Safety Assessment of Docosahexaenoic Acid in X-Linked Retinitis Pigmentosa: The 4-Year DHAX Trial

Dianna K. Hughbanks-Wheaton,^{1,2} David G. Birch,^{1,2} Gary E. Fish,³ Rand Spencer,³ N. Shirlene Pearson,⁴ Alison Takacs,¹ and Dennis R. Hoffman^{1,2}

¹Retina Foundation of the Southwest, Dallas, Texas, United States

²Department of Ophthalmology, UT Southwestern Medical Center, Dallas, Texas, United States

³Texas Retina Associates, Dallas, Texas, United States

⁴Pearson Statistical Consulting & Expert Testimony, Richardson, Texas, United States

Correspondence: Dianna K. Hughbanks-Wheaton, Retina Foundation of the Southwest, Suite 200, 9600 North Central Expressway, Dallas, TX 75231, USA; dwheaton@retinafoundation.org.

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PURPOSE. Docosahexaenoic acid (DHA) continues to be evaluated and recommended as treatment and prophylaxis for various diseases. We recently assessed efficacy of high-dose DHA supplementation to slow vision loss in patients with X-linked retinitis pigmentosa (XLRP) in a randomized clinical trial. Because DHA is a highly unsaturated fatty acid, it could serve as a target for free-radical induced oxidation, resulting in increased oxidative stress. Biosafety was monitored during the 4-year trial to determine whether DHA supplementation was associated with identifiable risks.

METHODS. Males ($n = 78$; 7–31 years) meeting entry criteria were enrolled. The modified intent-to-treat cohort (DHA = 33; placebo = 27) adhered to the protocol ≥ 1 year. Participants were randomized to an oral dose of 30 mg/kg/d DHA or placebo plus a daily multivitamin. Comprehensive metabolic analyses were assessed for group differences. Treatment-emergent adverse events including blood chemistry metabolites were recorded.

RESULTS. By year 4, supplementation elevated plasma and red blood cell-DHA 4.4- and 3.6-fold, respectively, compared with the placebo group ($P < 0.00001$). Over the trial duration, no significant differences between DHA and placebo groups were found for vitamin A, vitamin E, platelet aggregation, antioxidant activity, lipoprotein cholesterol, or oxidized LDL levels (all $P > 0.14$). Adverse events were transient and not considered severe (e.g., gastrointestinal [GI] irritability, blood chemistry alterations). One participant was unable to tolerate persistent GI discomfort.

CONCLUSIONS. Long-term, high-dose DHA supplementation to patients with XLRP was associated with limited safety risks in this 4-year trial. Nevertheless, GI symptoms should be monitored in all patients taking high dose DHA especially those with personal or family history of GI disturbances. (ClinicalTrials.gov number, NCT00100230.)

Keywords: biosafety, fatty acids, adverse events

Retinitis pigmentosa (RP) is a retinal degenerative disease characterized by night blindness and visual field constriction¹ with four underlying inheritance patterns. The X-linked form of RP (XLRP) is among the most severe with night blindness often detectable by age 5 years^{2–4} and legal blindness by the second or third decade. Gene defects are known to cause retinal degeneration, yet factors such as environment, diet, stress, and/or metabolism may modify disease severity. Many patients with RP have lower plasma and red blood cell (RBC) levels of the n3 polyunsaturated fatty acid docosahexaenoic acid (DHA; 22:6n3) than normally-sighted controls.⁵ Blood DHA was significantly correlated with age-adjusted ERG responses in XLRP such that patients with lower RBC-DHA tended to have lower ERG amplitudes.⁶ These findings were similarly documented in approximately 70% of female XLRP carriers (Hoffman DR, et al. *IOVS* 1998;39:ARVO Abstract 725). A reduction in DHA biosynthesis was demonstrated in XLRP using stable isotopes to assess in vivo metabolism⁷ suggesting that downregulation of hepatic Δ^5 desaturase may contribute to

subnormal blood DHA levels. Thus, daily supplementation with DHA may bypass any decrease in DHA biosynthesis.

Docosahexaenoic acid comprises 1% to 5% of membrane fatty acids in most human tissues; however, it is the most abundant fatty acid in the retina.⁸ This n3 fatty acid can increase membrane fluidity and modify the mobility of vital proteins and activities of retinal enzymes,^{9,10} promote photoreceptor differentiation,¹¹ and antiapoptotic activity.¹² The highly unsaturated nature of DHA makes it a potential target for free radical oxidative damage. Increased polyunsaturated fatty acid (PUFA) intake, particularly long-chain PUFAs (LCPUFAs; >18 carbons), may lead to elevated oxidative stress and subsequent membrane damage.^{13,14} The n3 LCPUFAs demonstrate metabolic competition with arachidonic acid (ARA; 20:4n6) leading to alterations in prostaglandins and leukotrienes and reportedly diminish platelet aggregation and increase bleeding times.¹⁵ Numerous n3-supplementation studies report elevations in low- and high-density lipoprotein (LDL and HDL)-cholesterol,¹⁶ and high doses of DHA and eicosapentaenoic acid (EPA; 20:5 n3)

derived from fish oil have been utilized to decrease plasma triacylglycerols.¹⁷

Long-term clinical trials with XLRP¹⁸ or RP^{19,20} patients receiving DHA doses ranging from 400 mg/d²¹ to 1200 mg/d²² have reported minimal safety risks. The current DHAX trial²³ employed DHA doses based on bodyweight resulting in intakes up to 3600 mg/d. The 30 mg/kg/d DHAX dose is within the range (3–84 mg DHA/kg/d) received by nursing infants^{24–26} and less than the 50–100 mg/kg/d doses used in three recent clinical trials.^{27–29} The DHAX trial provides the opportunity to assess the biological safety and metabolic consequences of long-term, high dose DHA supplementation in patients with XLRP.

METHODS

Subjects

Seventy-eight males diagnosed with XLRP (ages 7–31 years) were randomized to DHA ($n = 41$) or placebo ($n = 37$) supplements for a 4-year duration; DHAX recruitment and eligibility criteria were described previously.²³ Sixteen non-adherent participants dropped from the study during year 1, two additional participants were determined genetically to be non-XLRP. The resulting modified intent-to-treat (mITT) cohort consisted of 60 participants (33 DHA; 27 placebo) that completed ≥ 1 year. Subsequently, eight participants (three DHA; five placebo) dropped before year 4, primarily due to apathy and/or continued vision loss.

