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Omega-3 supplementation lowers inflammation and anxiety in medical students: A randomized controlled trial

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ABSTRACT

Observational studies have linked lower omega-3 (*n*-3) polyunsaturated fatty acids (PUFAs) and higher omega-6 (*n*-6) PUFAs with inflammation and depression, but randomized controlled trial (RCT) data have been mixed. To determine whether *n*-3 decreases proinflammatory cytokine production and depressive and anxiety symptoms in healthy young adults, this parallel group, placebo-controlled, double-blind 12-week RCT compared *n*-3 supplementation with placebo. The participants, 68 medical students, provided serial blood samples during lower-stress periods as well as on days before an exam. The students received either *n*-3 (2.5 g/d, 2085 mg eicosapentaenoic acid and 348 mg docosahexanoic acid) or placebo capsules that mirrored the proportions of fatty acids in the typical American diet. Compared to controls, those students who received *n*-3 showed a 14% decrease in lipopolysaccharide (LPS) stimulated interleukin 6 (IL-6) production and a 20% reduction in anxiety symptoms, without significant change in depressive symptoms. Individuals differ in absorption and metabolism of *n*-3 PUFA supplements, as well as in adherence; accordingly, planned secondary analyses that used the plasma *n*-6:*n*-3 ratio in place of treatment group showed that decreasing *n*-6:*n*-3 ratios led to lower anxiety and reductions in stimulated IL-6 and tumor necrosis factor alpha (TNF-α) production, as well as marginal differences in serum TNF-α. These data suggest that *n*-3 supplementation can reduce inflammation and anxiety even among healthy young adults.

The reduction in anxiety symptoms associated with *n*-3 supplementation provides the first evidence that *n*-3 may have potential anxiolytic benefits for individuals without an anxiety disorder diagnosis. ClinicalTrials.gov identifier: NCT00519779.

1. Introduction

1.1. Inflammation, mental and physical health, and omega-3

Chronic inflammation has been linked to a broad spectrum of health problems including cardiovascular disease, stroke, and rheumatoid arthritis (Shelton and Miller, 2010; Wall et al., 2010; Wilson, 2008). Large population studies suggest that greater fish consumption may help control or protect against the onset of these and other inflammatory conditions (Breslow, 2006; Larsson et al., 2011; Wall et al., 2010). Fish oil is the prime source for the two key omega-3 (*n*-3) polyunsaturated fatty acids (PUFAs), eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA). Diets high in *n*-3 PUFAs may also benefit mood and behavior, particularly depression.

Epidemiological data have demonstrated inverse relationships between annual fish consumption and depression—the more fish eaten, the lower the prevalence of serious clinical depression as well as depressive symptoms (Golding et al., 2009; Hibbeln, 1998; Tanskanen et al., 2001). Observational studies have also associated lower *n*-3 PUFA plasma levels with depressed mood in both psychiatric and nonpsychiatric populations (Kiecolt-Glaser et al., 2007; Lin et al., 2010; Tiemeier et al., 2003). However, a recent meta-analysis of randomized controlled trials concluded that *n*-3 PUFA supplementation benefited clinically depressed individuals, but not those with less severe depressed mood (Appleton et al., 2010).

Comorbid depressive and anxiety disorders occur frequently, and some symptoms are common to both (Ross, 2009); thus,

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n-3's relationship to anxiety is also of interest. Although lower *n*-3 PUFA plasma levels have been reported in patients with social anxiety disorder (Green et al., 2006), the results from two small placebo-controlled trials were mixed (Buydens-Branchey et al., 2008; Fux et al., 2004).

Both depression and anxiety can enhance the production of proinflammatory cytokines (Glaser and Kiecolt-Glaser, 2005; Raison et al., 2006; Steptoe et al., 2007). Inflammatory mechanisms have been implicated in the pathophysiology of depression, and stressful experiences that sometimes precipitate depression can also boost proinflammatory cytokine production (Raison et al., 2006). The *n*-3 PUFA's antidepressant properties may be related to its ability to dampen inflammatory responses (Maes et al., 2000; Sijben and Calder, 2007).

1.2. PUFAs and inflammation, mechanistic pathways

Dietary intakes of both the *n*-3 and omega-6 (*n*-6) PUFAs influence inflammation. Arachidonic acid (AA) is an *n*-6 polyunsaturated fatty acid derived from linoleic acid. The eicosanoids produced from enzymatic hydroxylation of AA increase proinflammatory cytokine production (Maes and Smith, 1998). In contrast, the eicosanoids derived from *n*-3 PUFAs curb the production of AA-derived eicosanoids (Maes and Smith, 1998; Pischon et al., 2003). Thus, both higher plasma levels of *n*-3 PUFAs as well as lower plasma *n*-6:*n*-3 ratios should restrain proinflammatory cytokine production (Ferrucci et al., 2006). However, the evidence has been decidedly mixed; epidemiological and observational studies have provided more consistent support for *n*-3 PUFA's anti-inflammatory properties than RCTs (Sijben and Calder, 2007).

In a provocative study addressing stress-related inflammatory change, unsupplemented medical students who had higher *n*-6:*n*-3 ratios (above the mean at baseline, several weeks before exams) demonstrated greater increases in lipopolysaccharide (LPS) stimulated tumor necrosis factor alpha (TNF-α) production during exams than those with lower levels (Maes et al., 2000). These findings suggest that relatively modest differences in baseline *n*-3 PUFA dietary status could influence stress-related changes in proinflammatory cytokine production.

Data from older adults suggested that depressive symptoms and *n*-6:*n*-3 ratios can work together to enhance inflammation beyond the contribution provided by either variable alone (Kiecolt-Glaser et al., 2007). Although cytokine levels were fairly consistent across *n*-6:*n*-3 ratios when depressive symptoms were low, higher *n*-6:*n*-3 ratios were associated with progressively elevated TNF-α and interleukin 6 (IL-6) levels as depressive symptoms increased. These observational studies (Kiecolt-Glaser et al., 2007; Maes et al., 2000) suggest that PUFAs may influence the magnitude of inflammatory responses to stress and depression.

