ONLINE SUPPLEMENTAL TEXT

Subjects and Methods

Study Design

Inclusion and exclusion criteria: The following conditions were cause for exclusion: fasting triglyceride concentration > 500 mg/dL; fasting LDL cholesterol > 160 mg/dL, hemoglobin < 12 g/dL for females or < 14 g/dL for males, arthritis, asthma, stroke, heart attack, clinical depression or fish allergy. Use of prescription or over-the-counter medications that could have affected principal study endpoints were also cause for exclusion, including any use of fish-oil or high-dose antioxidant supplements, > 2 servings per week of oily fish (e.g., herring, salmon, trout and tuna) or shellfish, >1 serving per week of flaxseed or flaxseed oil, > 2 servings per week of walnuts or walnut oil, >1 α-linolenic acid-enriched egg per day. Other exclusionary conditions included smoking > 14 cigarettes per week, consuming > 14 alcoholic drinks per week, BMI < 18.5 or >35 kg/m^2, pregnancy, being the first-degree relative of a study participant, and donation of blood within one month of baseline blood draw.

Randomization to treatment groups within genotypes: The genotype groups and treatment groups were not known to study investigators or participants. The human studies manager at the Western Human Nutrition Research Center (WHNRC) acted as an intermediary between the lab conducting the genotyping (Dr. Allayee) and study personnel during the study and initial data analysis. Coded treatment lists within each genotype were generated by the study pharmacist, from the University of California, Davis Investigative Pharmacy. After completion of the study, genotyping was repeated on new DNA samples from peripheral blood to confirm genotypes. Three subjects had been misidentified. These subjects were initially identified as

"33", "35", and "55" but were found to be "34" (placebo), "55" (fish oil), and "35" (fish oil), respectively.

Fish oil and placebo treatments: Bottling was performed by the UC Davis Investigational Pharmacy. Subjects each received one bottle containing 252 capsules as determined by weight, sufficient for 50 d. The fatty acid profile for the 40/20 EE capsules consisted of a minimum 40% EPA and 20% DHA by analysis. The composition of the corn/soybean oil capsules include the following: 0% DHA, 0% EPA, 25% oleic acid, 53% linoleic acid, 5% α-linolenic acid, 10% palmitic acid, and 7% other fatty acids. Each capsule contained 3 mg/g vitamin E (as mixed tocopherols) as an antioxidant. The fatty acid composition, color and capsule size were the same for all 40/20 EE and placebo capsules. However, the taste of the capsules varied between lots. Lots 10524 (omega-3) and 8980 (placebo) were flavored with vanilla while lots 10525 (omega-3) and 8981 (placebo) were flavored with citrus. Fatty acid analysis was performed quarterly on stored capsules to monitor stability of omega-3 fatty acid levels. No significant differences were seen.

Instructions to subjects: Trained staff instructed subjects to take 1 or 2 capsules per meal (total 5 capsules/day), and to record the number of capsules taken each day on a calendar supplied by the study. Dietary guidelines were provided at week 0 to remind subjects to maintain their usual diets and to list food items and supplements identified as exclusionary. Biweekly e-mails or telephone calls were made to the subjects to inquire about adverse events. During the study visit at week 6, the calendars were examined and unused capsules counted to assess compliance.

Laboratory Methods

Western blots: Whole-cell lysis buffer consisted of 1% NP-40, 30mM Tris-HCl, 0.5 mM EDTA, 150 mM NaCl and 10% Glycerol (pH 7.5); each 10 mL contained 1 tablet of protease inhibitor complete cocktail (Roche Diagnostics GMBH, Germany) and 0.5 mM phenylmethanesulfonyl fluoride (PMSF; Sigma-Aldrich Corp, St. Louis, MO). Cell extracts were resolved by SDS-PAGE on 10% polyacrylamide gels for 1 hour at 175 volts using Bio-Rad Mini-PROTEAN II cell and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) in transfer buffer, 25 mmol/L 0.25M Tris, 200 mmol/L glycine, and 20% methanol for 1.5 hours at 150 volts. Membranes were blocked at room temperature for 2 hours in a blocking solution of Tris-buffered saline (TBS), pH of 7.6, with 5% dry skimmed milk (Nestle-Carnation, Wilkes-Barre, PA). Molecular weight standards were run on each gel (BenchMark Pre-stained Protein Ladder; Invitrogen, Carlsbad, CA). After blocking the membranes, primary mouse monoclonal antibodies to 5-LO (Product 610695 BD Biosciences, San Jose, CA) in blocking solution were incubated overnight (Dilution 1:250) at 4°C. After washing blots were incubated with sheep anti-mouse horseradish peroxidase-conjugated IgG (Product NA931V, GE Health Care, Piscataway, NJ) for 2 hours at room temperature (Dilution 1:5000 in blocking solution). After each antibody application, blots were washed 5 times for 5 min each, in TBS. Antibody binding was visualized by chemiluminescence using SuperSignal ECL (ThermoScientific, Waltham, MA) by both ECL film (Amersham Bioscience, Arlington Heights, IL, USA) and AlphaEase FC imager (FluorChem8800, Alpha Innotech, San Leandro, CA). Blots were incubated with ECL solution for 1 minute. Membranes were then washed 5 times for 5 minutes each, with TBS and reincubated with primary monoclonal mouse antibodies to β-actin (Product A2228 Sigma, Saint Louis, MO) in blocking solution overnight at 4°C (Dilution 1:40, 000). Secondary antibodies