Informed consent was obtained from participants and/or parents of minors. Research adhered to the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board of The University of Texas Southwestern Medical Center at Dallas. Study oversight was conducted by a Data and Safety Monitoring Committee (see Acknowledgments).

Age-matched, healthy males ($n = 29$) with normal acuity and visual fields were recruited to provide blood samples for normative biochemistry measures.

Trial Design

A computer-generated, varied block size, randomization schedule was stratified by age and vitamin A supplementation. Visual function, blood samples, and bodyweight were assessed at baseline and annually (Supplementary Table S1). Individualized daily capsule intakes to achieve DHAX dosage were adjusted annually. Treatment-emergent adverse events (TEAE), including self-reported and blood analysis-derived events, were recorded.

Intervention

Gelatin capsules contained DHA-enriched (DHASCO) or corn/soy oil (placebo) triglycerides (DSM Nutritional Products, Columbia, MD, USA). The DHA content of DHASCO capsules provided 200 mg DHA in each 500 mg capsule. Fatty acid profiles of capsules are given in Supplementary Table S2. Study capsules were indistinguishable from placebo in appearance, smell, or taste and contained small amounts of vitamins E and C as antioxidants and a food-grade orange extract as flavoring. Toxicological outcomes of DHASCO consumption were considered minor and not of clinical significance.³⁰

Initial bodyweights were between 22 and 123 kg. At 30 mg/kg, the corresponding capsule intake ranged from 3 to 18 capsules/d providing a total DHA dose of 600 to 3600 mg/d. The corresponding total amount of fat (oil) ranged from 1.5 to 9 g/d and is comparable to 1/2 to 2 pats of butter.

Commercial multivitamins were provided to all participants to maintain a minimum antioxidant status. An over-the-counter multivitamin (CVS “Daily Multiple Essential,” SKU: 41656; CVS Corp., Lowell, MA, USA) provided 100% of the recommended daily amounts of vitamins A, C, D, E, B₆, and B₁₂.

Compliance

Capsule counts were conducted annually to assess protocol adherence. Docosahexaenoic acid in RBCs provided a more precise compliance measure; however, during the trial this information was only available to the monitoring committee. Capsule use was followed with a self-report diary completed every 3 months by the adult participant or with parental assistance in children.

Blood Samples

Fasting blood samples were drawn from an antecubital vein at baseline, 6 months, and annual visits; a total of 14.3 mL per blood draw was collected. Plasma and RBC fatty acids were assessed every 6 months and biosafety assays conducted annually. Antioxidant defense mechanisms were indexed by plasma concentrations of: the antioxidant vitamin E; the retinal chromophore precursor, vitamin A; oxidized low density lipoprotein (oxLDL), and a total plasma antioxidant capacity analysis. Whole blood platelet aggregation was monitored to address concerns of increased bleeding due to inactivation of platelet activation responses. A comprehensive blood panel assessed metabolic modifications and lipoprotein lipids were also measured.

Fatty Acid Analysis

Fatty acids were analyzed using previously detailed methodology.³¹ Plasma and RBCs were separated by centrifugation, lipids were solvent extracted, total lipids transmethylated, and methyl esters quantified by capillary column gas chromatography with flame ionization detection. Upon trial conclusion, fatty acids from placebo and DHASCO capsules were similarly analyzed. Fatty acid content was expressed as relative weight percentage of total fatty acids.

Biosafety Analysis

Vitamin A (retinol) and vitamin E (α -tocopherol) were quantified by C18 reverse-phase high-pressure liquid chromatography following lipid-soluble vitamin extraction.³² Retinol and α -tocopherol concentrations were expressed as μ M.

Total antioxidant capacity of plasma was assessed using the total peroxyl radical trapping parameter.³³ Quenching capacity of plasma as measured by chemiluminescence was compared with a standard curve of the synthetic vitamin E derivative, Trolox. Antioxidant activity was expressed as μ M “Trolox equivalents.”

The oxLDL analysis utilized a commercially available ELISA kit (#10-1143-01; Mercodia AB, Uppsala, Sweden). Normal plasma levels of oxLDL were reported to range from 25 to 120 U/L.³⁴

Platelet aggregation was measured in citrate-anticoagulated whole blood using type I collagen as agonist.³⁵ Results are expressed as impedance (ohms) to aggregation.

Lipoprotein Lipid Profiles

Total cholesterol, HDL-cholesterol, and triacylglycerides (TG) were measured using assay-specific disposable test cassettes