1.3. The present study

We hypothesized that *n*-3 PUFA supplementation would decrease proinflammatory cytokine production in contrast to placebo. Moreover, we also expected that supplementation would be protective during stressors, blunting stress-related increases in proinflammatory cytokine production. As a secondary hypothesis, *n*-3 PUFA supplementation would lower anxiety and depressive symptoms, and would dampen the heightened negative mood symptoms frequently observed during exams as well. We also compared the utility of statistical analyses that used changes in the plasma and peripheral blood mononuclear cell (PBMC) PUFA *n*-6:*n*-3 ratio as continuous measures with those that simply used *n*-3 PUFA supplementation vs. placebo.

2. Methods

2.1. Participants

The 68 first- and second-year medical students (38 men and 30 women) ranged in age from 21 to 29 (mean = 23.65, SD = 1.87). The study included five cohorts with 9–17 students per cohort; data collection began in August, 2007 and ended in December, 2009. Exclusion criteria included high fish intake, fish oil or flaxseed supplements, smoking, alcohol or drug abuse, any chronic illness with an inflammatory or endocrine component, lipid-altering drugs, beta blockers, steroids, ACE-inhibitors, regular use of non-steroidal anti-inflammatories, and use of psychoactive drugs or mood altering medications. The institutional review board approved this study, and each participant provided informed consent.

2.2. Study design

This double-blind RCT compared 2.496 g *n*-3/day (2085 mg of eicosapentaenoic acid (EPA) and 348 mg of docosahexaenoic acid (DHA) with placebo, a mixture of palm, olive, soy, canola, and coco butter oils that approximated the saturated:monounsaturated: polyunsaturated (SMP) ratio consumed by US adults, 37:42:21 (USDA Continuing Survey of Food Intake by Individuals, 1994–1996). We chose the 7:1 EPA/DHA balance because of evidence that EPA has relatively stronger anti-inflammatory and antidepressant effects than DHA (Lin et al., 2010; Sijben and Calder, 2007). OmegaBrite (Waltham, MA) supplied both the *n*-3 and the matching placebo; all pills were coated with a fuchsia coloring. OmegaBrite added a mild fish flavor to the placebo to help disguise any differences between the *n*-3 PUFA pills and the placebo, and we told participants about the fish flavoring to promote blindness (Stoll et al., 2001). Table 1 shows the results of our independent analysis of the fatty acid profile of the *n*-3 and placebo pills.

Blood samples were collected in the Ohio State Clinical Research Center at a lower-stress or non-exam baseline (visit 1) and again on the day before a major exam (visit 2). The next four data points assessed how 12 weeks of supplementation affected responses both during lower and higher stress periods, as students were evaluated twice between exams, visits 3 and 5, and twice on the day before a major exam, visits 4 and 6 (Fig. 1). Sample 4 was collected roughly 6 weeks after supplementation had been initiated, while sample 6 was collected about 3 months after supplementation, providing data on the kinetics of change. Students fasted overnight prior to each visit; a standardized prepackaged breakfast was provided outside the lecture room before classes, so that the blood samples collected midday (11:00 AM to 1:00 PM) would not reflect recent dietary variations.

Table 1
Fatty acid composition of the placebo and *n*-3 dietary supplements given to study participants as determined by independent analysis.

		Placebo % Fatty acid	*n*-3 Supplement % Fatty acid
C14:0	Myristic acid	3.1	0.0
C16:0	Palmitic acid	16.4	0.1
C18:0	Stearic acid	3.2	0.5
C18:1n9	Oleic acid	48.7	0.7
C18:1n7	Vaccenic acid	1.6	0.3
C18:2n6	Linoleic acid	21.5	0.2
C18:3n3	Alpha linolenic acid	3.3	0.2
C18:4n3	Stearidonic acid	0.1	6.4
C20:4n6	Arachidonic acid	0.1	3.2
C20:4n3	Eicosatetraenoic acid	0.0	1.0
C20:5n3	Eicosapentaenoic acid	1.0	76.8
C22:6n3	Docosahexaenoic acid	0.1	8.5

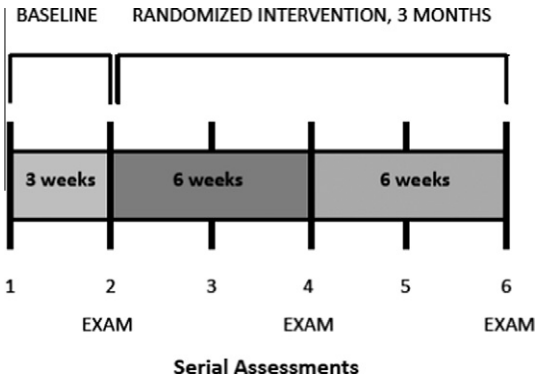

Fig. 1. Schematic of the study protocol. Blood samples were collected at a lower-stress or non-exam baseline (visit 1) and again on the day before a major exam (visit 2). The next four data points assessed how 12 weeks of supplementation affected responses both during lower and higher stress periods, as students were evaluated twice between exams, visits 3 and 5, and twice on the day before a major exam, visits 4 and 6.

2.3. Randomization

At the end of the second session, students were randomized to *n*-3 or placebo using a computer-generated permuted block randomization sequence, with six students per block. The data manager who prepared and maintained the randomization sequence had no involvement in other aspects of the research, including data collection and biological laboratory analyses, and she was the only person who had the randomization list. The active and placebo study medications were packaged according to the randomization sequence, so that the student was randomized when s/he was assigned to the next available individual supply of study medication. At each subsequent visit, students returned unused pills and received the next set.

2.4. Health-related behaviors

At baseline, nurses assessed height, weight, and central adiposity. Adipose tissue in the abdomen may secrete up to three times as much IL-6 as other subcutaneous fat tissues (Browning, 2003). Sagittal abdominal diameter (SAD) measurements provided data on abdominal fat. Validational studies using computerized axial tomography and dual-energy X-ray absorptiometry have demonstrated SAD's utility as a noninvasive central adiposity measure (Clasey et al., 1999).

