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Omega-3 Fatty Acids, Oxidative Stress, and Leukocyte Telomere Length: A Randomized Controlled Trial

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Abstract

Shorter telomeres have been associated with poor health behaviors, age-related diseases, and early mortality. Telomere length is regulated by the enzyme telomerase, and is linked to exposure to proinflammatory cytokines and oxidative stress. In our recent randomized controlled trial, omega-3 (*n*-3) polyunsaturated fatty acid (PUFA) supplementation lowered the concentration of serum proinflammatory cytokines. This study assessed whether *n*-3 PUFA supplementation also affected leukocyte telomere length, telomerase, and oxidative stress. In addition to testing for group differences, changes in the continuous *n*-6:*n*-3 PUFA ratio were assessed to account for individual differences in adherence, absorption, and metabolism. The double-blind 4-month trial included 106 healthy sedentary overweight middle-aged and older adults who received (1) 2.5 g/day *n*-3 PUFAs, (2) l.25 g/day *n*-3 PUFAs, or (3) placebo capsules that mirrored the proportions of fatty acids in the typical American diet. Supplementation significantly lowered oxidative stress as measured by F2-isoprostanes (p=0.02). The estimated geometric mean log-F2-isoprostanes values were 15% lower in the two supplemented groups compared to placebo. Although group differences for telomerase and telomere length were nonsignificant, changes in the *n*-6:*n*-3 PUFA plasma ratios helped clarify the intervention's impact: telomere length increased with decreasing *n*-6:*n*-3 ratios, p=0.02. The data suggest that lower *n*-6:*n*-3 PUFA ratios can impact cell aging. The triad of inflammation, oxidative stress, and immune cell aging represents important pre-disease mechanisms that may be ameliorated through nutritional interventions. This translational research broadens our understanding of the potential impact of the *n*-6:*n*-3 PUFA balance. ClinicalTrials.gov identifier: NCT00385723

Keywords: omega-3, omega-6, telomeres, inflammation, cell aging, nutritional neuroscience, oxidative stress, F2-isoprostanes, fish oil

1. Introduction

1.1 Telomeres, Telomerase, Inflammation, and Oxidative Stress

Telomeres, the caps found at the ends of chromosomes, are essential for chromosomal stability and replication; the enzyme telomerase is important for telomere formation, maintenance, and restoration (Blackburn, 2005; Epel et al., 2004). A growing literature has linked shorter telomeres with health behaviors, age-related diseases, and earlier mortality (Brouilette et al., 2003; Epel et al., 2009; Kimura et al., 2008; Valdes et al., 2005).

Telomeres can be maintained or lengthened by telomerase, an intra-cellular enzyme that adds telomeric DNA to shortened telomeres (Chan and Blackburn, 2003). Telomere length is also linked to, and likely regulated by, exposure to proinflammatory cytokines and oxidative stress

(Aviv, 2006; Carrero et al., 2008; Damjanovic et al., 2007). Inflammation triggers T-cell proliferation, one known cause of telomere shortening (Aviv, 2004; Carrero et al., 2008; Gardner et al., 2005). Oxidative stress promotes telomere erosion during cellular replication *in vitro* and also stimulates the synthesis of proinflammatory cytokines (Aviv, 2006; Lipcsey et al., 2008).

1.2 Telomeres, Telomerase, and Omega-3 PUFAs

Although telomeres typically shorten with aging, shortening is not inevitable, and telomeres can also lengthen (Aviv et al., 2009; Ehrlenbach et al., 2009; Epel et al., 2009; Farzaneh-Far et al., 2010a; Nordfjall et al., 2009). It is important to identify malleable factors that might promote telomere stability over time. Based on theoretical and empirical reasons, it is possible that blood levels of polyunsaturated fatty acids (PUFAs) may be one of the factors that can prevent telomere shortening over time. The omega-3 (*n*-3) PUFAs can reduce inflammation and decrease oxidative stress (Calder, 2005; Kiecolt-Glaser et al., 2011; Mori et al., 1999; Nalsen et al., 2006), described below, and thus could buffer telomeres from their damaging effects.

In the Heart and Soul Study, which followed 608 people with stable coronary heart disease over five years, average telomere length increased in 23% of the individuals, shortened in 45%, and remained unchanged in 32% (Farzaneh-Far et al., 2010a). Slower telomere attrition was predicted by higher baseline levels of the two key *n*-3 PUFAs, eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA), which were the only significant predictors out of 16 clinical and behavioral factors examined (Farzaneh-Far et al., 2010b). Each standard deviation increase in the DHA+EPA total was associated with a 32% reduction in the odds of telomere attrition. In a different pilot study, an intensive three-month lifestyle change program that included *n*-3 PUFA supplementation significantly increased telomerase activity (Ornish et al., 2008).

Dietary intakes of both the *n*-3 and omega-6 (*n*-6) PUFAs influence inflammation. Arachidonic acid (AA) is an *n*-6 PUFA that may be derived from its precursor, linoleic acid, found in popular dietary oils such as corn, sunflower, and safflower oils. The eicosanoids produced from AA increase proinflammatory cytokine production (Calder, 2005). In contrast, the eicosanoids derived from EPA, a long chain *n*-3 PUFA found in cold water fish oils, curb the production of AA-derived eicosanoids (Calder, 2005). Thus, both higher plasma levels of *n*-3 PUFAs as well as lower plasma *n*-6:*n*-3 PUFA ratios restrain proinflammatory cytokine production (Kiecolt-Glaser et al., 2007). Accordingly, it is not surprising that both higher levels of *n*-3 PUFAs as well as lower *n*-6:*n*-3 PUFA ratios have been associated with lower proinflammatory cytokine production in epidemiological and observational studies (Farzaneh-Far et al., 2009; Ferrucci et al., 2006; Kalogeropoulos et al., 2010; Kiecolt-Glaser et al., 2007).