TABLE 1. Biochemical and Safety Outcome Measures of mITT Cohort at Trial Year 4

Characteristic	Normative Values* or Cutoff Threshold†	Year 0 to Year 4 Change (4-Y Mean Values; Mean ± SE)		
		Placebo (n = 22)	DHA (n = 30)	Effect of Treatment, P
Plasma DHA, %	1.42 ± 0.10*	−0.11 ± 0.07 (1.33 ± 0.08)	4.16 ± 0.29 (5.79 ± 0.29)	<0.00005
Plasma ARA, %	9.60 ± 0.28*	−0.20 ± 0.31 (8.16 ± 0.08)	−2.89 ± 0.32 (6.14 ± 0.05)	<0.00005
RBC DHA, %	2.91 ± 0.14*	−0.04 ± 0.13 (2.82 ± 0.03)	6.78 ± 0.51 (9.97 ± 0.09)	<0.00005
RBC ARA, %	16.8 ± 0.40*	−0.43 ± 0.21 (15.8 ± 0.06)	−4.25 ± 0.39 (12.1 ± 0.07)	<0.00005
Plasma antioxidant activity, mM TEq	279 ± 17*	20.9 ± 15.1 (308 ± 17)	40.2 ± 12.5 (316 ± 15)	0.33
Plasma oxidized LDL, U/L	54 ± 2*	−7.60 ± 2.75 (45.9 ± 2.3)	−2.19 ± 1.61 (50.3 ± 1.8)	0.08
Plasma vitamin A, μM	3.33 ± 0.15*	0.37 ± 0.11 (3.23 ± 0.12)	0.54 ± 0.16 (3.71 ± 0.16)	0.45
Plasma vitamin E, μM	26.1 ± 1.6*	6.79 ± 2.00 (28.4 ± 1.4)	8.24 ± 1.56 (32.5 ± 1.4)	0.56
Whole blood platelet aggregation, ohms	16 ± 6*	−2.50 ± 0.97 (12.1 ± 0.7)	−2.80 ± 0.74 (11.1 ± 0.4)	0.80
Plasma total cholesterol, mg/dL	<240†	6.23 ± 6.10 (160 ± 5)	4.10 ± 5.64 (176 ± 5)	0.80
Plasma LDL cholesterol, mg/dL	<160†	5.39 ± 4.60 (92 ± 5)	7.19 ± 5.46 (109 ± 5)	0.83
Plasma HDL cholesterol, mg/dL	>23†	−2.32 ± 2.71 (46 ± 3)	−1.03 ± 3.01 (52 ± 3)	0.76
Plasma triacylglycerols, mg/dL	<500†	12.0 ± 13.7 (118 ± 19)	−10.8 ± 9.2 (85 ± 9)	0.16
Glucose, mg/dL	<126†	5.95 ± 5.44 (97.5 ± 5.3)	−3.30 ± 2.07 (89.3 ± 1.4)	0.08
BUN, mg/dL	<36†	−0.73 ± 0.71 (13.3 ± 0.7)	−0.03 ± 0.73 (13.6 ± 0.5)	0.51
Creatinine, mg/dL	<2.1†	0.07 ± 0.02 (0.82 ± 0.04)	0.10 ± 0.03 (0.84 ± 0.03)	0.77
BUN/creatinine ratio	<38†	−3.23 ± 1.22 (16.6 ± 0.9)	−3.63 ± 1.35 (17.0 ± 0.8)	0.83
Sodium, mg/dL	<151†	0.09 ± 0.49 (140.7 ± 0.4)	−0.07 ± 0.64 (140.2 ± 0.5)	0.86
Potassium, mg/dL	<6.1†	0.04 ± 0.10 (4.65 ± 0.60)	0.15 ± 0.09 (4.69 ± 0.08)	0.40
Chloride, mg/dL	<153†	−0.45 ± 0.56 (102.7 ± 0.6)	−0.57 ± 0.52 (102.6 ± 0.4)	0.89
Carbon dioxide, mg/dL	<45†	−0.59 ± 0.44 (23.1 ± 0.4)	−0.50 ± 0.54 (23.1 ± 0.4)	0.90
Calcium, mg/dL	<11.6†	−0.17 ± 0.08 (9.70 ± 0.09)	0.07 ± 0.08 (9.85 ± 0.05)	0.034
Total protein, mg/dL	<12†	−0.09 ± 0.09 (7.26 ± 0.08)	0.08 ± 0.07 (7.36 ± 0.06)	0.14
Albumin, mg/dL	>2.9†	0.02 ± 0.05 (4.64 ± 0.05)	0.16 ± 0.06 (4.76 ± 0.04)	0.09
Globulin, mg/dL	<6.3†	−0.10 ± 0.06 (2.62 ± 0.06)	−0.08 ± 0.05 (2.59 ± 0.05)	0.75
Albumin/globulin ratio	<3.5†	0.07 ± 0.04 (1.79 ± 0.04)	0.12 ± 0.05 (1.87 ± 0.05)	0.39
Total bilirubin, mg/dL	<1.9†	−0.02 ± 0.04 (0.41 ± 0.06)	0.11 ± 0.06 (0.54 ± 0.07)	0.09
Alkaline phosphatase, IU/L	<1040†	−58 ± 21 (135 ± 20)	−33 ± 19 (160 ± 20)	0.40
SGOT, IU/L	<156†	−0.77 ± 2.66 (24.5 ± 2.9)	−1.80 ± 2.04 (23.7 ± 1.7)	0.76
SGPT, IU/L	<143†	7.05 ± 4.48 (29.6 ± 4.7)	4.13 ± 5.78 (26.5 ± 5.7)	0.71

Significant differences ($P < 0.05$) in bold. TEq, vitamin E derivative, Trolox equivalents; BUN, blood urea nitrogen; SGOT, serum glutamic-oxaloacetic transaminase (also AST, aspartate aminotransferase); SGPT, serum glutamic-pyruvic transaminase (also ALT, alanine aminotransferase).

* Normative values ($n = 29$ age-matched; mean ± SE).

† Threshold cutoff for moderate AE based on DAIDS criteria.³⁷

and an autoanalyzer. Low-density lipoprotein cholesterol was calculated using the Friedewald equation.³⁶ Concentrations were recorded as mg/dL.

Comprehensive Metabolic Panel

Serum was sent to a commercial clinical laboratory (LabCorp, Dallas, TX, USA) for blood chemistry analysis. Results were reported compared with age-appropriate normative ranges and outlying results were flagged. Normative metabolite values and cutoff thresholds are given in Table 1.

Treatment-Emergent Adverse Events

All related, possibly related, or unrelated adverse events were recorded. Only TEAEs judged to be related or possibly related were analyzed. Treatment-emergent adverse events were self-reported at annual visits as well as in quarterly diaries. Blood chemistry anomalies were identified by Division of AIDS (DAIDS³⁷) criteria. Serious adverse events requiring hospitalization were to be reported immediately to study investigators.

Statistical Analysis

All mITT data for the 4-year trial were used for analysis. Two-tailed t -tests were used to compare study groups at baseline and for comparing overall change attributable to intervention (i.e., year 4 minus year 0 data). Values are given as mean ± SE. Statistical software (SPSS version 22; IBM Corporation, Armonk, NY, USA; and Statistica version 12; StatSoft, Inc., Tulsa, OK, USA) were used for statistical analyses.

RESULTS

Baseline Demographics, Anthropometry, and Blood Chemistry

Demographic, anthropometric, and blood chemistry measures were similar between groups at baseline (Supplementary Table S3). The DHA-supplemented group was slightly older (mean = 16.1 vs. 14.9 years), had marginally higher plasma-DHA (1.57 vs. 1.47%), RBC-DHA (3.12% vs. 2.86%), and plasma total cholesterol (168.3 vs. 151.7 mg/dL) compared with placebo. The 18 participants who dropped from the study were no different according to demographics, anthropometrics, and

FIGURE 1. Docosahexaenoic acid and ARA levels in plasma lipids as a function of treatment intervention. Mean $\pm$ SE plasma levels of DHA ($\square$, $\blacksquare$) and ARA ($\triangle$, $\blacktriangle$) in participants of the high-dose DHA-supplementation arm (filled symbols) or the placebo arm (open symbols), respectively. Gray dashed lines are average plasma DHA (lower section of plot; $1.42\% \pm 0.1\%$) and average plasma ARA (upper section of plot; $9.60\% \pm 0.27\%$) from 29 age-matched individuals with normal visual function; grayed area demarks 95% CI (DHA range, 1.22% – 1.62% ; ARA range, 9.1% – 10.1%).