At visits 1 and 6, students completed the Women's Health Initiative Food Frequency Questionnaire (FFQ), providing data on the type, frequency, and quantity of foods and beverages consumed in the past 90 days (Patterson et al., 1999). The precision is similar to other FFQs, and means are within 10% of dietary records or recalls. Software calculations estimated dietary intake of energy, macro- and micronutrients, as well as intake of key food groups using the Nutrition Data Systems for Research. Participants were asked not to take any other omega-3 supplements other than those provided by the study, and not to change their diet during the trial.

The Pittsburgh Sleep Quality Index, administered at visits 1 and 6, assessed sleep quality and disturbances over a one-month interval; it has good diagnostic sensitivity and specificity (Buysse et al., 1989). A tailored version of the Pittsburgh Sleep Diary that students completed daily for four days before each visit provided data on sleep, caffeine, alcohol, exercise, and medications (Monk et al., 1994).

The Seven-Day Physical Activity Recall assessed the weekly frequency and duration of various physical activities at visits 1 and 6 (Taylor et al., 1984). One of the most widely used physical activity assessments in epidemiological research as well as exercise science; it has an excellent history of validation and testing.

The modified version of the Health Review, administered at each visit, provided reliable data on infectious illness symptoms as well as possible supplementation side effects (Jenkins et al., 1980; Orts et al., 1995). Developed to provide a simple reliable and valid method for periodic assessment of infectious illness, the symptoms assessed also include the primary gastrointestinal side effects described for *n*-3 PUFA supplementation.

2.5. Depressive and anxiety symptoms

The Center for Epidemiological Studies Depression Scale (CES-D), administered at all visits, has been used extensively to measure depressive symptomatology (Basco et al., 1997; Radloff, 1977). Studies have shown acceptable test–retest reliability and excellent construct validity (Basco et al., 1997).

The Beck Anxiety Inventory assesses both cognitive and physiological symptoms (Beck et al., 1988). Developed to discriminate anxiety from depression while displaying convergent validity (Beck et al., 1988), the measure appears to be better at discriminating anxiety from depression than other self-report anxiety scales, and it has good test–retest reliability. The scale was administered at each visit.

2.6. Fatty acid analyses

Lipids were extracted from plasma and PBMCs using chloroform:methanol (2:1, v/v) with 0.2 vol. 0.88% KCl (Bligh and Dyer, 1959). Fatty acid methyl esters of the fractions were prepared by incubating the fractions with tetramethylguanidine at 100 °C (Shantha et al., 1993) and analyzed by gas chromatography (Shimadzu, Columbia, MD) using a 30-m Omegawax 320 (Supelco-Sigma) capillary column. The helium flow rate was 30 ml/min and oven temperature ramped beginning at 175 °C and held for 4 min then increased to 220 °C at a rate of 3 °C/min as previously described (Belury and Kempa-Steczko, 1997). Retention times were compared to authentic standards for fatty acid methyl esters (Supelco-Sigma, St. Louis, MO and Matreya, Inc., Pleasant Gap, PA).

2.7. Cytokine assays

Our primary outcome was changes in serum and stimulated production of IL-6 and TNF-α because elevated levels of these cytokines are associated with an activated inflammatory response. Serum and lipopolysaccharide (LPS) stimulated production levels of interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) were multiplexed and measured using an electrochemilluminescence method with Meso Scale Discovery kits, and read using the Meso Scale Discovery Sector Imager 2400. The stored serum and culture supernatant samples for each subject were assayed for all the cytokine markers in one run, thus using the same controls for all six time points for each person. Sensitivity for the serum cytokines is 0.3 pg/ml. The intra-assay coefficient of variation for IL-6 is 2.8%, and the inter-assay coefficient of variation is 12.5%; corresponding values for TNF-α are 4.3% and 12.1%.

LPS-induced culture supernatants have been widely used to assess the *n*-3 PUFA's immunomodulatory effects, in part because T- and B-cell proliferation and differentiation is an important aspect of the adaptive immune response (Fritsche, 2006), To assess LPS-stimulated cytokine production, PBMC cultures, 1×10^6 cells/ml, were incubated for 24 h in 3 ml RPMI 1640 media containing 10% human male serum either with or without 1.0 μg/ml LPS. After 24 h the cells were pelleted by centrifugation

(2000 rpm for 5 min) and the supernatants removed and stored at −80 °C. The dose and duration were based on evidence that the effects of dietary *n*-3 supplementation are best demonstrated through the use of low LPS concentrations to stimulate PBMCs (Calder, 2001; Fritsche, 2006). Sensitivity for LPS-stimulated cytokines is 2.4 pg/ml. The intra-assay coefficient of variation for IL-6 is 4.56% and the inter-assay coefficient of variation is 13.67%. Corresponding values for TNF-α are 3.16% and 9.08%.

2.8. Sample size

Sample size was based on detecting changes in the primary endpoint (cytokine levels), with the assumptions of a two-sided 5% significance level and 10% loss to follow-up. Maes et al. (2000) found stress-related increases in cytokine levels in a low *n*-3 group to be up to six times lower than a high *n*-3 group; we based our sample size on a conservative target of a threefold difference. To detect a stress-related increase in cytokine levels in the placebo group that was three times higher than the control group, the target sample size of 30 subjects per treatment group would have 90% power.

2.9. Statistical methods

Mixed models were used to test the effects of supplementation (Diggle et al., 2002). This type of model treats the responses from each subject as repeated measures, accounting for the within-subject correlation. An unstructured variance–covariance structure was used to estimate error variance, since the study design of alternating stress and non-stress visits did not support an assumption of compound symmetry, using the PROC MIXED procedure in SAS 9.1 with a REPEATED statement (SAS Institute, Cary, NC). In models for serum cytokines a random effect for the assay plate was also included. To test for treatment group differences, models included the effects of visit, treatment group, and their interaction, adjusting for baseline levels as covariates. Across all outcomes and treatment groups there were no significant differences between the stress (visit 1) and non-stress (visit 2) time points, so the average response at visits one and two was taken as the baseline measurement. Outcome variables were natural log transformed when residual analyses suggested violation of the normality assumption.