were then again applied for 2 hours (Dilution 1:10, 000 in blocking solution) at room temperature and membranes were visualized as described above. A fixed amount of cells (500,000 per well) and volume (25 uL) were added in each lane to normalize for loading. The human erythromyeloblastoid leukemia cell line K-562, which expresses high amounts of 5-LO, was used as a positive control to control for gel to gel differences. The quality of proteins and efficacy of protein transfer were evaluated by Ponceau Red (Sigma-Aldrich, St. Louis, USA) staining (not shown). The density of the two ALOX5 bands summed together was divided by the density of the β-actin band to provide a value for statistical analysis.

Results

Baseline Data

Subject characteristics: The placebo and fish oil groups did not differ with respect to age (placebo, 30.5 y [24.0/47.0 y]; fish oil, 35.0 y [27.0/46.0 y]; p = 0.48), BMI (placebo, 27.5 ± 4.5 kg/m^2; fish oil, 27.7 ± 4.8 kg/m^2; p = 0.83), or sex (placebo, 19 F/39 M; fish oil, 18 M/40 F; p = 1.0). BMI differed (p = 0.022) by genotype using 6 (but not 3) genotype groups but pair-wise comparisons were not significant. Age and sex did not differ by genotype. Age and BMI did not differ between males and females. These data indicate that the genotype and treatment groups were comparable with regard to sex and age but significant heterogeneity was seen among the six genotype groups for BMI.

Effect of Intervention

Compliance with intervention assessed by pill counts at 6 wk visit: The median (25th/75th percentiles) duration of the intervention in all subjects was 42 d (42/47 d; range, 37 – 65 d). The median duration did not differ by treatment group (placebo, 42 d [42/47 d]; fish oil, 42 d [42/44 d]; p = 0.075) or genotype (data not shown). The median reported intake of

capsules, expressed as a percentage of expected intake based on the number of days between visits, was 93.6% (81.4/100%) for all subjects for whom counts of returned capsules were available (45 fish oil and 44 placebo). No differences in reported intake were seen by treatment group (fish oil, 94.8% [77.9/100%]; placebo, 92.8% [85.7/98.6%]; $p = 0.88$) or by genotype (data not shown).

Online Supplemental Table 1. Demographic data on all subjects screened for enrollment.

Variable*	Screened N = 783	Not Enrolled N = 667	Enrolled N = 116	p-value**
Female; %	72.1% (557/772)	72.9% (478/656)	68.1% (79/116)	0.35
Age (y); median $25^{th}/75^{th}$ %iles	41 28/50	42 30/51	33, 25/46	<0.001
BMI (kg/m^2); median $25^{th}/75^{th}$ %iles	28 24.8/32.3	28 25/33	26.5 23.6/29.8	<0.001

* data are from screening questionnaire

** enrolled vs. not enrolled

Online Supplemental Table 2. Age, BMI and sex for subjects completing and not completing the study.

Variable	Completing N = 98	Not Completing N = 18	p-value
Age (years); median $25^{th}/75^{th}$ percentiles	34 25/47	31.5 27/41	0.94
BMI (kg/m^2); mean ± SD	27.8 ± 4.7	26.4 ± 4.30	0.25
Female; %	69 % (68/98)	61% (11/18)	0.68

Online Supplemental Table 3. Genotype and treatment group of subjects completing study with oxylipid metabolite data available.

	Genotype	Fish Oil	Placebo	Total
	"33"	4	5	9
	"34"	6	4	10
	"44"	2	2	4
Subtotal	"dd"	12	11	23
	"35"	14	13	27
	"45"	10	12	22
Subtotal	"d5"	24	25	49
Subtotal	"55"	13	12	25
Total		49	48	97

Online Supplemental Table 4. Mean (± SE) oxylipid concentrations (nmol/L) from A23187-stimulated monocyte supernatants by genotype group at the baseline visit.