RBC-DHA but on average were slightly older (mean = 19.6 years) than the mITT cohort.

DHA and ARA

Mean plasma DHA levels in mITT participants at baseline were not significantly different from age-matched normative males ($1.52\% \pm 0.06\%$ vs. $1.43\% \pm 0.10\%$; $P = 0.38$; Supplementary Table S4). Red blood cell-DHA at baseline was similar to mean

normal ($3.00\% \pm 0.10\%$ vs. $2.91\% \pm 0.15\%$; $P = 0.59$; Supplementary Table S5). The placebo group showed little variation in either plasma (Fig. 1) or RBC (Fig. 2) DHA levels throughout the trial consistent with protocol recommendations to abstain from over-the-counter capsules containing DHA or altering consumption of cold water n3-rich fish.

The DHA group reached a plateau level at 6 months for plasma DHA that averaged $5.90\% \pm 0.30\%$ for the remainder of the trial. Similarly, an average 6-month to 4-year plateau level of

FIGURE 2. Docosahexaenoic acid and ARA levels in RBC lipids as a function of treatment intervention. Mean $\pm$ SE RBC levels of DHA ($\square$, $\blacksquare$) and ARA ($\triangle$, $\blacktriangle$) in participants of the high-dose DHA-supplementation arm (filled symbols) or the placebo arm (open symbols), respectively. Gray dashed lines are average RBC-DHA (lower section of plot; $2.9\% \pm 0.3\%$) and average RBC-ARA (upper section of plot; $16.8\% \pm 0.4\%$) from 29 age-matched individuals with normal visual function; grayed area demarks 95% CI (DHA range, 2.3% – 3.5% ; ARA range, 16.0% – 17.6%).

TABLE 2. Fatty Acid Profile of mITT Cohort at Year 4 of DHAX Clinical Trial as Percentage of Total Plasma Lipids*

	Placebo <i>n</i> = 22	DHA <i>n</i> = 30	<i>P</i> Value
n3 fatty acids			
α-LNA	0.70 ± 0.06	0.66 ± 0.04	0.54
EPA	0.41 ± 0.05	1.08 ± 0.11	<0.0005
DPAn3	0.57 ± 0.02	0.36 ± 0.02	<0.0005
DHA	1.33 ± 0.08	5.79 ± 0.29	<0.0005
n6 fatty acids			
LA	32.1 ± 1.1	32.1 ± 0.8	0.98
20:3n6	2.00 ± 0.08	1.49 ± 0.07	<0.0005
ARA	8.16 ± 0.37	6.14 ± 0.27	<0.0005
22:4n6	0.37 ± 0.02	0.17 ± 0.02	<0.0005
DPAn6	0.25 ± 0.01	0.07 ± 0.01	<0.0005
Totals			
Total saturates†	35.3 ± 0.7	35.3 ± 0.7	0.98
Total monounsaturates‡	18.0 ± 0.5	16.3 ± 0.4	0.016
Total n3 PUFA	3.06 ± 0.13	7.94 ± 0.38	<0.0005
Total n6 PUFA	43.6 ± 4.8	40.4 ± 0.8	0.019
Total n3 LCPUFA	2.32 ± 0.13	7.26 ± 0.37	<0.0005
Total n6 LCPUFA	11.0 ± 0.4	8.09 ± 0.35	<0.0005
Ratios			
DPAn6/DHA ratio	0.189 ± 0.012	0.019 ± 0.005	<0.0005
n6/n3 PUFA ratio	14.8 ± 0.7	5.59 ± 0.41	<0.0005
n6/n3 LCPUFA ratio	4.88 ± 0.16	1.33 ± 0.17	<0.0005
Unsaturation index	141 ± 2	156 ± 2	<0.0005

Unsaturation index is the sum of (number of double bonds × percentage of each fatty acid). Statistical significance determined by *t*-test at *P* < 0.05 and given in bold. DPAn3, docosapentaenoic acid, 22:5n3; DHA, docosahexaenoic acid, 22:6n3; LA, linoleic acid, 18:2n6; ARA, arachidonic acid, 20:4n6; DPAn6, docosapentaenoic acid, 22:5n6.

* Values are mean ± SE for relative percentage of total fatty acids in plasma.

† Includes 14:0, 16:0, 17:0, 18:0, 20:0, 22:0, and 24:0.

‡ Includes 16:1, 18:1, 20:1, 22:1, and 24:1.

10.44% ± 0.44% was attained in RBCs. Participants receiving DHA had mean plasma and RBC-DHA levels 4.4- and 3.6-fold higher at year 4 than in the placebo group (both *P* < 0.00001; Table 1).

Consistent with metabolic competition between n3 and n6 fatty acids, mean plasma ARA levels were reduced 28% in the first 6 months and remained depressed throughout the trial in DHA-supplemented participants (Fig. 1). The 6-month to 4-year average plasma ARA level in the DHA group was 6.27% ± 0.18% compared with 8.07% ± 0.29% in the placebo group. The plasma ARA level in DHA participants was significantly reduced compared with both the placebo and normative groups at year 4 (both *P* < 0.00001; Table 1). In a similar manner, the average 6-month to 4-year level of ARA in RBCs was reduced 24% compared with the placebo group (Fig. 2; 11.8% ± 0.26% vs. 15.5% ± 0.18%, respectively; *P* < 0.00001).

The highest plasma and RBC-DHA levels were attained by a participant at the 6-month interval (10.1% and 14.9%, respectively). The lowest levels of plasma and RBC-ARA were 3.31% and 8.64% at the 3.5- and 2-year intervals, respectively, but in different participants. After trial termination and unmasking, one DHA participant was determined to be noncompliant throughout most of the trial with an RBC-DHA level at baseline of 3.3% and 3.4% at year 4.

