL-1β and TNF-α by treatment group and study time points. The within-group analysis of the EPA + DHA Group showed that plasma IL-6 levels were significantly lower at Week 4 ($p = 0.008$) and at Week 8 ($p < 0.001$) relative to Week 0. Similarly, IL-1β levels were significantly lower at Week 4 ($p < 0.001$) and Week 8 ($p < 0.001$) versus Week 0, and at Week 8 versus Week 4 ($p = 0.008$). Following this same pattern, levels of TNF-α were also lower at Week 4 ($p < 0.001$) and at Week 8

Table 3
Treatment effects (EPA + DHA vs. control)[a] on cytokines (natural log-transformed).

	Week 0 (Baseline)	Week 4	Week 8	Change week 0–4	Difference in change vs. control[a]	*P*-value[b]	Change week 0–8	Difference in change vs. control[a]	*P*-value[b]
***Log* (IL-6)**									
EPA + DHA	2.45 (0.14)	2.15 (0.15)	1.90 (0.17)	−0.31 (0.04)	−0.37 (0.12)	0.003	−0.55 (0.10)	−0.48 (0.12)	0.001
Control	1.89 (0.11)	1.96 (0.14)	1.83 (0.11)	0.06 (0.10)			−0.07 (0.08)		
***Log* (IL-1β)**									
EPA + DHA	2.52 (0.13)	1.78 (0.10)	1.39 (0.10)	−0.74 (0.12)	−0.83 (0.14)	< 0.001	−1.12 (0.17)	−1.15 (0.18)	< 0.001
Control	1.25 (0.12)	1.34 (0.13)	1.83 (0.11)	0.09 (0.08)			0.02 (0.06)		
***Log* (TNF-α)**									
EPA + DHA	3.90 (0.18)	3.45 (0.21)	3.01 (0.20)	−0.45 (0.09)	−0.41 (0.11)	0.001	−0.89 (0.14)	−0.85 (0.15)	< 0.001
Control	3.00 (0.20)	2.96 (0.20)	2.96 (0.87)	−0.04 (0.06)			−0.04 (0.07)		

[a] Least squares means (SE).
[b] Comparison to control, derived from multilevel modeling.

($p < 0.001$) versus Week 0, and at Week 8 compared to Week 4 ($p = 0.005$). No significant within-group changes for levels of IL-6, IL-1β and TNF-α were detected in the Control Group over the study interval.

The between-group comparisons showed that levels of IL-6, IL-1β and TNF-α were significantly higher in the EPA + DHA Group at Week 0 compared to the Control Group ($p = 0.024$, $p < 0.001$ and $p < 0.001$, respectively). After adjusting for baseline difference, the treatment group had significant lower levels of IL-6 ($p = 0.008$), IL-1β ($p < 0.001$), and TNF-α ($p < 0.001$) at Week 4 compared to the control group. The treatment group also had significantly lower IL-6 ($p = 0.007$), IL-1β ($p < 0.001$), and TNF-α ($p < 0.001$) at Week 8 than the control group, after adjusting for the baseline differences.

4. Discussion

We examined changes in fasting blood plasma levels of proinflammatory cytokines IL-6, IL-1β, and TNF-α in aging adults before and after supplementing diets with EPA + DHA or placebo. In support of our primary hypothesis, we found that EPA + DHA therapy had a significant lowering effect on levels of all inflammatory factors quantified after 4 weeks of therapy and an even greater lowering effect after 8 weeks of therapy. Specifically, IL-6 decreased by 12% (0–4 weeks) and 22% (0–8 weeks) in the EPA + DHA Group, compared to a 3% and 4% increase, respectively, in the Control Group. Similarly, the EPA + DHA group showed a 29% and a 44% decrease in IL-β by 4 and 8 weeks, respectively, compared to 8% and 2% increases in the Control Group. TNF-α also decreased in the EPA + DHA Group by 12% and 23% (4 and 8 weeks, respectively), compared to a modest 1% decrease at both time points in the Control Group. As expected, levels of IL-6, IL-1β, and TNF-α were all significantly lower in the EPA + DHA Group than the Control Group at both the 4- and 8-week time points.

High levels of circulating proinflammatory cytokines are characteristic of inflammaging, the chronic low-grade inflammation that occurs with aging and is in part due to the culminating manifestation of immunosenescence, a low functioning immune system [2]. For many older adults, immunosenescence eventually leads to higher levels of proinflammatory than anti-inflammatory cytokines in systemic circulation and thus greater risk for accelerated aging [29], age-related diseases [30], and a reduced life expectancy [2,31,32]. Lowering chronically high levels of circulating proinflammatory cytokines may temper systemic inflammation and thus help prevent, delay the onset, or reduce symptoms of many common age-related conditions. In a recent meta-analysis of 68 randomized clinical trials, marine-derived n-3 PUFAs EPA and DHA was shown to have a significant lowering effect on IL-6 and TNF-α levels in adults of various ages with chronic non-autoimmune diseases and healthy people [21]. We hypothesized that EPA + DHA therapy might be particularly effective in lowering levels of IL-6, IL-1β, and TNF-α in older adults at risk for high levels of circulating proinflammatory factors because of: (1) advanced age, (2) a chronic inflammatory condition (chronic venous leg ulcers), and (3) typical Western diets. Here, we demonstrate that a combined dose of 2.5 g/d of EPA + DHA significantly lowers levels of IL-6, IL-1β, and TNF-α in blood plasma after just 4 weeks of therapy in older adults who made no other dietary changes.