Secondary analyses used changes in plasma and PBMC *n*-6:*n*-3 ratios as continuous measures in place of group assignment; these analyses were preplanned and no adjustment was made for multiple analyses as these were considered exploratory analyses. Differences in the proportions experiencing nonserious adverse events between treatment groups were evaluated using Fisher's exact test. James' blinding indices were calculated using participant and experimenter guesses about treatment assignment at the final study visit (James et al., 1996). Alpha was set to 0.05, and two-sided tests were conducted.

3. Results

3.1. Study population, diet, and health behaviors

Randomized groups were equivalent on key dimensions (Table 2). Randomization produced balanced groups that did not differ on age, weight, BMI, baseline FFQ dietary variables, and health-related variables (including sleep and medications), $p > 0.1$ for all tests.

Analyses of FFQ data at the last visit (week 6) revealed no significant changes in self-reported dietary intake of saturated-, mono-, and *n*-6 poly-unsaturated fatty acids, though subjects generally reported lower intakes of macronutrients at visit 6

Table 2
Baseline characteristics.

	Placebo (*N* = 34)	Supplement (*N* = 34)
Age (years)	23.4 (1.7)	23.9 (2.0)
Female	14 (41%)	16 (47%)
Race		
White	25 (74%)	21 (62%)
Black	0 (0%)	2 (6%)
Asian	5 (15%)	7 (21%)
Other	4 (12%)	4 (12%)
Sagittal abdominal diameter (cm)	18.2 (2.7)	17.7 (2.2)
Beck anxiety[a]		
Median (IQR)	2.5 (1–4.5)	3.5 (2–6)
Range	0–20	0–14
CES-D[a]		
Median (IQR)	5.25 (3.5–7.5)	6.25 (4–8)
Range	1–16.5	1–15.5

Data are mean (SD) or *n* (%) except where noted. There were no significant group differences on any of the participant characteristics ($p > 0.1$ for all tests).
[a] Average of the two baseline visits.

Table 3
Self-reported diet (Food Frequency Questionnaire), physical activity, and sleep[a].

	Baseline (week 1)	Change visits 1–6	*P* for change[b]	*P* for comparison of changes[c]
Calories (g)				
Placebo	2297 ± 144	−266 ± 100	0.01	0.61
Supplement	2114 ± 145	−193 ± 98	0.05	
Fiber (g)				
Placebo	24 ± 1.9	−1.9 ± 1.1	0.10	0.51
Supplement	24 ± 1.9	−0.8 ± 1.1	0.47	
Fat (g)				
Placebo	79 ± 6.3	−7.2 ± 4.2	0.09	0.86
Supplement	72 ± 6.4	−6.2 ± 4.1	0.14	
Protein (g)				
Placebo	102 ± 6.2	−11 ± 5.0	0.04	0.99
Supplement	91 ± 6.3	−11 ± 4.9	0.04	
SFA (g)				
Placebo	27 ± 2.3	−2.8 ± 1.5	0.07	0.70
Supplement	24 ± 2.3	−2.0 ± 1.5	0.20	
MUFA (g)				
Placebo	30 ± 2.5	−2.6 ± 1.7	0.12	0.73
Supplement	27 ± 2.5	−1.8 ± 1.6	0.27	
PUFA (g)				
Placebo	15 ± 1.2	−1.2 ± 0.9	0.17	0.79
Supplement	14 ± 1.2	−1.6 ± 0.9	0.08	
Omega-3 FA (g)				
Placebo	1.5 ± 0.12	−0.1 ± 0.1	0.15	0.59
Supplement	1.4 ± 0.12	−0.2 ± 0.1	0.03	
Linoleic acid (g)				
Placebo	14 ± 1.1	−1.1 ± 0.8	0.20	0.82
Supplement	12 ± 1.1	−1.3 ± 0.8	0.10	
Hours of exercise per week				
Placebo	4.5 ± 0.5	−2.2 ± 0.6	0.001	0.35
Supplement	3.3 ± 0.5	−1.3 ± 0.6	0.04	
Sleep score (PSQ)				
Placebo	3.8 ± 0.3	0.1 ± 0.3	0.62	0.79
Supplement	4.0 ± 0.3	0.3 ± 0.3	0.39	
Weight (lb)				
Placebo	162 ± 5.8	−0.8 ± 0.8	0.35	0.60
Supplement	152 ± 5.8	−0.1 ± 0.8	0.86	

[a] All values are means ± SEMs from mixed effects models. Tests are for orthogonal contrasts in the models. Fatty acids are from self-reported diet only as measured by the FFQ, and do not reflect plasma or PBMC changes.
[b] Within-group tests.
[c] Between-group tests.