TABLE 3. Fatty Acid Profile of mITT Cohort at Year 4 of DHAX Clinical Trial as Percentage of Total RBC Lipids*

	Placebo <i>n</i> = 22	DHA-Supplemented <i>n</i> = 30	<i>P</i> Value
n3 fatty acids			
α-LNA	0.14 ± 0.01	0.13 ± 0.01	0.29
EPA	0.35 ± 0.03	0.85 ± 0.08	<0.0005
DPAn3	4.44 ± 0.10	1.23 ± 0.06	<0.0005
DHA	2.82 ± 0.14	9.97 ± 0.50	<0.0005
n6 fatty acids			
LA	13.3 ± 0.28	12.7 ± 0.20	0.07
20:3n6	1.85 ± 0.07	1.46 ± 0.04	<0.0005
ARA	15.8 ± 0.3	12.1 ± 0.39	<0.0005
22:4n6	4.44 ± 0.10	2.20 ± 0.15	<0.0005
DPAn6	0.79 ± 0.03	0.26 ± 0.04	<0.0005
Totals			
Total saturates†	41.4 ± 0.17	42.1 ± 0.16	0.002
Total monounsaturates‡	16.5 ± 0.32	16.6 ± 0.14	0.79
Total n3 PUFA	5.50 ± 0.19	12.23 ± 0.52	<0.0005
Total n6 PUFA	36.6 ± 0.31	29.0 ± 0.51	<0.0005
Total n3 LCPUFA	5.33 ± 0.19	12.07 ± 0.52	<0.0005
Total n6 LCPUFA	23.3 ± 0.30	16.3 ± 0.6	<0.0005
Ratios			
DPAn6/DHA ratio	0.294 ± 0.016	0.038 ± 0.009	<0.0005
N6/N3 PUFA ratio	6.83 ± 0.26	2.61 ± 0.20	<0.0005
n6/n3 LCPUFA ratio	4.47 ± 0.16	1.53 ± 0.15	<0.0005
Unsaturation index	165 ± 1	176 ± 1	<0.0005

Statistical significance determined by *t*-test at *P* < 0.05 and given in bold.

* Values are mean ± SE for relative percentage of total fatty acids in red blood cells.

† Includes 14:0, 16:0, 17:0, 18:0, 20:0, 22:0, and 24:0.

‡ Includes 16:1, 18:1, 20:1, 22:1, and 24:1.

Other Fatty Acids

Although statistically significant, a 1.7% increase in total saturated fatty acids in RBCs of the DHA group at trial year 4 is not of clinical significance and contrasts with no increase in saturates in plasma (Tables 2, 3). Similarly, the significant 10% increase in total monounsaturated fatty acids in plasma (*P* = 0.006) contrasts with no change in monounsaturates in RBCs. The n3 and n6 essential fatty acids, α-linolenic (α-LNA) and linoleic acid (LA), respectively, were unchanged by DHA supplementation. Retroconversion of DHA to EPA was evident by 1.7- and 1.5-fold increases compared with placebo in plasma and RBC levels of EPA at year 4. A 40% to 50% reduction in both plasma and RBC content of n3docosapentaenoic acid (DPAn3) in DHA-supplemented participants was observed compared to placebo.

Competition between n3 and n6 fatty acids was most evident by the significant 70% decreases of the n6 metabolic end-product, DPAn6 (*P* < 0.0005) in plasma and RBCs; similarly, 20:3n6 and 22:4n6 were reduced by DHA supplementation. Over the 4-year interval, total n3 LCPUFAs in plasma and RBCs were significantly increased by 2- to 3-fold (*P* < 0.0005) while total n6 LCPUFAs decreased 30% by DHA supplementation (*P* < 0.0005; Tables 2, 3). Total PUFA of the n3 and n6 series include the essential fatty acids, LA and α-LNA, and are more reflective of dietary fat intake. Docosahexaenoic acid supplementation diminished n6 PUFA in plasma lipids by only 7%, whereas the n6 LCPUFA content was decreased 28% compared with placebo. Nevertheless, the plasma n6 to n3

TABLE 4. Cumulative TEAEs in mITT Cohort ($n = 60$) for the 4-Year DHAX Trial

Self-Reported TEAEs (Related or Possibly Related)	Placebo Group ($n = 27$)	DHA Group ($n = 33$)
Stomach ache/flu/pain	4	6
Dehydration	0	2
Increased sensitivity to allergens	0	1
Hypertension	1	1
Hypothyroidism	0	1
Hypercholesterolemia	0	1
Cataract	0	1*
Corneal abrasion, due to ERG lens	1	0
Total	6	13
Blood Chemistry TEAEs (Related or Possibly Related)	Placebo Group ($n = 27$)	DHA Group ($n = 33$)
Low HDL cholesterol	5	2
Elevated LDL cholesterol	0	2
Elevated total cholesterol	1	2
Elevated SGPT	0	1
Elevated potassium†	4	0
Elevated glucose	3	0
Total	13	7

* Reported by participant noncompliant with trial protocol.

† Clinical lab (LabCorp) considered due to incomplete serum separation.

PUFA ratio was decreased 62% in DHA participants compared with placebo; 5.6 vs. 14.8 (Table 2). In RBCs, total n6 PUFAs were reduced 21% and the n6-to-n3 PUFA ratio was diminished 62% by DHA-supplementation compared with placebo; 2.6 vs. 6.8 (Table 3). The unsaturation indices (number of double bonds $\times$ percentage of each fatty acid) in plasma and RBC were significantly elevated in DHA-supplemented participants ($P < 0.0005$) by year 4.

Treatment-Related Changes in Biosafety Measures

Despite marked elevations in plasma and RBC-DHA levels and reductions in ARA levels ($P < 0.00005$; Table 1), no group differences over the 4-year trial were found for anthropometrics, platelet aggregation, plasma antioxidant status, oxLDL or vitamins A and E ($P > 0.08$). Significant differences from placebo were found for DHA-supplemented participants for blood calcium ($P = 0.034$); however, this was judged not of clinical relevance as plasma levels were within the normal range (<11.6 mg/dL; Table 1). No other blood elements differed between groups ($P > 0.05$). No group differences were evident for lipoproteins including total ($P = 0.80$), LDL- ($P = 0.99$), or HDL-cholesterol ($P = 0.76$). Although DHA supplementation decreased TG content over the 4-year trial, this was not statistically significant ($P = 0.16$). An effect of age on biochemistry outcomes was not evident; divided by median age there were no differences in the year 0 to year 4 change values between the younger (≤ 14 years) and older (>14 years) placebo and DHA-supplemented groups for any of the above safety measures (data not shown).

Treatment-Emergent Adverse Events

Eighteen individuals dropped before completing year 1; three of these individuals reported one TEAE each: gastrointestinal (GI) upset, dehydration, and visual disturbance (i.e., loss of vision, increased floaters, and light sensitivity). Among the mITT cohort, 23 individuals had a total of 39 TEAEs (Table 4).