1.3 The present study

In our recent 4-month randomized controlled trial (RCT), serum interleukin 6 (IL-6) decreased by 10% and 12% in our low (1.25 g/day) and high (2.5 g/day) dose *n*-3 PUFA

groups, respectively, compared to a 36% increase in the placebo group (Kiecolt-Glaser et al., 2012). Similarly, low and high dose *n*-3 PUFA groups showed modest 0.2% and –2.3% changes in serum TNF-α, in contrast to the 12% increase in the control group. Depressive symptoms, the other primary trial outcome, were low at baseline and did not change. This study assessed the impact of *n*-3 PUFA supplementation, and consequent changes in the *n*-6:*n*-3 PUFA ratio, on secondary outcomes in our RCT: leukocyte telomere length, telomerase, and oxidative stress.

2. Methods

2.1 Participants

Blood samples were obtained from 106 men and women, ages 40–85 (Table 1) who were part of the 138 participants in the parent *n*-3 PUFA RCT (ClinicalTrials.gov identifier: NCT00385723); no blood samples were available from the early participants for these analyses. Campus and community print and web-based announcements were used for recruitment. The Ohio State University biomedical institutional review board approved this study, and each participant provided written informed consent.

Table 1

Baseline characteristics of study population, by intervention group

	Placebo (n=31)	**1.25 g/day *n*-3 (n=40)**	**2.5 g/day *n*-3 (n=35)**
Age, mean (SD), years	51.2 (8.9)	50.3 (7.8)	50.6 (6.5)
Female, No. (%)	23 (74%)	24 (60%)	22 (63%)
Race, No. (%)			
•White	23 (74%)	33 (83%)	27 (77%)

•Black	4 (13%)	5 (13%)	7 (20%)
•Asian	2 (6%)	1 (3%)	1 (3%)
•Other	2 (6%)	1 (3%)	0 (0%)
Body mass index, mean (SD), kg/m^2	31.1 (4.8)	31.7 (4.5)	30.7 (3.8)
Sagittal abdominal diameter, mean (SD), cm	23.1 (3.1)	23.4 (3.1)	22.9 (2.4)
Telomere length at baseline, base pairs			
•N	31	40	35
•Mean (SD)	5866 (430)	5809 (374)	5916 (432)
•Median (IQR)	5810 (5588–6257)	5782 (5566–6092)	5838 (5681–6280)
•Range	4981–6531	4963–6612	5042–6764
Telomerase at baseline, activity/10,000 cells			
•N	27	36	31
•Mean (SD)	8.5 (4.2)	8.2 (4.4)	7.9 (3.3)
•Median (IQR)	7.0 (5.8–10.9)	7.1 (5.8–9.8)	7.4 (5.2–10.5)
•Range	2.0–20.3	2.1–25.5	2.6–16.6
F2-isoprostanes at baseline, ng/ml			

•N	27	38	32
•Mean (SD)	0.036 (0.017)	0.037 (0.015)	0.031 (0.012)
•Median (IQR)	0.032 (0.025–0.044)	0.033 (0.026–0.045)	0.032 (0.024–0.036)
•Range	0.017–0.087	0.01–0.069	0.011–0.081

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The online screening form assessed health history, medications, and health behaviors. Exclusions included psychoactive drugs or mood altering medications, lipid-altering drugs, cardiovascular medications, steroids, prostaglandin inhibitors, heparin, warfarin, regular use of non-steroidal anti-inflammatory drugs other than a daily aspirin, and alcohol/drug abuse. We also excluded pregnant or nursing women, vegetarians, diabetics, people who routinely took fish oil or flaxseed supplements or ate more than two portions of oily fish per week, smokers, and individuals with recurrent digestive problems, convulsive disorders, and autoimmune and/or inflammatory diseases. Individuals were excluded if they typically engaged in 2 or more hours of vigorous physical activity per week or had a body mass index (BMI) below 22.5 or over 40.

In addition, we used participants' ability to follow the supplementation regimen as a criterion for study entry. Participants received a 7-day supply of placebo capsules (single blind) at the in-person screening session, and those who had taken less than 80% of the capsules a week later were dropped before randomization. The most common medications among our 106 subjects were, in order, nonprescription NSAIDs (n=14; 8 aspirin, 4 ibuprofen, 2 naproxen), levothyroxine (n=8), estrogen with or without progesterone (n=8), and antacids (n=7), and these did not differ by group. Individuals were excluded if they typically engaged in 2 or more hours of vigorous physical activity per week or had a body mass index (BMI) below 22.5 or over 40.

2.2 Design and Study Components

Data collection for this double-blind placebo-controlled four month RCT began in September, 2006 and ended in February, 2011. At baseline and at 4 months we assessed telomere length, telomerase, and oxidative stress. Blood samples were collected between 7:00 and 9:00 AM to control for diurnal variation. Samples were obtained in the Ohio State Clinical Research Center, a hospital research unit. Attrition was minimal; only 5 of 138 subjects in the parent study failed to complete the full trial (Kiecolt-Glaser et al., 2012).