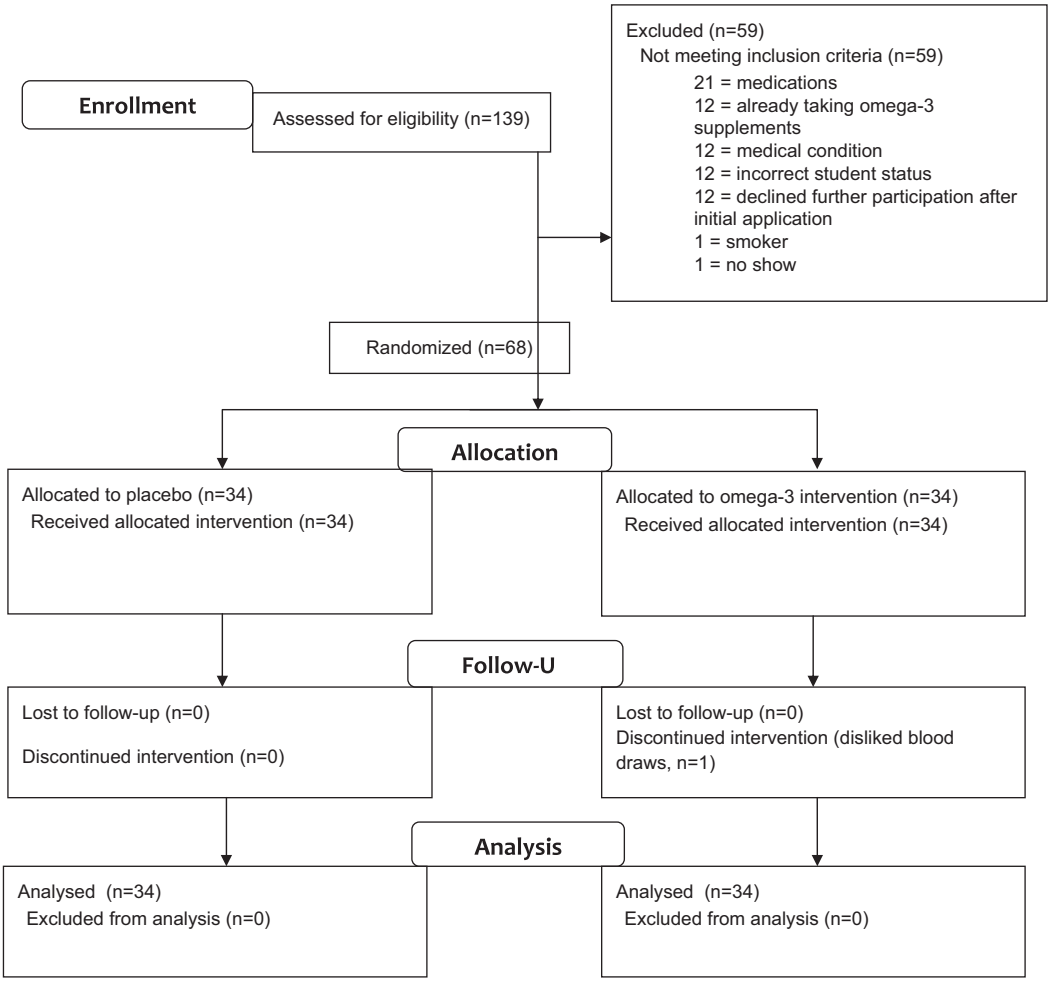

Fig. 2. Screening, randomization, and participant flow by group.

(Table 3). Of these nutrients, levels of protein and Calories were significantly reduced in both treatment groups and supplemented participants (but not placebo) reported lower intake of *n*-3 PUFAs at visit 6 (vs. visit 1). Both groups reported less physical activity. Importantly, there were no significant differences between treatment groups in the changes in dietary intake for any of the macronutrients or in physical activity or sleep.

Very few students reported taking any medication at any point during the study, and the numbers did not differ between groups. The most common medications were multivitamins ($n = 22$), birth control pills ($n = 20$), antihistamines ($n = 11$), and antibiotics ($n = 6$).

3.2. Protocol adherence and blinding

Of the 68 randomized students, 67 (99%) completed all six visits, and the remaining student completed five visits (Fig. 2). Protocol adherence was measured by the number of pills returned at each study visit. There was no difference in adherence between the active and placebo groups, with less than 5% of pills returned in each study arm (4.4% vs. 4.2%, $p = 0.91$). The mean number of pills taken per day was 5.7 in both groups.

The James' blinding index for participants at the end of the study was 0.55 (95% CI: 0.43–0.66, $n = 67$) (James et al., 1996). For primary experimenters the James' blinding index was 0.80 (95% CI: 0.71–0.90, $n = 67$). Blinding is considered adequate if the index is greater than 0.5.

3.3. Safety and tolerability

Nonserious adverse events reported by at least one subject are summarized in Table 4. There were no significant group differences on any dimension.

3.4. Plasma and PBMC fatty acid analyses

Baseline levels of EPA or DHA were similar in plasma and PBMCs (EPA, ~0.5% and DHA, ~1.6%) in young adult samples from collected for this study. By visit 3, plasma levels of EPA and DHA (Fig. 3 A and B) were approximately 6-fold and 1/2-fold higher

Table 4
Nonserious adverse events reported by at least one study subject during trial.

	Placebo ($n = 34$)	Supplement ($n = 34$)	*p*-Value
Sore throat	8 (24%)	8 (24%)	1.00
Nasal symptoms	6 (18%)	7 (21%)	1.00
Stomach pain	5 (15%)	6 (18%)	1.00
Tachycardia	6 (18%)	5 (15%)	1.00
Diarrhea	3 (9%)	4 (12%)	1.00
Skin rash	5 (15%)	2 (6%)	0.43
Nausea	2 (6%)	4 (12%)	0.67
Heartburn	4 (12%)	2 (6%)	0.67
Earache	1 (3%)	1 (3%)	1.00

Data are *n* (%).