Our finding of greater lowering effects on IL-6, IL-1β, and TNF-α with longer durations of EPA + DHA therapy (8 weeks versus 4 weeks) is understandable if we consider that changing EPA and DHA composition of cell membrane and tissues versus serum or plasma requires a relatively longer interval of therapy, which was shown by Katan et al. [33]. In their study, changes in fatty acid composition in serum, erythrocyte membranes and adipose tissue were assessed over 18 months in response to fish oil intake. They found that levels of EPA plateaued after 4–8 weeks in serum, but that the plateau in erythrocyte membranes and gluteal fat tissue was not seen until supplementation for 6 months and 12 months, respectively, suggesting that the greatest lowering effect on inflammatory mediators produced by some cell types may not be seen until therapy has been administered for several months. Thus, the results observed in the present study that the lowering effect of EPA + DHA therapy on IL-6, IL-1β, and TNF-α is stronger with the extension of therapy is consistent with the study by Katan et al. [33].

Of additional interest are our findings that although the average BMI for both study groups was $>40\ kg/m^2$, the lowering effects of EPA + DHA therapy on IL-6, IL-1β, and TNF-α levels were still significant, which differs from findings from a recent meta-analysis reporting that lowering effects were weakened when BMIs were greater than $30\ kg/m^2$ [21]. Total n-6 PUFAs and arachidonic acid levels in adipose tissue have been shown to increase in tandem with BMI and as known, arachidonic acid generates numerous proinflammatory eicosanoids through the cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) pathways that upregulate proinflammatory cytokine synthesis [34]. However, most studies in the meta-analysis used a much lower n-3 PUFA dose than what was used in the current study, which may explain why we found the significant lowering effect on IL-6, IL-1β, and TNF-α levels in our participants with high BMIs receiving EPA + DHA therapy. Moreover, collective findings from previous human *ex vivo* models suggest that EPA + DHA supplementation in a dose ≥ 2 g/d is needed to reduce proinflammatory cytokine production [1]. The collective data suggest that although n-3 PUFAs preferentially compete with n-6 PUFAs

for COX and 5-LOX and are incorporated into cell membrane phospholipids at the expense of arachidonic acid, a higher EPA + DHA dose may be needed for obese versus non-obese people to adequately replace high levels of arachidonic acid in adipose tissue and ultimately reduce levels of proinflammatory factors. Our EPA + DHA dose of 2.5 g/d was sufficient to significantly increase plasma levels of EPA and DHA, and significantly reduce the AA:EPA ratios and levels of proinflammatory factors in plasma after 4 weeks of therapy in obese, older adults. Moreover, the study dose fell within the FDA's approval for safety and thus would appear to be a good choice for future studies.

Although we expected our older adult participants to have elevated plasma levels of the proinflammatory cytokines (IL-6, IL-1β, TNF-α) relative to healthy younger adults, the values were higher than what some previous studies have reported for similarly aged people with chronic conditions such as depression and/or mild cognitive impairment [35,36]. Moreover, the levels of the proinflammatory factors measured in the current study were higher than reported in a study by Kim and colleagues who assessed 22 cytokines, chemokines, and growth factors in a gender-matched, healthy cohort of young (< 65 years of age) and older adults (≥65 years of age) [37]. In their study, no significant differences were found in levels of IL-6, IL-1β or TNF-α between the two groups. The high levels we detected in both of our study groups are likely due to the presence of multiple factors associated with elevated levels of circulating proinflammatory elements; aging, multiple inflammatory-related diseases, high BMIs and high plasma n-6:n-3 PUFA ratios reflective of typical Western diets. Moreover, the significantly higher baseline levels of IL-6, IL-1β and TNF-α in the EPA + DHA versus the Control Group could have contributed to the strength of the lowering effect of EPA + DHA therapy on these levels over time. These findings are in line with previous studies reporting greater lowering effects of EPA + DHA supplementation on levels of proinflammatory cytokines in people with higher baseline levels than those with lower levels; characteristic of older adults versus younger adults and people with inflammatory diseases versus healthy people [21].