2.21 Supplement and Placebo

This RCT compared responses to A) 2.5 g/day *n*-3 PUFA, B) 1.25 g/day *n*-3 PUFA, or C) placebo. We chose a 7:1 EPA:DHA balance because of evidence that EPA has relatively stronger anti-inflammatory and antidepressant effects than DHA (Ariel and Serhan, 2007; Lin et al., 2010b; Sijben and Calder, 2007); thus, the high dose group received 2085 mg/d of EPA and 348 mg/d of DHA. The placebo was a mixture of palm, olive, soy, canola, and coco butter oils that approximated the saturated:monounsaturated:polyunsaturated ratio consumed by US adults, 37:42: 21 (USDA Continuing Survey of Food Intake by Individuals, 1994–1996). OmegaBrite (Waltham, MA) supplied both the *n*-3 PUFA and the matching placebo; all supplements were coated with a fuchsia coloring. OmegaBrite added a mild fish flavor to the placebo to help disguise any differences between the *n*-3 PUFAs and the placebo, and we told participants about the fish flavoring to promote blindness (Stoll et al., 2001). Both the placebo and the *n*-3 PUFA pills included 1 IU of vitamin E. In order to ensure integrity of the oil supplements, dietary oils were analyzed every 6–8 months by gas chromatography of methylated fatty acids prepared in the Belury lab. Table 2 shows a typical fatty acid composition of the dietary supplements (% fatty acid of total fatty acids detected for analysis).

Table 2

Fatty Acid Composition of Dietary Oil Supplements (% Fatty Acid)

Fatty Acid	Name	Placebo	Supplement
C14:0	myristic acid	6.4	0.1
C16:0	palmitic acid	19.6	0.1
C16:1n7	palmitoleic acid	0.4	0.1
C18:0	stearic acid	3.1	0.5
C18:1n9	oleic acid	43.5	0.7
C18:1n7	vaccenic acid	1.4	0.4

C18:2n6	linoleic acid	20.4	0.2
C18:3n6	gamma linolenic acid	0.0	0.7
C18:3n3	alpha linolenic acid	3.3	0.2
C18:4n3	stearidonic acid	0.1	6.9
C20:1n9	cetoleic acid	0.2	0.4
C20:2n6	eicosadienoic acid	0.0	0.1
C20:3n6	dihomo-gamma-linolenic acid	0.0	0.1
C20:4n6	arachidonic acid	0.1	3.5
C20:4n3	eicosatetraenoic acid	0.0	1.5
C20:5n3	eicosapentaenoic acid	1.2	75.0
C22:4n6	adrenic acid	0.0	0.0
C22:5n3	docosapentaenoic acid	0.0	0.8
C22:6n3	docosahexaenoic acid	0.2	8.9
Total Reported		100	100
Saturated Fatty Acids[1]		29.1	0.7
Monounsaturated Fatty Acids[2]		45.3	1.2
Omega 3 Fatty Acids[3]		4.8	93.3

Omega 6 Fatty Acids[4]	20.5	4.6
n-6/n-3ratio[5]	4.27	0.05

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[1]C14:0, C16:0, C18:0

[2]C16:1n7, C18:1n7, C18:1n9

[3]Sum of (C18:3n3+C18:4n3+C20:4n3+C20:5n3+C22:5n3+C22:6n3)

[4]Sum of (C18:2n6+C18:3n6+C20:2n6+C20:3n6+C20:4n6+C22:4n6)

[5]Sum of (C18:2n6+C18:3n6+C20:2n6+C20:3n6+C20:4n6+C22:4n6)/Sum of (C18:3n3+C18:4n3 +C20:4n3+C20:5n3+C22:5n3+C22:6n3)

2.22 Randomization and Masking

Before a participant left the baseline session, s/he was randomly assigned to one of three treatment groups using a permuted block randomization sequence prepared and maintained by the data manager who had no involvement in data collection or biological laboratory analyses; she was the only person who had access to the randomization list. The active and placebo study supplements were packaged according to the randomization sequence, so that a participant was randomized to the next available individual supplement package. At each subsequent study visit, participants returned unused supplements and received the next month's supply. Adherence was excellent and did not differ between active and placebo groups, with 3.3%, 2.0%, and 2.6% percent of unused supplements returned in the placebo, low, and high dose groups, respectively (p=0.31). Analysis of data from both participants and experimenters showed adequate blinding as previously described (Kiecolt-Glaser et al., 2012)

2.3 Anthropometry and Health-Related Behaviors

Sagittal abdominal diameter (SAD) measurements at baseline provided data on abdominal fat. Validational studies have demonstrated SAD's utility as a noninvasive central adiposity measure (Clasey et al., 1999).

Participants completed the Women's Health Initiative Food Frequency Questionnaire at the screening session and at 4 months. It provided data on foods and beverages consumed in the past 90 days (Patterson et al., 1999).

The Pittsburgh Sleep Quality Index, administered at baseline and 4 months, assessed sleep quality and disturbances over a one-month interval (Buysse et al., 1989). It has good diagnostic sensitivity and specificity (Buysse et al., 1989).

The Community Healthy Activities Model Program for Seniors Questionnaire assessed the frequency and duration of various physical activities at baseline and 4 months. An excellent instrument for middle-aged and older populations, it is sensitive to relatively small changes in physical activity (Stewart et al., 2001).

2.4 Depressive Symptoms and Syndromal Mood Disorders

The Center for Epidemiological Studies Depression Scale is a widely-used measure of depressive symptomatology (Radloff, 1977). The Structured Clinical Interview for DSM-IV (SCID) mood disorder modules, administered during screening, provided baseline data on both lifetime and current mood disorders (First et al., 1996); the visit 4 SCID assessed the subsequent development or resolution of symptoms.