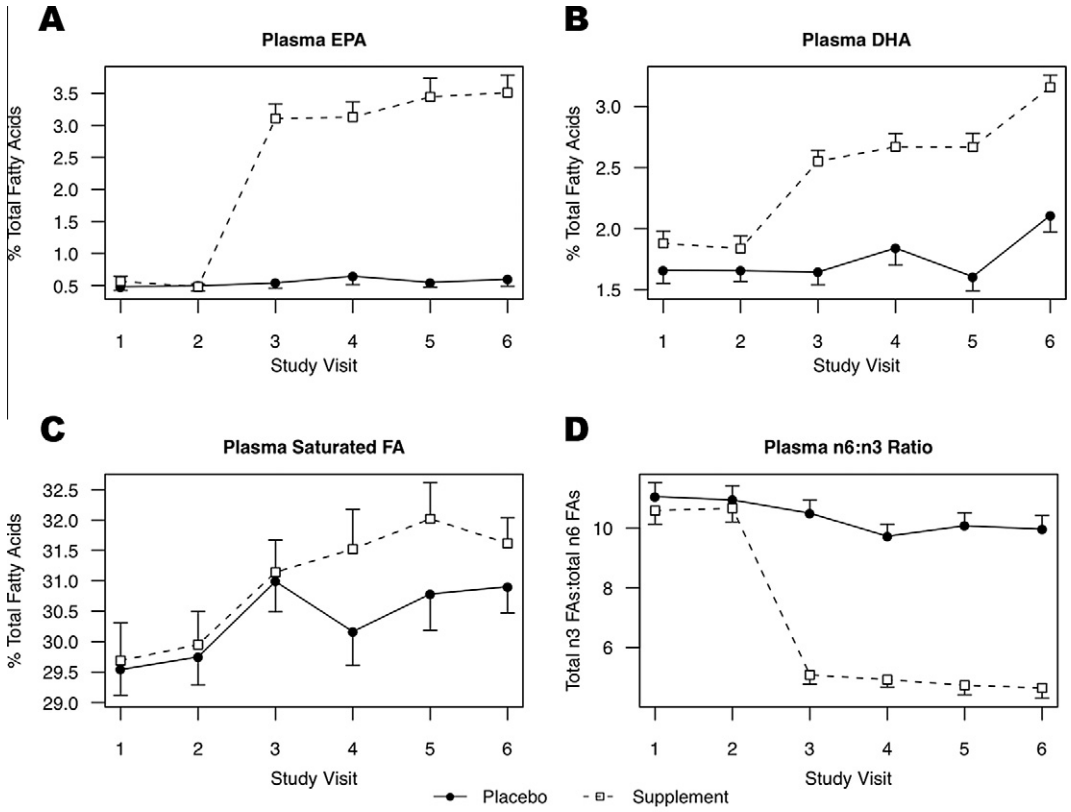

Fig. 3. Mean plasma concentrations (±SEM) of eicosapentaenoic acid (EPA; A), docosahexaenoic acid (DHA; B), total saturated fatty acids (C), and *n*-3:*n*-6 ratios (D) across six study visits for 68 subjects ($n = 34$ per arm).

Table 5
Group effects on primary and secondary outcomes (natural log-transformed).

	Covariate-adjusted least squares mean (SE) during trial[a]				
	Placebo ($n = 34$)	Supplement ($n = 34$)	Group difference (SE)	95% CI	*p*-Value
Primary outcomes					
Serum cytokines					
log(IL-6)	0.054 (0.070)	0.051 (0.069)	−0.0033 (0.090)	−0.18 to 0.18	0.97
log(TNF-α)	0.61 (0.028)	0.54 (0.027)	−0.076 (0.039)	−0.15 to 0.002	0.06
Stimulated cytokines					
log(IL-6)	11.1 (0.051)	10.9 (0.050)	−0.15 (0.072)	−0.30 to −0.009	0.04
log(TNF-α)	7.4 (0.061)	7.3 (0.060)	−0.15 (0.086)	−0.33 to 0.018	0.08
Secondary outcomes					
Mood					
Beck Anxiety, log	1.2 (0.075)	0.93 (0.076)	−0.23 (0.11)	−0.44 to −0.011	0.04
CES-D, log	1.6 (0.097)	1.6 (0.098)	0.012 (0.14)	−0.26 to 0.29	0.93

Note: All outcomes are natural log-transformed.
[a] Least squares means adjusted for baseline value, visit, and gender. Cytokine models additionally adjusted for SAD.

compared to levels prior to supplementation with *n*-3 oils (visits 1 and 2) and the levels remained elevated until the end of the study. The levels of EPA and DHA in PBMCs rose by visit 3 but were not as dramatic in increase, 3-fold and 1/3-fold, respectively for EPA and DHA, compared to the rise of EPA and DHA observed in plasma (data not shown). Supplementation with the placebo oil resulted in no changes of EPA, DHA, saturated fatty acids or the ratio of *n*-6:*n*-3 fatty acids in either plasma or PBMCs. The *n*-6:*n*-3 ratio was significantly decreased after *n*-3 supplementation in plasma and PBMCs where there was generally less variability in plasma compared to variability in PBMCs for EPA and DHA.

3.5. Primary and secondary outcomes

Results for primary (inflammatory) and secondary (mood) outcomes are summarized in Table 5. Across all outcomes and treatment groups there were no significant differences between stress time points (visits 4 and 6) and non-stress time points (visits 3 and 5), thus the ability of *n*-3 supplementation to dampen stress responses could not be tested. Results are displayed as the average effect across the four post-supplementation study visits.

After adjusting for baseline values, gender, and SAD, stimulated cytokines showed stronger treatment effects than serum cytokines

(Table 5). The estimated mean stimulated log-IL-6 value was 0.15 units lower in the supplemented group than the placebo group, corresponding to 14% decrease in the geometric mean of IL-6 ($p = 0.04$). A similar decrease was seen for stimulated TNF-α levels, though it was of borderline significance (14% decrease, $p = 0.08$). There were no significant effects of *n*-3 supplementation on serum IL-6, and a borderline effect on serum TNF-α (7% decrease, $p = 0.06$).

At baseline, females had lower levels of both serum and stimulated IL-6 and TNF-α ($p < 0.1$ for all cytokines). Even after controlling for baseline levels, females had lower serum TNF-α than males during the follow-up period ($p = 0.02$). Higher SAD at baseline was associated with higher levels of both serum and stimulated TNF-α ($p = 0.08$, $p = 0.04$, respectively). After controlling for baseline levels, there was no effect of SAD on cytokines post-supplementation. Neither gender nor SAD modified the effect of the supplement for any cytokine.

Analyses of secondary outcomes revealed a significant effect of *n*-3 supplementation on anxiety but not depression (Table 5). In this young and healthy population, anxiety scores were low at baseline (see Table 2), so there was not much room for reduction. However, the *n*-3 supplemented group had 20% lower geometric mean anxiety scores ($p = 0.04$) post-randomization, adjusting for baseline anxiety scores, study visit, and gender.

After supplementation, there was a significant negative correlation between plasma *n*-3 levels and anxiety in the treated group ($r = -0.39$, $p = 0.0006$). This relationship was also seen as a positive association between *n*-6:*n*-3 ratio and anxiety in the treated group ($r = 0.34$, $p = 0.001$). No significant associations were seen in the placebo group.

Post-supplementation, IL-6 levels did not significantly correlate with anxiety or depression scores in either the treated or untreated groups. However, the trend for serum IL-6 was for a positive association among the untreated and no association among the treated for both anxiety ($r = 0.12$ vs. $r = 0.01$) and depression ($r = 0.29$ vs. $r = -0.02$).