Nineteen TEAEs were self-reported (13 DHA, 6 placebo). Twenty TEAEs were identified by blood chemistry (7 DHA, 13 placebo). Two self-reported TEAEs in the placebo group and one in DHA group were considered related to intervention as abstinence from capsules alleviated symptoms. The latter experienced GI symptoms sporadically for 2 years (three AE reports) including diarrhea, flatulence, and eructation; symptoms were alleviated by capsule discontinuation. This participant dropped at year 2 as it was determined he had a family history of inflammatory bowel disease (Crohn). No serious TEAEs requiring hospitalization were reported. Further TEAE details are given in the Supplementary Results.

Overall, TEAEs were minor and sporadic in occurrence and duration, except one participant with recurrent GI symptoms. No trends were identified in self-reported TEAEs or blood chemistry.

DISCUSSION

Supplementation of DHA at 30 mg/kg/d resulted in 4.4- and 3.6-fold increases in mean plasma and RBC DHA levels within 6 months and remained similarly elevated throughout the trial. Mean plasma and RBC DHA levels attained over the 4 years were 5.9% and 10.4% with individual maximums of 10.1% and 14.9% at the 6-month time point. Whether blood DHA levels reached a ceiling is not known. In other studies, children with cystic fibrosis supplemented 6 months with 50 mg DHA/kg/d had an increase in RBC-DHA levels from $2.8\% \pm 0.1\%$ to $11.1\% \pm 0.3\%$ (mean $\pm$ SE); two patients reached an RBC-DHA level of 13.1%.²⁷ In a 1-year trial, children provided with 100 mg DHA/kg/d had an average 5-fold elevation in plasma DHA.²⁹

The DHA provided in this trial was high dose compared with many cardiovascular studies using fish oil.³⁸ However, n3 fatty acids are building blocks of membranes throughout the body and DHA, specifically, is present in abundance in neural tissues including the retina, emphasizing importance in this tissue. The DHAX trial dose was based on bodyweight, thus the heaviest participant had a daily capsule intake of 18 capsules or 9 g oil/d, which is less than the fat (13 g) in a single doughnut. However, the DHA provided by this intervention is considerably more than found in the typical, n6-rich Western diet. The mean daily DHA intake in the US population is 70 mg³⁹ compared with the mean 2200 mg/d DHA supplemented in the DHAX trial; furthermore, the average DHAX dose represents only 2.8% of the typical US daily fat intake (79 g/d).⁴⁰ Thus, supplementation with DHA should not be considered a pharmaceutical approach to treating XLRP, but provision of dietary macronutrients necessary for optimizing cell membrane structure and function.

Competitive inhibition by DHA resulted in 24% to 28% reductions of ARA in plasma and RBCs, subsequently modifying the ARA/DHA baseline ratios from 5.2 to 1.0 in plasma and 5.8 to 1.2 in RBC by year 4. The ARA/DHA ratio in the placebo group remained at 5.6 through 4 years. Similarly, DHA supplementation resulted in 8- to 10-fold differences in the ratios of end products of n6 and n3 metabolism, namely DPAn6-to-DHA in plasma and RBCs (Tables 2, 3). Of particular interest is the 62% lower plasma n6-to-n3 PUFA ratio at year 4 in DHA-supplemented participants compared with placebo (5.6 vs. 14.8). The typical n6/n3 ratio associated with Western diets is 16.7⁴¹; thus, desirable target ratios of 2⁴² and 4⁴³ have been recommended for maintenance of a healthy fatty acid profile. Reducing the n6/n3 ratio can be achieved by either n3 supplementation or reduction in n6 PUFA intake. Alaskan Eskimos with a high n3-rich fish intake have plasma PUFA ratios of 3.5.⁴⁴ High intake of n3 fatty acids and low n6/n3 ratios are associated with lower rates of cardiovascular

disease and atherosclerosis.⁴¹ The American Heart Association recommends that individuals with coronary artery disease take at least 1g/d of DHA plus EPA.³⁹

Despite altered blood fatty acid profiles, comprehensive metabolic analyses revealed no consistent changes of clinical relevance during the DHAX trial (Table 1). As a tissue with high oxygen utilization, the retina is susceptible to oxidative stress¹³; nevertheless, group differences in antioxidant status were nonsignificant, but directionally favoring lower oxidative stress in the DHA group. Total plasma antioxidant activity was 2-fold greater, plasma vitamin A and E levels were marginally elevated, and oxLDL slightly decreased at year 4 in the DHA-supplemented group. Studies of DHA supplementation in RP have failed to demonstrate that DHA acts as a target for free radical-induced oxidative stress resulting in exacerbation of disease progression.^{18–20} In *in vitro* studies, the double bonds of DHA are susceptible to oxidation, yet once incorporated into tissue lipids, DHA appears to possess antioxidative action.^{45,46} Supplementation in Rett syndrome (72.9 mg DHA/kg/d) significantly reduced ($P < 0.001$) isoprostane-indexed oxidative stress and lessened disease severity ($P = 0.02$).^{28,47} In the DHAX trial, daily multivitamin supplementation as well as DHA may have provided adequate antioxidants to support the body's natural defense system against oxidative stress. Furthermore, the specialty oils derived from algae (such as DHASCO) are less susceptible to peroxidation than fish oil fatty acid preparations as determined by rancimat analysis.⁴⁸

Early n3 fatty acid supplementation studies reported decreased platelet aggregation resulting in modestly prolonged bleeding times.^{15,49} A meta-analysis of eight fish oil supplementation trials (1.72–4.60 g fish oil/d) found no influence to incidence of major bleeding episodes.³⁸ A literature survey by Harris⁵⁰ concluded that “the risk for clinically significant bleeding was virtually nonexistent” among fish oil supplementation studies and is similarly echoed in a recent systematic review.⁵¹ Over the 4-year DHAX trial, 30 mg/kg/d DHA supplementation was without a significant effect on platelet aggregation ($P = 0.80$; Table 1); bleeding times were not measured.