2.5 Fatty Acid Analyses

Lipids were extracted from plasma 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). We report fatty acids that were greater than 0.01% of peaks detected; these included myristate (14:0), palmitate (16:0), palmitoleate (16:1n7), stearate (18:0), oleate (18:1n9), vaccenate (18:1n7), linoleate (18:2n6), gamma-linolenate (18:3n6), alpha-linolenate (18:3n3), stearidonate (18:4n3), catoleate (20:1n9), eicosadienoate (20:2n6), dihomo-gamma-linolenate (20:3n6), arachidonate (20:4n6), eicosapentaenoate (20:5n3), adrenate (22:4n6), docosapentaenoate (22:5n3), and docosahexaenoate (22:6n3). For calculating the *n*-6:*n*-3 ratio, all identified *n*-6 and *n*-3 fatty acids were used.

2.6 Telomere Length

Peripheral blood lymphocytes (PBL) were purified from whole blood by density-gradient centrifugation in Lymphocyte Separation Medium (Mediatech, Inc.). PBLs (2 x 10^6 cells) were lysed in 300ul lysis buffer (included in Gentra Puregene cell kit from Qiagen) and genomic DNA isolation was performed following the company's instruction. The quality and

quantity of the genomic DNA were determined using 260/280 UV spectrophotometery and Picogreen Assay. The telomere length measurement assay is adapted from the published original method by Cawthon.(Cawthon, 2002; Lin et al., 2010a) The telomere thermal cycling profile consists of: Cycling for T(telomic) PCR: denature at 96°C for 1 second, anneal/extend at 54°C for 60 seconds, with fluorescence data collection, 30 cycles. Cycling for S (single copy gene) PCR: denature at 95°C for 15 seconds, anneal at 58°C for 1 second, extend at 72° C for 20 seconds, 8 cycles; followed by denature at 96°C for 1 second, anneal at 58°C for 1 second, extend at 72°C for 20 seconds, hold at 83°C for 5 seconds with data collection, 35 cycles.

The primers for the telomere PCR are *tel1b* [5′-CGGTTT$(GTTTGG)_5$GTT-3′], used at a final concentration of 100 nM, and *tel2b* [5′-GGCTTG$(CCTTAC)_5$CCT-3′], used at a final concentration of 900 nM. The primers for the single-copy gene (human beta-globin) PCR are *hbg1* [5′ GCTTCTGACACAACTGTGTTCACTAGC-3′], used at a final concentration of 300 nM, and *hbg2* [5′-CACCAACTTCATCCACGTTCACC-3′], used at a final concentration of 700 nM. The final reaction mix contains 20 mM Tris-HCl, pH 8.4; 50 mM KCl; 200 μM each dNTP; 1% DMSO; 0.4x Syber Green I; 22 ng E. coli DNA per reaction; 0.4 Units of Platinum Taq DNA polymerase (Invitrogen Inc.) per 11 microliter reaction; 0.5 – 10 ng of genomic DNA. Tubes containing 26, 8.75, 2.9, 0.97, 0.324 and 0.108ng of a reference DNA (from Hela cancer cells) are included in each PCR run so that the quantity of targeted templates in each sample can be determined relative to the reference DNA sample by the standard curve method. Each concentration of the reference DNA is run as quadruplets and samples are run as triplicates.

To control for inter-assay variability, 8 control DNA samples from cancer cell lines (including 293T, H1299, UMUC3, and UMUC3 cells infected with a lentiviral construct containing the telomerase RNA gene to extent telomeres, harvested at various population doublings after infection) are included in each run. In each batch, the T/S ratio of each control DNA is divided by the average T/S for the same DNA from 10 runs to get a normalizing factor. This is done for all 8 samples and the average normalizing factor for all 8 samples is used to correct the participant DNA samples to get the final T/S ratio. The T/S ratio for each sample is measured twice, each time in triplicate wells. When the duplicate T/S value and the initial value vary by more than 7%, the sample is run the third time and the two closest values will be reported. The formula to convert the T/S ratio to base pairs is base pairs = 3,274+ 2,413*(T/S). The inter-assay coefficient of variation for telomere length measurement was 4.3% for this study.

2.7 Telomerase Activity

PBLs were purified from whole blood as above. Cells were lysed with 1XCHAPS buffer (10 mM Tris.HCl, pH 7.5, 1mM MgCl2, 1 mM EGTA, 0.1 mM Benzamidine, 5 mM b-mercaptoethanol, 0.5% CHAPS, 10% glycerol) on ice for 30 minutes and spun at 4°C at 14k rpm for 20 minutes to generate an extract corresponding to 10000 cells/ul. Extracts were stored at −80 degree for batch analysis of telomerase activity. Telomerase activity was measured by the TRAPeze Telomerase detection kit (Millipore, Cat# S7700) using a modified protocol developed by the Blackburn lab.(Lin et al., 2010a) Three concentrations (2000, 5000 and 10000 cells) were used for TRAP reactions to ensure that the assay was in the linear range. Details of the method are published elsewhere.(Lin et al., 2010a) The inter-assay coefficient of variation (CV) was 6.8%.

2.8 Oxidative Stress

F_2-isoprostanes provide the most reliable index of *in vivo* oxidative stress when compared to other well-known biomarkers (Milne et al., 2007). Plasma samples were analyzed by Vanderbilt's Eicosanoid Core Laboratory, following their published protocol (Milne et al., 2007).