To assess the possibility that analyses that used *n*-6:*n*-3 ratios as continuous measures would show stronger relationships with outcomes than those that used treatment assignment, pre-planned secondary analyses were conducted using plasma *n*-6:*n*-3 ratio in place of treatment group indicator in the mixed models. Results from these analyses are displayed in Table 6. Overall, the continuous *n*-6:*n*-3 ratio showed slightly stronger associations with both inflammatory and mood outcomes than did the group assignment. There were significant effects of the *n*-6:*n*-3 ratio on stimulated IL-6 and TNF-α levels as well as anxiety, with increasing *n*-6:*n*-3 ratios leading to increased stimulated inflammatory cytokine production and increased anxiety. The effect of the *n*-6:*n*-3 ratio on serum TNF-α was small and of borderline significance, and there was no effect on serum IL-6 or depressive symptoms.

The variability observed in PBMCs both before and after *n*-3 supplementation was substantially greater than the variability in plasma, making it harder to see significant relationships in PBMCs. The PBMC plasma *n*-6:*n*-3 ratio was not significantly associated with any primary or secondary outcome, though the effects on stimulated cytokines were in the expected direction with $p < 0.1$ (data not shown). In addition, the magnitude of change was different following supplementation; at 12 weeks the total plasma *n*-3 PUFA concentration was 122% higher than baseline in the supplemented group on average, compared to a 55% average increase in PBMCs.

Table 6
Results from models using continuous *n*-6:*n*-3 plasma ratio to predict primary and secondary outcomes (natural log-transformed).*

	Estimate (SE)	95% CI	*p*-Value
Primary outcomes			
Serum cytokines			
log(IL-6)	0.0095 (0.012)	−0.014 to 0.033	0.43
log(TNF-α)	0.0089 (0.0047)	−0.0004 to 0.018	0.06
Stimulated cytokines			
log(IL-6)	0.021 (0.010)	0.0016 to 0.040	0.03
log(TNF-α)	0.021 (0.010)	0.0006 to 0.042	0.04
Secondary outcomes			
Mood			
Beck anxiety, log	0.039 (0.015)	0.0098 to 0.069	0.01
CES-D, log	0.0061 (0.017)	−0.027 to 0.039	0.72

*Least squares means adjusted for baseline value, visit, and gender. Cytokine models additionally adjusted for SAD.
Note: All outcomes are natural log-transformed.

4. Discussion

4.1. Intervention-related reductions in inflammation and anxiety

Students who received *n*-3 PUFAs showed a 14% decrease in stimulated IL-6 production and a 20% reduction in anxiety symptoms compared to controls. Additional analyses that used changes in the plasma *n*-6:*n*-3 ratio as a continuous measure enhanced the magnitude of the effects seen by group assignment; in addition to anxiety, these analyses demonstrated significant effects for both stimulated TNF-α and IL-6 production by PBMCs, as well as a borderline effect for serum TNF-α levels. Individuals can differ in absorption and metabolism of the *n*-3 PUFA supplements, as well as in adherence, and these analyses helped to clarify the intervention's impact.

The differences in inflammation are particularly striking because our medical students had higher levels of *n*-3 and lower *n*-6 than anticipated, based on population data (Simopoulos, 2002). Indeed, their average dietary *n*-6:*n*-3 ratio at baseline was 10.82, substantially lower than the typical North American dietary ratios of 15:1 to 17:1 (Simopoulos, 2002). Despite this fact, we nonetheless saw significant decrements in inflammation related to changes in their plasma *n*-6:*n*-3 ratios in response to the *n*-3 PUFA supplements.

The fatty acid composition of the modern Western diet has changed dramatically the last century, and these changes are thought to be related to increases in inflammatory-related diseases (Hallahan and Garland, 2005; Weber and Leaf, 1991). For example, the early hunter-gatherer diet had an *n*-6:*n*-3 PUFA ratio of 2:1 to 3:1 (Cordain et al., 2005). However, during the early 1900s, the typical Western diet underwent fundamental alterations with the enormous growth in refined vegetable oil use, a central *n*-6 source that replaced *n*-3 PUFAs from fish, wild game, and leaves (Cordain et al., 2005; van West and Maes, 2003), leading to the contemporary North American *n*-6:*n*-3 ratio of 15:1 to 17:1 (Hibbeln et al., 1997; Simopoulos, 2002). It has been suggested that these dramatic shifts in the modern Western diet's fatty acid composition are related to the increases in depression and cardiovascular disease (Hallahan and Garland, 2005; Hibbeln, 1998; van West and Maes, 2003; Wall et al., 2010).

4.2. Randomized PUFA trials

Randomized trials with long-chain *n*-3 PUFAs have produced mixed results, with more consistent decreases in inflammation in at-risk groups including the elderly, diabetics, and hypertriglyceridemic participants, all of whom had relatively high baseline inflammation (Fritsche, 2006). Dietary data from the National Health and Nutrition Examination Survey (NHANES) show a mean

daily EPA + DHA intake of 0.1 g/d among adults ages 20–39 (Ervin et al., 2004); at baseline our students' average intake was 70% higher than their national age peers, based on their FFQ reports of 0.17 g/d. The combination of low baseline inflammation and higher than expected *n*-3 PUFA levels limited our ability to detect change. Accordingly, the fact that both anxiety and inflammation were nonetheless altered by supplementation is notable.

We found stronger and more reliable relationships in our stimulated cytokine data compared to serum. The discordance between stimulated and serum *n*-3 PUFA-related inflammatory changes is likely related to the very low baseline levels of serum cytokines in this healthy, young, and relatively thin population.

4.3. Anxiety and depressive symptoms

The reduction in anxiety symptoms associated with *n*-3 supplementation provides the first evidence that *n*-3 may have potential anxiolytic benefits for individuals without an anxiety disorder diagnosis. Proinflammatory cytokines promote secretion of corticotropin-releasing hormone (CRH), a primary gateway to hormonal stress responses; CRH also stimulates the amygdala, a key brain region for fear and anxiety (Raison et al., 2006). Accordingly, alterations in inflammation could also influence anxiety.