Docosahexaenoic acid supplementation did not significantly alter lipoprotein cholesterol or TG profiles in the DHAX trial, nor were there alterations in the majority of blood chemistry metabolites. Although a disruption of glycemic regulation has been associated with n3 fatty acid treatment,⁵² mean fasting glucose levels were marginally reduced after the 4-year DHA intervention compared with placebo ($P = 0.08$; Table 1). Fish oil n3 fatty acid doses of 2 to 4 g/d (750–1500 mg DHA; 930–1860 mg EPA) are prescribed for patients with hypertriglyceridemia.¹⁷ For an average 80 kg adult, the 4 g daily dose of n3 fatty acid would equate to approximately 50 mg/kg/d. In contrast, DHAX trial participants received the long-term dose of 30 mg/kg/d and averaged 2200 mg DHA/d, yet only exhibited a modest, nonsignificant reduction in plasma TG (33 mg/dL compared with placebo; $P = 0.10$; Table 1). Similarly, no overall changes in total, LDL-, or HDL-cholesterol were found in DHAX participants receiving DHA.

A phase I clinical trial using 400 mg/d in XLRP showed that neither visual function nor biosafety was compromised by long-term DHA supplementation.²¹ In a clinical trial providing 1200 mg DHA/d to adult RP patients, no negative effects on visual function were found.¹⁹ In three meta-analyses of fish oil studies providing up to 4.6 g/d of EPA and DHA to over 5000 subjects, no severe adverse events were documented.^{17,38,51} Mild GI distress characterized as flatulence, eructation, and loose stools were reported for a few individuals with a rate not significantly different from those receiving placebo. The occurrence of TEAEs in the DHAX trial was consistent with these safety outcomes.

Docosahexaenoic acid continues to be evaluated and recommended as treatment and prophylaxis for various disease states. Since minimal metabolic anomalies and no severe adverse events occurred in the 4-year DHAX trial, the 30 mg/kg/d DHA dose does not pose a safety risk to the majority of individuals. Patients deciding to take high doses of DHA should be monitored closely, particularly those with personal or family history of GI problems.

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References

- Heckenlively JR. *Retinitis Pigmentosa*. Philadelphia, PA: Lippincott; 1988.
- Bird AC. X-linked retinitis pigmentosa. *Br J Ophthalmol*. 1975; 59:177–199.
- Fishman GA, Farber MD, Derlacki DJ. X-linked retinitis pigmentosa. Profile of clinical findings. *Arch Ophthalmol*. 1988;106:369–375.
- Birch DG, Anderson JL, Birch EE. Early abnormalities of rod function in children with X-linked retinitis pigmentosa. *Clin Vision Sci*. 1993;8:329–335.
- Hoffman DR. Fatty acids and visual dysfunction. In: Chow CK, ed. *Fatty Acids in Foods and Their Health Implications*. New York, NY: Marcel Dekker; 2000:817–842.
- Hoffman DR, Birch DG. Docosahexaenoic acid in red blood cells of patients with X-linked retinitis pigmentosa. *Invest Ophthalmol Vis Sci*. 1995;36:1009–1018.
- Hoffman DR, DeMar JC, Heird WC, Birch DG, Anderson RE. Impaired synthesis of DHA in patients with X-linked retinitis pigmentosa. *J Lipid Res*. 2001;42:1395–1401.
- Fliesler SJ, Anderson RE. Chemistry and metabolism of lipids in the vertebrate retina. *Prog Lipid Research*. 1983;22:79–131.
- Treen M, Uauy RD, Jameson DM, Thomas V, Hoffman DR. Effect of docosahexaenoic acid on membrane fluidity and function in intact cultured Y-79 retinoblastoma cells. *Arch Biochem Biophys*. 1992;294:564–570.
- Litman BJ, Mitchell DC. A role for phospholipid polyunsaturation in modulating membrane protein function. *Lipids*. 1996; 31:S193–S197.
- Rotstein NP, Avelano MI, Barrantes FJ, Politi LE. Docosahexaenoic acid is required for the survival of rat retinal photoreceptor *in vitro*. *J Neurochem*. 1996;66:1851–1859.