2.9 Statistical Methods

Baseline subject characteristics and outcomes at baseline were compared across supplementation groups using ANOVA and chi-square tests as appropriate. Continuous variables that were right-skewed were natural logarithm transformed (telomerase, F2-isoprostanes) to better approximate normality of residuals. Where appropriate, Pearson correlations and Spearman correlations (skewed variables) were used to describe cross-sectional associations between continuous measures. Analysis of covariance was used to separately model change in telomerase (N=94), telomere length (N=106), and F2-isoprostanes (N=97) from baseline to 4 months, adjusting for baseline levels, using all subjects with available four-month follow-up data. In order to control Type I error, the Tukey-Kramer method was used for between-group comparisons. Although supplementation group was the main predictor of interest, secondary analyses used change in continuous *n*-6:*n*-3 PUFA ratio in place of group because individuals differ in absorption and metabolism of *n*-3 PUFA supplements, as well as in adherence. Despite the relative balance between the groups due to the randomization, analyses were repeated controlling for age, gender, and sagittal abdominal diameter (SAD). Alpha was set to 0.05, and two-sided tests were conducted. All analyses were carried out in SAS version 9.3 (SAS Institute, Cary, NC).

3. Results

3.1 Study population, baseline data

Table 1 shows baseline characteristics of the analysis sample (N = 106), with 31 subjects in the placebo arm, 40 in the low dose fish oil arm, and 35 in the high dose fish oil arm. Randomization produced groups that did not differ on age, baseline FFQ dietary variables, sleep quality, depressive symptoms, and history of major depressive disorder, $p > 0.19$ for all tests. There were no baseline group differences on SAD or BMI ($p > 0.60$ for both). Using BMI cut points of 25 and 30 kg/m^2, 100 participants (94%) were overweight, and 54 (51%) were obese. Groups were similar on telomere length, telomerase, and F2-isoprostanes at baseline ($p > 0.26$ for all tests).

3.2 Changes in fatty acids

Baseline levels of plasma fatty acids and changes over time are shown in Table 3. EPA and DHA levels were low (~0.5% and ~1.5% respectively), and there were no group differences in EPA, DHA, total *n*-3 PUFA, or *n*-6:*n*-3 PUFA ratio at baseline. There were small differences in total *n*-6 PUFA across groups ($p = 0.01$) with slightly lower levels at baseline in the 1.25 g/d n-3 PUFA group. By month four plasma levels of EPA were approximately 3.5-fold higher in the 1.25 g/d n-3 group and 6-fold higher in the 2.5 g/d n-3 group ($p < 0.0001$ for both), and plasma DHA levels were approximately 1.7-fold higher in the 1.25 g/d n-3 PUFA group and 2-fold higher in the 2.5 g/d n-3 PUFA group ($p < 0.0001$ for both). The placebo group experienced a small increase in EPA, with plasma EPA levels approximately 1.5-fold higher ($p = 0.001$), but had no change in DHA ($p = 0.47$). The *n*-6:*n*-3 PUFA ratio was significantly decreased after supplementation for both low and high dose groups, dropping from 10 to 6.8 in the 1.25 g/d *n*-3 PUFA group and from 11 to 5.0 in the 2.5 g/d *n*-3 PUFA group ($p < 0.0001$ for both). The placebo group experienced a much smaller decrease of borderline significance, dropping from 11 to 10 ($p = 0.06$).

Table 3

Plasma fatty acids[a], by intervention group

	Baseline	*P* Value[2]	4 months	*P* Value[2]	Change, 0 to 4 months	*P* for change[3]	comparison of changes[2]
Saturated Fatty Acids							
•myristic acid c14:0		0.29		0.94			0.21
••Placebo	0.92 (0.38)		0.91 (0.33)		–0.006 (0.42)	0.93	
••1.25 g/day n-3	1.0 (0.47)		0.89 (0.33)		–0.14 (0.45)	0.06	

••2.5 g/day n-3	0.86 (0.52)		0.91 (0.49)	0.054 (0.56)	0.58	
•palmitic acid c16:0		0.04		0.93		0.07
••Placebo	23 (2.6)		23 (3.0)	0.28 (3.3)	0.64	
••1.25 g/day n-3	24 (2.7)		23 (2.5)	–0.55 (3.2)	0.28	
••2.5 g/day n-3	22 (2.8)		23 (3.0)	1.2 (3.3)	0.04	
•palmitoleic acid c16:1n-7		0.33		0.06		0.03
••Placebo	1.7 (0.56)		1.8 (0.61)	0.13 (0.59)	0.23	
••1.25 g/day n-3	1.8 (0.64)		1.5 (0.56)	–0.28 (0.72)	0.02	
••2.5 g/day n-3	1.5 (0.80)		1.5 (0.60)	–0.012 (0.60)	0.91	
Omega-6 Fatty Acids						
•linoleic acid c18:2n-6		0.005		0.69		0.06
••Placebo	30 (3.9)		30 (5.3)	–0.47 (5.5)	0.63	
••1.25 g/day n-3	28 (4.1)		29 (4.4)	0.7 (4.3)	0.29	

••2.5 g/day n-3	31 (4.7)		29 (4.0)		–2.1 (5.4)	0.03		
•gamma linolenic acid c18:3n-6		0.58		0.26				0.08
••Placebo	0.45 (0.16)		0.47 (0.22)		0.019 (0.19)	0.57		
••1.25 g/day n-3	0.49 (0.18)		0.44 (0.18)		–0.06 (0.2)	0.05		
••2.5 g/day n-3	0.47 (0.13)		0.40 (0.16)		–0.074 (0.17)	0.01		
•dihomo-gamma linolenic acid c20:3n-6		0.43		0.001				0.0002
••Placebo	1.7 (0.36)		1.8 (0.46)		0.12 (0.43)	0.14		
••1.25 g/day n-3	1.9 (0.40)		1.7 (0.46)		–0.20 (0.40)	0.00		
••2.5 g/day n-3	1.8 (0.54)		1.4 (0.37)		–0.31 (0.44)	0.0002		
•arachidonic acid c20:4n-6		0.52		0.64				0.05