In contrast to the significant effects observed for anxiety, depressive symptoms were not responsive to the intervention. These data are in accord with a recent meta-analysis of randomized controlled trials which concluded that *n*-3 PUFA supplementation benefited clinically depressed individuals, but not those with less severe depressed mood (Appleton et al., 2010).

4.4. Self-reported diet and exercise changes

At the onset of the study, we had asked subjects to refrain from making any substantial changes to dietary or exercise behaviors. Yet, according to reports of food intake and exercise, there were trends for reductions in all macronutrient categories and exercise in both groups. We believe this reflects a general observation reported in other studies: intraindividual variability of *reported* food intake in sequential dietary assessments generally declines after the first assessment, especially for Calories and macronutrient intakes (Arab et al., 2010; Bidulescu et al., 2009).

Students who received *n*-3 PUFA supplements also reported a significantly lower dietary *n*-3 PUFA intake at the end of the study than at baseline, while the placebo group did not show a significant change. However, whether or not this group of students actually changed their diet, the 122% increase in plasma *n*-3 PUFAs at the trial's conclusion objectively documents the desired *n*-3 PUFA increments.

4.5. Dosage and risks

Controlled inflammatory responses are a necessary part of host defense mechanisms; if inflammatory responses are suppressed in normal individuals, could this actually be a negative consequence of supplementation? Both epidemiological and clinical studies suggest that infectious disease susceptibility is not enhanced by either *n*-3 supplementation or a high dietary fish intake enhance (Sijben and Calder, 2007). For example, responses to a measles epidemic among Greenland Inuits were quite similar to previous measles epidemics in other native populations, suggesting that the extremely high levels of *n*-3 PUFAs in their diet did not worsen their viral defenses (Calder, 2004; Sijben and Calder, 2007). Similarly, the risk of community-acquired pneumonia was 32% lower among men in the top quintile of α-linoleic acid intake than in men in the bottom quintile, and pneumonia risk fell by 31% for every 1 g/d increment in α-linoleic acid intake; moreover, although EPA and DHA were not associated with pneumonia risk, high fish consumption was linked with lower risk of pneumonia among men with low dietary *n*-3 and *n*-6 from plant sources (Merchant et al., 2005).

A number of surgical studies have demonstrated significant reductions in infection in postsurgical patients who receive enterally-delivered *n*-3 PUFAs vs. those who do not; although the salutary effects cannot be ascribed solely to the *n*-3 PUFAs because the supplements typically included other nutrients, the overall advantage is clear (Heyland et al., 2001; Yaqoob, 2004). Thus, these epidemiological and surgical studies suggest that *n*-3 PUFAs have positive benefits related to infectious disease risks.

The FDA has concluded that intakes of up to 3 g/d of marine *n*-3 PUFAs are "Generally Recognized As Safe" (Kris-Etherton et al., 2002), and our 2.5 g/d dose fell within that range. The most common risks associated with PUFA doses between 1 and 3 g/d are gastrointestinal upset, fishy aftertaste, and rise in LDL-C (the latter usually only in patients with hypertriglyceridemia) (Kris-Etherton et al., 2002). For the GISSI Prevention study which supplemented 5666 myocardial infarction patients for 3.5 years with1 g/d of *n*-3 PUFAs, both compliance and the safety profile were excellent; 3.8% of PUFA-supplemented patients discontinued the pills, compared to 2.1% who received vitamin E (Valagussa et al., 1999). Aside from a fishy aftertaste, the most common side effects reported were gastrointestinal disturbances (4.9%) and nausea (1.4%), compared to 2.9% and 0.4%, respectively, in the vitamin E group (Valagussa et al., 1999). Among 275 patients who were given a much higher dose, 6.9 g/d of EPA + DHA, the *n*-3 PUFA patients did not differ from controls who received corn oil on any adverse event, with gastrointestinal upset reported by 8% of *n*-3 PUFA and 7% of corn oil patients (Leaf et al., 1994).

The possibility that very high doses of *n*-3 PUFAs may be associated with an increased risk of hemorrhagic stroke comes from studies with Greenland Eskimos who have excess mortality from hemorrhagic stroke when compared to Danish whites (Iso et al., 2001). However, the average EPA + DHA intake for Greenland Eskimos is 10.5 g/d, or more than four times as large as the dose used in this study. Prospective data from the 79,839 women in the Nurses Health cohort who were followed for 14 years found no association between fish or *n*-3 PUFA intake and risk of hemorrhagic stroke (Iso et al., 2001). Indeed, risk of thrombotic infarction was reduced by 48% among women who ate fish 2–4 times per week (Iso et al., 2001).

Some studies suggest that bleeding time may be prolonged when *n*-3 PUFAs exceed 3 g/d (Iso et al., 2001). On the other hand, studies of Alaskan Eskimos who depended on fish and marine animals as primary dietary components and consequently had *n*-3 plasma FA levels that were 4.3 times as high as non-native controls showed no increase in mean bleeding times compared to controls (Parkinson et al., 1994). Moreover, even among patients who were treated with aspirin or anticoagulants there was little clinical evidence of increased risk of bleeding (Eritsland, 2000; Schmidt et al., 2005).

4.6. Strengths and limitations

We did not find reliable exam-related increases in inflammation or distress, in contrast to prior academic stress studies (Glaser et al., 1990; Maes et al., 2000; Marshall et al., 1998). As a consequence, we were unable to test the hypothesis that *n*-3 supplemented students would show smaller exam-related increments in inflammation and distress among compared to the placebo group. Unfortunately, the absence of systematic stress-related changes in the placebo group was clearly problematic in this regard, one limitation of the study.

Strengths of this RCT include minimal attrition, as well as careful assessment of variables known to influence inflammation including smoking, medication use, physical activity, and abdominal adiposity. In accord with the low incidence reported in large *n*-3 PUFA studies described above, side effects were infrequent and did not differ between groups. Our 2.5 g/d study dose produced differences in inflammation and anxiety in this young and healthy population. Thus, the risks appear minimal in the face of potential benefits.

Inflammation is a prominent feature in the major age-associated sources of death and disability (Sijben and Calder, 2007). The results of our RCT suggest that the simple dietary intervention of increasing *n*-3 PUFAs could have important benefits.

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