	Genotype			
Metabolite	"33"	"35"	"55"	P-value[1]
N	14	30	30	
Group 1[2]				
LTB4[3]	6.50 ± 1.12	7.20 ± 0.78	6.39 ± 0.55	0.686
LTB5	0.15 ± 0.033	0.12 ± 0.023	0.29 ± 0.19	0.597
LTD4[3]	1.81 ± 0.26	1.37 ± 0.20	1.03 ± 0.16	0.056
LTE4[3]	3.45 ± 0.86	3.24 ± 0.60	3.48 ± 0.75	0.848
Group 2				
6-trans LTB4	9.20 ± 2.59	12.02 ± 1.87	17.80 ± 4.33	0.137
5-oxo-ETE	1.75 ± 0.38	2.21 ± 0.38	2.87 ± 0.58	0.297
5-HETE	[a]328 ± 63	424 ± 57	539 ± 60	0.031
5-HEPE	[a]20.2 ± 8.25	32.2 ± 4.90	38.7 ± 6.47	0.009
15-HETE	[a]6.64 ± 1.78	[a]11.23 ± 1.80	17.53 ± 2.80	0.002
15-HEPE	[a]1.02 ± 0.30	[a]1.38 ± 0.22	2.09 ± 0.36	0.014
5,15-DiHETE	[a]2.34 ± 0.82	4.80 ± 0.92	5.95 ± 1.24	0.025
9-HETE	[a]6.10 ± 1.92	12.18 ± 2.29	16.65 ± 2.85	<0.001
Lipoxin A4	1.40 ± 0.27	1.90 ± 0.23	2.02 ± 0.32	0.281
17-HDoHE	[a]4.00 ± 2.11	6.19 ± 1.07	10.56 ± 2.30	0.012
Group 3				
8,15-DiHETE	0.47 ± 0.98	0.83 ± 0.19	1.25 ± 0.38	0.291

[1] One-factor ANOVA. Letter superscripts indicate a significant difference from the “55” genotype group by post-hoc comparison.

[2] Groups 1, 2 and 3 based on correlations among metabolites as seen in Table 3.

[3] One of these 14 subjects with "33" genotype was excluded as outlier for LTB4, LTD4 and LTE4 analysis.

Online Supplemental Table 5. Mean (± SE) oxylipid concentrations (nmol/L) from A23187-stimulated monocyte supernatants by genotype group at the baseline visit.

	Genotype			
Metabolite	"44"	"45"	"55"	P-value[1]
N	4	23	30	
Group 1[2]				
LTB4	3.31 ± 0.79	6.78 ± 0.77	6.39 ± 0.55	0.115
LTB5	0.10 ± 0.056	0.17 ± 0.029	0.29 ± 0.19	0.130
LTD4	0.93 ± 0.26	1.27 ± 0.23	1.03 ± 0.16	0.790
LTE4	3.99 ± 1.16	3.68 ± 0.70	3.48 ± 0.75	0.269
Group 2				
6-trans LTB4	5.63 ± 1.43	[a]7.34 ± 1.56	17.80 ± 4.33	0.011
5-oxo-ETE	1.71 ± 0.71	1.53 ± 0.26	2.87 ± 0.58	0.105
5-HETE	308 ± 93	[a]285 ± 60	539 ± 60	0.002
5-HEPE	[a]11.44 ± 3.01	[a]14.25 ± 2.91	38.7 ± 6.47	<0.001
15-HETE	6.47 ± 2.54	[a]7.50 ± 2.01	17.53 ± 2.80	0.002
15-HEPE	1.00 ± 0.63	[a]1.21 ± 0.25	2.09 ± 0.36	0.025
5,15-DiHETE	1.32 ± 0.34	[a]2.19 ± 0.86	5.95 ± 1.24	<0.001
9-HETE	5.89 ± 3.05	[a]7.47 ± 2.19	16.65 ± 2.85	0.002
Lipoxin A4	1.10 ± 0.22	1.28 ± 0.26	2.02 ± 0.32	0.104
17-HDoHE	3.44 ± 1.73	[a]3.97 ± 1.07	10.56 ± 2.30	0.007
Group 3				
8,15-DiHETE	0.11 ± 0.020	0.76 ± 0.14	1.25 ± 0.38	0.163

[1] One-factor ANOVA. Letter superscripts indicate a significant difference from the “55” genotype group by post-hoc comparison.