••Placebo	7.7 (2.7)		7.5 (2.5)		–0.25 (1.8)	0.44	
••1.25 g/day n-3	7.3 (1.9)		7.2 (1.7)		–0.115 (1.1)	0.53	
••2.5 g/day n-3	7.9 (2.5)		7.0 (2.0)		–0.95 (1.6)	0.001	
Omega-3 Fatty Acids							
•alpha linolenic acid c18:3n-3		0.26		0.81			0.12
••Placebo	0.69 (0.23)		0.68 (0.18)		–0.01 (0.21)	0.78	
••1.25 g/day n-3	0.76 (0.27)		0.65 (0.15)		–0.11 (0.26)	0.009	
••2.5 g/day n-3	0.67 (0.24)		0.65 (0.26)		–0.016 (0.23)	0.67	
•stearidonic acid c18:4n-3		0.45		0.80			0.95
••Placebo	0.38 (0.15)		0.39 (0.20)		0.012 (0.25)	0.80	
••1.25 g/day n-3	0.41 (0.17)		0.44		0.0252	0.63	

			(0.39)	(0.33)		
••2.5 g/day n-3	0.44 (0.24)		0.44	–0.0016	0.98	
			(0.36)	(0.44)		
•eicosatetraenoic acid c20:4n-3		0.83		0.07		0.50
••Placebo	0.17 (0.10)		0.16	–0.010	0.68	
			(0.08)	(0.14)		
••1.25 g/day n-3	0.18 (0.09)		0.19	0.01 (0.1)	0.65	
			(0.10)			
••2.5 g/day n-3	0.18 (0.08)		0.21	0.027	0.23	
			(0.09)	(0.13)		
•eicosapentaenoic acid (EPA) c20:5n-3		0.52		<.0001		<.0001
••Placebo	0.48 (0.17)		0.74	0.26 (0.40)	0.001	
			(0.45)			
••1.25 g/day n-3	0.53 (0.21)		1.86	1.3 (0.73)	<.0001	
			(0.81)			
••2.5 g/day n-3	0.55 (0.36)		3.42	2.9 (1.4)	<.0001	
			(1.33)			

•docosapentaenoic acid (DPA) c22:5n-3		0.19	<.0001		<.0001
••Placebo	0.53 (0.14)	0.59 (0.12)	0.056 (0.15)	0.05	
••1.25 g/day n-3	0.60 (0.13)	1.01 (0.31)	0.42 (0.31)	<.0001	
••2.5 g/day n-3	0.56 (0.17)	1.19 (0.31)	0.62 (0.31)	<.0001	
•docosahexaenoic acid (DHA) c22:6n-3		0.66	0.0001		<.0001
••Placebo	1.5 (0.58)	1.6 (0.64)	0.061 (0.47)	0.47	
••1.25 g/day n-3	1.4 (0.59)	2.0 (0.70)	0.59 (0.46)	<.0001	
••2.5 g/day n-3	1.5 (0.62)	2.2 (0.48)	0.71 (0.67)	<.0001	
Totals and n-6:n-3 Ratio					
•Total *n*-3		0.74	<.0001		<.0001

••Placebo	3.8 (0.82)		4.1 (0.93)		0.37 (0.77)	0.01		
••1.25 g/day n-3	3.9 (0.8)		6.2 (1.6)		2.3 (1.4)	<.0001		
••2.5 g/day n-3	3.9 (1.1)		8.2 (1.8)		4.2 (2.1)	<.0001		
•Total *n*-6		0.01		0.43				0.01
••Placebo	41 (5.4)		40 (6.8)		–0.55 (6.2)	0.62		
••1.25 g/day n-3	38 (5.0)		39 (5.0)		0.27 (4.7)	0.72		
••2.5 g/day n-3	42 (5.9)		39 (5.1)		–3.6 (5.9)	0.001		
•*n*-6:*n*-3 Ratio		0.07		<.0001				<.0001
••Placebo	11 (2.0)		10 (2.8)		–0.97 (2.7)	0.06		
••1.25 g/day n-3	10 (2.0)		6.8 (2.1)		–3.4 (2.2)	<.0001		
••2.5 g/day n-3	11 (3.1)		5.0 (1.6)		–6.4 (3.4)	<.0001		

Open in a separate window

Units: Percent of total fatty acids

[1]All values are mean (SD)

[2]ANOVA

[3]Paired t-tests within group

3.3 Changes in F2-isoprostanes

Table 4 shows the significant group differences in changes in log-F2-isoprostanes after supplementation. The estimated mean change in log-F2-isoprostanes was 0.073 for the placebo group, corresponding to an 8% increase in geometric mean. In contrast, the estimated mean change in log-F2-isoprostanes was –0.094 for the low dose group and –0.086 for the high dose group, corresponding to decreases in the geometric mean of 9% and 8%, respectively. For both doses these changes were significantly different than the placebo group (Tukey-Kramer adjusted p = 0.02; p = 0.04, respectively), resulting in the intervention groups having a 15% lower geometric mean F2-isoprostanes at 4 months compared to control. There was not a significant difference between the two supplemented groups (p = 0.99). These results remained the same in analyses additionally controlling for age, gender and SAD.

Table 4

Estimated change in outcomes (4 months minus baseline) by intervention group, adjusted for baseline outcome levels.