EPA + DHA supplementation is a promising low-risk, low-cost therapy for moderating the significant negative clinical effects of inflammaging in aging adults because they may reduce elevated circulating levels of proinflammatory factors such as IL-6, IL-1β and TNF-α. The mechanisms of action by which EPA + DHA assuage these proinflammatory factors involve: (1) generating eicosanoids that are weak chemoattractants for cells that secrete proinflammatory cytokines, and (2) blocking key transcription factors (e.g., NFkB) that upregulate gene expression of proinflammatory cytokines. For this study, we hypothesized that EPA + DHA therapy would significantly reduce levels of these proinflammatory factors in plasma over an 8-week interval. We also hypothesized that the laboratory analyses of plasma PUFAs would show low levels of EPA + DHA and high total n-6:n-3 ratios at baseline, indicating study participants had been consuming a typical Western diet, and indeed, the data confirmed our hypotheses. On entry into the study, the average plasma n-6:n-3 ratio for the group as a whole was 14:1. Further, plasma levels of EPA + DHA were only 2% of the total fatty acids. These findings are in line with previous reports that on average adults in the U.S. consume diets with scant amounts of EPA and DHA (90 mg/d) [38] and high n-6:n-3 ratios (>10:1) that are linked to high levels of systemic inflammation [8,9]. Given what is known about the dietary PUFA intake of U.S. adults and the positive relationship between high n-6:n-3 ratios and inflammation, we can surmise that Western diets are potentiating the effects of obesity and aging on inflammaging, and thus contributing to several inflammation-related conditions commonly experienced by aging adults such as nonhealing wounds which often do not progress through the healing stages because of chronic inflammation.

Traditionally, chronological age alone has not been a useful indicator of morbidities and mortality since there is considerable evidence that people age very differently in terms of age-related diseases and disabilities [39]. Efforts to develop an aging metric based on genetic profiles, epigenetic changes and telomere length have met with limited success [40]. Measuring biomarker signatures of inflammation, such as IL-6, TNF-a, and IL-1β that change with aging may be an effective way to assess for inflammaging in older adults and thus risk for inflammatory diseases with high morbidity and mortality rates [41]. Equally important is developing low-risk interventions that effectively moderate or prevent age-related changes in inflammation and immune function. Data from the current study suggest that EPA + DHA therapy may be one intervention to consider for tempering the harmful effects of inflammaging. In addition to their anti-inflammatory effects, EPA + DHA also have antiplatelet actions, but without affecting the risk of clinically significant bleeding [42]. Although the FDA recommends periodic monitoring of people taking higher doses of EPA + DHA (e.g., Lovaza) in conjunction with the anticoagulant warfarin, most studies indicate that EPA + DHA doses of even 3–6 g/d do not significantly affect the anticoagulant status of patients taking warfarin [43].

Limitations of this study are that the intervention timeline was relatively short (8 weeks) and the sample size was small. Moreover, circulating anti-inflammatory cytokines were not quantified and thus we could not determine potential differences in anti-inflammatory responses between the groups which may have somewhat reduced or balanced proinflammatory responses. Additionally, the generalizability of the findings is restricted to obese adults in middle to late adulthood with multiple chronic inflammatory conditions. As such, it is not known if the same outcomes would emerge in a sample of aging adults of normal weight with fewer comorbidities consuming the same EPA + DHA dose for the same interval of time. Despite these limitations, our findings are persuasive for EPA + DHA therapy lowering elevated levels of circulating proinflammatory factors IL-6, IL-1β and TNF-α in aging adults. These results warrant a larger clinical trial given the rapidly growing aging population, the increasing rates of inflammation-related diseases, and the continued consumption of foods with high amounts of n-6 PUFAs relative to n-3 PUFAs.

5. Conclusion

The current study found that EPA + DHA supplementation of 2.5 g/d resulted in significantly reduced n-6:n-3 PUFA ratios, AA:EPA ratios and levels of select proinflammatory cytokines in the circulation of aging adults with CVLUs after 4 and 8 weeks of therapy. Importantly, participants reported no unfavorable symptoms related to the therapy, which is in agreement with the low-incidence described in larger clinical trials of EPA + DHA supplementation [44,45]. Currently, the recommended daily intake of EPA and DHA varies by organization and expert group, ranging from 250 to 500 mg/d for healthy adults [46]. However, unless several meals containing fatty fish are consumed each week it is difficult to reach these recommended intake levels with diet alone. Further, the general consensus is that higher combined EPA + DHA intake levels are needed for anti-inflammatory effects to occur [1]. In future studies, we recommend that investigators test EPA + DHA therapy for longer intervals of time in both obese and non-obese samples. We also advise evaluating proposed genetic variation in PUFA metabolism to determine if EPA + DHA supplementation should be individualized. Long-term EPA + DHA therapy may be a safe, relatively lost-cost dietary intervention to help prevent or assuage inflammaging and the associated diseases that greatly diminish quality of life for aging adults.

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Supplementary materials

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