[2] Groups 1, 2 and 3 based on correlations among metabolites as seen in Table 3.

Online Supplemental Table 6. Mean (± SE) oxylipid concentrations (nmol/L) from A23187-stimulated monocyte supernatants by genotype group at the baseline visit.

	Genotype			
Metabolite	"33"	"34"	"44"	P-value[1]
N	14*	14	4	
Group 1[2]				
LTB4[3]	6.50 ± 1.12	6.17 ± 0.91	3.31 ± 0.79	0.190
LTB5	0.15 ± 0.033	0.39 ± 0.27	0.10 ± 0.056	0.700
LTD4[3]	1.81 ± 0.26	1.44 ± 0.22	0.93 ± 0.26	0.170
LTE4[3]	3.45 ± 0.86	5.71 ± 1.37	3.99 ± 1.16	0.590
Group 2				
6-trans LTB4	9.20 ± 2.60	10.09 ± 2.83	5.63 ± 1.43	0.780
5-oxo-ETE	1.75 ± 0.38	1.40 ± 0.25	1.71 ± 0.71	0.740
5-HETE	328 ± 63	330 ± 78	308 ± 93	0.930
5-HEPE	20.2 ± 8.25	15.22 ± 4.32	11.44 ± 3.01	0.940
15-HETE	6.64 ± 1.78	8.04 ± 1.90	6.47 ± 2.54	0.897
15-HEPE	1.02 ± 0.30	0.92 ± 0.15	1.00 ± 0.63	0.925
5,15-DiHETE	2.34 ± 0.82	3.57 ± 1.33	1.32 ± 0.34	0.930
9-HETE	6.10 ± 1.92	8.26 ± 2.43	5.89 ± 3.05	0.890
Lipoxin A4	1.40 ± 0.27	2.18 ± 0.60	1.10 ± 0.22	0.710
17-HDoHE	4.00 ± 2.11	3.36 ± 0.97	3.44 ± 1.73	0.430
Group 3				
8,15-DiHETE	0.47 ± 0.98	0.63 ± 0.16	0.11 ± 0.020	0.170

[1] One-factor ANOVA.

[2] Groups 1, 2 and 3 based on correlations among metabolites as seen in Table 3.

[3] One of the 14 "33" subjects was excluded as outlier for LTB4, LTD4 and LTE4 analysis.

Online Supplemental Table 7. Mean (± SE) oxylipid concentrations (nmol/L) from A23187-stimulated monocyte supernatants by genotype group at the baseline visit.

	Genotype		
Metabolite	"35"	"45"	P-value[1]
N	30	23	
Group 1[2]			
LTB4	7.20 ± 0.78	6.78 ± 0.77	0.705
LTB5	0.12 ± 0.023	0.17 ± 0.029	0.118
LTD4	1.37 ± 0.20	1.27 ± 0.23	0.713
LTE4	3.24 ± 0.60	3.68 ± 0.70	0.418
Group 2			
6-trans LTB4	12.02 ± 1.87	7.34 ± 1.56	0.046
5-oxo-ETE	2.21 ± 0.38	1.53 ± 0.26	0.153
5-HETE	424 ± 57	285 ± 60	0.038
5-HEPE	32.2 ± 4.90	14.25 ± 2.91	0.004
15-HETE	11.23 ± 1.80	7.50 ± 2.01	0.032
15-HEPE	1.38 ± 0.22	1.21 ± 0.25	0.647
5,15-DiHETE	4.80 ± 0.92	2.19 ± 0.86	0.012
9-HETE	12.18 ± 2.29	7.47 ± 2.19	0.007
Lipoxin A4	1.90 ± 0.23	1.28 ± 0.26	0.022
17-HDoHE	6.19 ± 1.07	3.97 ± 1.07	0.109
Group 3			

8,15-DiHETE	0.83 ± 0.19	0.76 ± 0.14	0.771

[1] Student's t-test.

[2] Groups 1, 2 and 3 based on correlations among metabolites as seen in Table 3.

Online Supplemental Figure 1. Scatter plots (with regression lines and confidence intervals) of oxylipid metabolites 60 min after A23187 stimulation of monocyte cultures vs. ALOX5 RNA levels measured on unstimulated cells at baseline. Data from all 99 subjects for which both variables were available are included except for 5-oxo ETE where one data point (17.3 nmol/L 5-oxo ETE and 0.33 ru ALOX5 RNA) was excluded to allow a better display of the data.

Online Supplemental Figure 1