				P*-values for group comparisons		
Outcome	**Placebo**	**1.25 g/day *n*-3**	**2.5 g/day *n*-3**	**Low Dose vs Placebo**	**High Dose vs Placebo**	**High vs Low Dose**
Change in Telomere length	–43 (–131, 46)	21 (–57, 99)	50 (–33, 133)	0.53	0.29	0.87
Change in ln (Telomerase)	0.066 (–0.11, 0.24)	0.10 (–0.045, 0.25)	0.040 (–0.12, 0.20)	0.94	0.97	0.83
Change in ln(F2-	0.073	–0.094	–0.086	0.02	0.04	0.99

isoprostanes)	(–0.021, 0.17)	(–0.17, –0.014)	(–0.17, 0.0009)

Units: Telomere length = base pairs; Telomerase = activity/10000 cells; F2-isoprostanes = ng/ml

Numbers in parentheses are 95% confidence intervals.

*Tukey-Kramer adjustment for multiple comparisons

Sample sizes: Telomerase, n=94; Telomere length, n=106; F2-isoprostanes, n=97

3.4 Changes in telomere length and telomerase

The adjusted mean change in telomere length, expressed in base pairs (bp), was an increase of 21 bp for the low dose group and an increase of 50 bp in the high dose group compared to a decrease of 43 bp for placebo (Table 4); however; differences between the groups were not significant. Telomere lengthening (defined as a positive change) was observed in 54% (n=19) of the 2.5 g/d n-3 PUFA group and 53% (n=21) of the 1.25 g/d n-3 PUFA group, but only 39% (n=12) of the placebo group, though these differences were not significant ($p = 0.39$). There were no differences among the groups in change in telomerase activity at four months (Table 4). Models additionally controlling for age, gender, and SAD produced similar results.

3.5 Changes in telomere length based on n-6:n-3 PUFA plasma ratios

Secondary analyses explored the effect of changes in plasma *n*-6:*n*-3 PUFA ratios on changes in telomere length, since individuals differ in absorption and metabolism of *n*-3 PUFA supplements. Table 5 shows the resulting linear regression analysis, controlling for baseline telomere length and baseline *n*-6:*n*-3 PUFA ratio. A one unit decrease in *n*-6:*n*-3 PUFA ratio was associated with an estimated 20 bp increase in telomere length ($p = 0.02$).

Table 5

Linear regression analysis for change in telomere length with change in n-6:n-3 fatty acid plasma ratio, controlling for baseline telomere length.

Effect	Estimate	95% CI	P-value
Intercept	1040	(296, 1785)	0.007
Telomere length, baseline	–0.15	(–0.27, –0.031)	0.01
n-6:n3 fatty acids, baseline	–21	(–44, 2.0)	0.07
Decrease in n6:n3 fatty acids[a]	20	(4, 36)	0.02

Units: telomere length = base pairs.

Regression model with change in telomere length (4 months minus baseline) as the outcome

[a]Decrease in *n*-6:n-3 PUFA ratio is calculated as baseline minus 4 months so that a positive value is a decrease in *n*-6:*n*-3 PUFA ratio.

The analysis was repeated using the change in AA:(EPA+DHA) ratio in place of *n*-6:*n*-3 PUFA ratio; the AA:(EPA+DHA) ratio is favored by some researchers because of a more direct tie to eicosanoid metabolism. Since the change in AA:(EPA+DHA) ratio was highly correlated with the change in *n*-6:*n*-3 PUFA ratio (r = 0.90, $p < 0.001$), results were similar, with a one unit decrease in AA:(EPA+DHA) ratio associated with a 35 bp increase in telomere length (p = 0.08). Similarly, when the change in the sum of EPA, DHA, and DPA was used in place of either ratio, results were again comparable, with a one unit increase in EPA+DHA+DPA associated with a 22 bp increase in telomere length ($p = 0.07$). All results were similar after adjusting for age, gender, and SAD.

3.6 Changes in telomere length related to IL-6

Since supplementation reduced serum IL-6 in both low and high dose groups in the parent study (Kiecolt-Glaser et al., 2012), as a secondary analysis we investigated the association between change in telomere length and change in IL-6 for the 101 subjects in the present study who had IL-6 data available at baseline and four months. There was a significant negative correlation between change in telomere length and change in IL-6 (Spearman r = –0.20, $p = 0.05$). Of the 51 subjects who experienced telomere lengthening, 61% (n=31) had lowered IL-6, compared to 34% (n=17) of the 50 who did not experience telomere lengthening ($p = 0.007$).

4. Discussion

4.1 Intervention-related changes

As previously reported, *n*-3 PUFA supplementation significantly reduced inflammation in this trial (Kiecolt-Glaser et al., 2012). In these new data, analyses that compared the changes among the *n*-3 PUFA groups revealed that supplementation also lowered F2-isoprostanes but showed no significant effects related to telomere length or telomerase. However, individuals differ in absorption and metabolism of *n*-3 PUFA supplements, as well as in adherence, and thus additional planned analyses that used actual changes in the *n*-6:*n*-3 PUFA plasma ratios clarified the intervention's impact: lower *n*-6:*n*-3 PUFA ratios were associated with longer telomeres at the trial's end. These randomized trial data complement the observational findings from the Heart and Soul Study which showed that slower telomere attrition was reliably related to higher levels of the two key *n*-3 PUFAs (Farzaneh-Far et al., 2010a). Our data are also in accord with evidence linking higher dietary intake of *n*-6 PUFAs with shorter telomeres (Cassidy et al., 2010; Kang, 2010).

Telomerase activity level did not change in our sample, in contrast to the changes observed following an intensive three-month lifestyle change program in a different study group - men with early prostate cancer - that included 3 g/day of fish oil (Ornish et al., 2008). That intervention also included dietary change (low-fat and high plant-based), aerobic exercise, and stress management. Further, as that previous study had no non-intervention control group, those data must be interpreted cautiously as regards involvement of *n*-3 PUFAs.