12. Kim H-Y, Akbar M, Lau A, Edsall L. Inhibition of neuronal apoptosis by docosahexaenoic acid (22:6n-3). Role of phosphatidylserine in antiapoptotic effect. *J Biol Chem*. 2000;275:35215-35223.
13. Anderson RE, Maude M, Alvarez RA, Acland G, Aguirre GD. A hypothesis to explain the reduced blood levels of docosahexaenoic acid in inherited retinal degenerations caused by mutations in genes encoding retina-specific proteins. *Lipids*. 1999;34:S235-S237.
14. Gu X, Meer SG, Miyagi M, et al. Carboxyethylpyrrole protein adducts and autoantibodies, biomarkers for age-related macular degeneration. *J Biol Chem*. 2003;278:42027-42035.
15. Knapp HR. Dietary fatty acids in human thrombosis and hemostasis. *Am J Clin Nutr*. 1997;65:1687S-1698S.
16. Jacobson TA, Glickstein SB, Rowe JD, Soni PN. Effects of eicosapentaenoic acid and docosahexaenoic acid on low-density lipoprotein cholesterol and other lipids: a review. *J Clin Lipidol*. 2012;6:5-18.
17. Bays H. Clinical overview of Omacor: a concentrated formulation of omega-3 polyunsaturated fatty acids. *Am J Cardiol*. 2006;98:711-761.
18. Hoffman DR, Locke KG, Wheaton DH, Fish GE, Spencer R, Birch DG. A randomized, placebo-controlled clinical trial of docosahexaenoic acid supplementation for X-linked retinitis pigmentosa. *Am J Ophthalmol*. 2004;137:704-718.
19. Berson EL, Rosner B, Sandberg MA, et al. Further evaluation of docosahexaenoic acid in patients with retinitis pigmentosa receiving vitamin A treatment. *Arch Ophthalmol*. 2004;122:1306-1314.
20. Berson EL, Rosner B, Sandberg MA, Weigel-DiFranco C, Willett WC. ω -3 intake and visual acuity in patients with retinitis pigmentosa receiving vitamin A. *Arch Ophthalmol*. 2012;130:707-711.
21. Wheaton DH, Hoffman DR, Locke KG, Watkins RB, Birch DG. Biological safety assessment of docosahexaenoic acid supplementation in a randomized clinical trial for X-linked retinitis pigmentosa. *Arch Ophthalmol*. 2003;121:1269-1278.
22. Berson EL, Rosner B, Sandberg MA, et al. Clinical trial of docosahexaenoic acid in patients with retinitis pigmentosa receiving vitamin A treatment. *Arch Ophthalmol*. 2004;122:1297-1305.
23. Hoffman DR, Hughbanks-Wheaton DK, Pearson NS, et al. Four-year placebo-controlled trial of docosahexaenoic acid in X-linked retinitis pigmentosa (DHAX trial): a randomized clinical trial. *JAMA Ophthalmol*. 2014;132:866-873.
24. Brenna JT, Varamini B, Jensen RG, Diersen-Schade DA, Boettcher JA, Arterburn LM. Docosahexaenoic and arachidonic acid concentrations in human breast milk worldwide. *Am J Clin Nutr*. 2007;85:1457-1464.
25. Hoffman DR, Theuer RC, Castañeda YS, et al. Maturation of visual acuity is accelerated in breast-fed term infants fed baby food containing DHA-enriched egg yolk. *J Nutrition*. 2004;134:2307-2313.
26. Lipillonne A, Groh-Wargo S, Gonzalez CHL, Uauy R. Lipid needs of preterm infants: updated recommendations. *J Pediatr*. 2013;162:S37-S47.
27. Lloyd-Still JD, Powers CA, Hoffman DR, et al. Bioavailability and safety of a high dose of docosahexaenoic acid triacylglycerol of algal origin in cystic fibrosis patients; a randomized controlled study. *Nutrition*. 2006;22:36-46.
28. DeFelice C, Signorini C, Durand T, et al. Partial rescue of Rett syndrome by ω -3 polyunsaturated fatty acids (PUFAs) oil. *Genes Nutr*. 2012;7:447-458.
29. Parker AM, Sunness JS, Brereton NH, et al. Docosahexaenoic acid therapy in peroxisomal diseases. *Neurology*. 2010;75:826-830.
30. Kyle DJ, Arterburn LM. Single cell oil sources of docosahexaenoic acid: clinical studies. In: Simopoulos AP, ed. *The Return of Omega-3 Fatty Acids Into the Food Supply. I. Land-Based Animal Food Products and Their Health Effects*. New York, NY: Basel Karger; 1998:116-131.
31. Hoffman DR, Birch EE, Birch DG, et al. Impact of early dietary intake and blood lipid composition of long-chain polyunsaturated fatty acids on later visual development. *J Ped Gastroenterol Nutr*. 2000;31:540-553.
32. Bui MH. Simple determination of retinol, α -tocopherol, and carotenoids (lutein, all-trans-lycopene, α - and β -carotenes) in human plasma by isocratic liquid chromatography. *J Chromatogr B Biomed Appl*. 1994;654:129-133.
33. Whitehead TP, Thorpe GHG, Maxwell SRJ. Enhanced chemiluminescent assay for antioxidant capacity in biological fluids. *Anal Chim Acta*. 1992;266:265-177.
34. Holvoet P, Mertens A, Verhamme P, et al. Circulating oxidized LDL is a useful marker for identifying patients with coronary artery disease. *Arterioscler Thromb Vasc Biol*. 2001;21:844-848.
35. Russell-Smith NC, Flower RJ, Cardinal DC. Measuring platelet and leucocyte aggregation/adhesion responses in very small volumes of whole blood. *J Pharmacol Meth*. 1981;6:315-333.
36. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem*. 1972;18:499-502.
37. Division of AIDS (DAIDS) Regulatory Support Center. Division of AIDS table for grading severity of adult and pediatric adverse events, version 1.0, December 2004; clarification August 2009. Available at: http://rsc.tech-res.com/document/safetyandpharmacovigilance/table_for_grading_severity_of_adult_pediatric_adverse_events.pdf. Accessed March 19, 2014.
38. Xin W, Wei W, Lin Z, et al. Fish oil and atrial fibrillation after cardiac surgery: a meta-analysis of randomized controlled trials. *PLoS One*. 2013;8:e72913.
39. Gebauer SK, Psota TL, Harris WS, Kris-Etherton PM. n-3 Fatty acid dietary recommendation and sources to achieve essentiality and cardiovascular benefits. *Am J Clin Nutr*. 2006;83:1526S-1535S.
40. National Health and Nutrition Examination Survey. Daily intake of nutrients by food source: 2007-2010. Available at: <http://www.ers.usda.gov/data-products/food-consumption-and-nutrient-intakes.aspx>. Accessed July 22, 2014.
41. Simopoulos AP. The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. *Exp Biol Med*. 2008;233:674-688.
42. Okuyama H, Kobayashi T, Watanabe S. Dietary fatty acids—the N-6/N-3 balance and chronic elderly diseases. Excess linoleic acid and relative N-3 deficiency syndrome seen in Japan. *Prog Lipid Res*. 1996;35:409-457.
43. Gomez Candela C, Bermejo Lopez LM, Loria Kohen V. Importance of a balanced omega 6/omega 3 ratio for the maintenance of health. Nutritional recommendations. *Nutr Hosp*. 2011;26:323-329.
44. Parkinson AJ, Cruz AL, Heyward WL, et al. Elevated concentrations of plasma ω -3 polyunsaturated fatty acids among Alaskan Eskimos. *Am J Clin Nutr*. 1994;59:384-388.
45. Rotstein NP, Politi LE, German OL, Girotti R. Protective effect of docosahexaenoic acid on oxidative stress-induced apoptosis of retina photoreceptors. *Invest Ophthalmol Vis Sci*. 2003;44:2252-2259.
46. Yavin E, Brand A, Green P. Docosahexaenoic acid abundance in the brain: a biodevice to combat oxidative stress. *Nutr Neurosci*. 2002;5:149-157.

47. Signorini C, de Felice C, Leoncini S, et al. F4-neuroprostanes mediate neurological severity in Rett syndrome. *Clinica Chimica Acta*. 2011;412:1399-1406.
48. Kyle DJ. Production and use of a single cell oil which is highly enriched in docosahexaenoic acid. *Lipid Technol*. 1996;8:106.
49. Kris-Etherton PM, Harris WS, Appel LJ. Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease. *Circulation*. 2002;106:2747-2757.
50. Harris WS. Expert opinion: omega-3 fatty acids and bleeding—cause for concern? *Am J Cardiol*. 2007;99:44c-46c.
51. Villani AM, Crotty M, Cleland LG, et al. Fish oil administration in older adults: is there potential for adverse events? A systematic review of the literature. *BMC Geriatrics*. 2013;13:41.
52. DeCaterina R, Madonna R, Bertolotto A, Schmidt EB. n-3 Fatty acids in the treatment of diabetic patients. *Diabetes Care*. 2007;30:1012-1026.