Depressive symptoms were quite low at baseline in this sample, and did not change (Kiecolt-Glaser et al., 2012). However, prior studies that have linked lower *n*-3 PUFA plasma levels and depression suggest a potential benefit for more distressed groups (Appleton et al., 2010; Hibbeln, 1998). Depression and chronic stress have been associated with shorter telomeres (Damjanovic et al., 2007; Epel et al., 2004; Wolkowitz et al., 2010). Depression and chronic stress boost inflammation (Glaser and Kiecolt-Glaser, 2005; Kiecolt-Glaser et al., 2003) as well as oxidative stress (Epel et al., 2004; Wolkowitz et al., 2010), and could speed telomere erosion through these pathways. Accordingly, *n*-3 PUFA supplementation might also slow telomere attrition by enhancing mood in more depressed samples.

Although age-related reductions in telomeres are the average situation, recent studies showed that telomeres can both shorten and elongate *in vivo*, and leukocyte telomere length can change within a period of months (Shlush et al., 2011; Svenson et al., 2011). Alterations in oxidative stress were highlighted as a potential mechanism in two recent reports (Shlush et al., 2011; Svenson et al., 2011). Our data suggest that a dietary intervention that reduces the joint burden of oxidative stress and inflammation may in turn have positive consequences for telomere length.

During aging there is a shift from naïve to memory T-cells, and the latter have shorter telomeres (Svenson et al., 2011). We do not know if the reductions in inflammation and oxidative stress in our *n*-3 PUFA supplemented participants reflected shifts in leukocyte subpopulations that contributed to the telomere changes observed, one limitation of the present study. In addition, a longer supplementation period with additional assessments would

have been helpful to better assess the full impact of the intervention on telomere length and telomerase.

It would have been desirable to examine *n*-6 and *n*-3 PUFAs in red blood cells (RBCs) in addition to plasma levels. Circulating PUFA levels reflect the interplay among dietary intake, absorption, and metabolism and are not always strongly correlated with dietary intake of fatty acids (Fusconi et al., 2003; Seierstad et al., 2005). RBC PUFA levels reflect longer-term PUFA consumption as the turn-over is slow and more reliable (Harris, 2008; Harris, 2009). For example, DHA levels in RBCs are thought to indicate dietary fat intake for the past four months, while levels in plasma may only mirror intake from the last few days (Arab, 2003; Sun et al., 2007). However, our intervention spanned four months and serum and plasma proinflammatory cytokines can change in hours (Glaser and Kiecolt-Glaser, 2005); for example, infusion of a fish oil-based lipid emulsion substantially reduced monocyte production of IL-6, TNF-α, IL-1, and IL-8 in response to endotoxin (Mayer et al., 2003). For these reasons, plasma PUFA data were essential to assess recent dietary influences on inflammatory markers in this study.

As described earlier, the *n*-6 and *n*-3 PUFAs compete for key enzymatic pathways, and thus the relative balance is of interest (Simopoulos, 2008). ATTICA, a large health and nutrition survey of healthy Greek adults, showed that higher *n*-6:*n*-3 PUFA plasma ratios were associated with higher TNF-α and IL-6 (Kalogeropoulos et al., 2010). In prior work from our lab, higher levels of depressive symptoms as well as higher *n*-6:*n*-3 PUFA ratios worked together to markedly enhance proinflammatory cytokines beyond the contribution provided by either variable alone (Kiecolt-Glaser et al., 2007).

Our participants' average *n*-6:*n*-3 PUFA ratio at baseline was 10.8 (SD=2.6, range=3.3–18.8), considerably lower than the 14:1 ratio derived from plasma data reported for both the large Nurses' Health Study and the ATTICA cohorts (Kalogeropoulos et al., 2010; Sun et al., 2007). Participants with higher *n*-6:*n*-3 PUFA ratios would have benefitted more because supplementation of poorer diets might have permitted greater positive change. Accordingly, our data may underestimate the potential impact of the *n*-3 PUFAs for the general population.

4.2 Health implications

We found that telomere length increased with decreasing *n*-6:*n*-3 PUFA ratios. These data suggest that rather than just considering the absolute amount of *n*-3 PUFA, the background levels of both the *n*-6 and the *n*-6:*n*-3 PUFAs should also be taken into account for clinical studies or for evaluation of nutritional interventions. For example, the *n*-6:*n*-3 PUFA ratio can be altered by increasing *n*-3 PUFA supplementation, but also by decreasing *n*-6 intake.

Several large studies have linked higher *n*-3 PUFA levels with lower all-cause mortality (Lee et al., 2009; Pottala et al., 2010) including a large 3.5 year trial (Marchioli et al., 2002). The *n*-3 PUFA's anti-inflammatory and antioxidant properties provide one obvious pathway for these reductions in mortality, consistent with the finding that decreases in IL-6 were associated with telomere lengthening in this study. Our data suggest that the *n*-3 PUFAs can impact cell aging in addition to inflammation and oxidative stress. This translational research broadens our understanding of the *n*-3 PUFA's potential therapeutic effects.

Short telomeres predict early disease, and slowing immune cell aging could have broad effects by slowing the onset of age-related diseases. Recent work has demonstrated the causal effect of telomerase deficiency and telomere shortening on cellular health and premature aging and mortality in rodents (Bernardes de Jesus et al., 2012; Jaskelioff et al., 2011; Sahin et al., 2011). In summary, the current study provides compelling initial evidence that lower *n*-6:*n*-3 PUFA ratios may be beneficial for slowing biological aging.

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Footnotes

Conflict of interest statement: Drs. Blackburn, Epel, and Lin are co-founders in Telome Health, Inc, a telomere measurement company.

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