

# A Randomized Multicenter Phase II Study of Docosahexaenoic Acid in Patients with a History of Breast Cancer, Premalignant Lesions, or Benign Breast Disease



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## Abstract

Obesity, a cause of subclinical inflammation, is a risk factor for the development of postmenopausal breast cancer and is associated with poorer cancer outcomes. Docosahexaenoic acid (DHA), an omega-3 fatty acid, possesses anti-inflammatory properties. We hypothesized that treatment with DHA would reduce the expression of proinflammatory genes and aromatase, the rate-limiting enzyme for estrogen biosynthesis, in benign breast tissue of overweight/obese women. A randomized, placebo-controlled, double-blind phase II study of DHA given for 12 weeks to overweight/obese women with a history of stage I–III breast cancer, DCIS/LCIS, Paget's disease, or proliferative benign breast disease was carried out. In this placebo controlled trial, the primary objective was to determine whether DHA (1,000 mg by mouth twice daily) reduced breast tissue levels of TNF $\alpha$ . Secondary objectives included

evaluation of the effect of DHA on breast tissue levels of COX-2, IL1 $\beta$ , aromatase, white adipose tissue inflammation, and gene expression by RNA-seq. Red blood cell fatty acid levels were measured to assess compliance. From July 2013 to November 2015, 64 participants were randomized and treated on trial (32 women per arm). Increased levels of omega-3 fatty acids in red blood cells were detected following treatment with DHA ( $P < 0.001$ ) but not placebo. Treatment with DHA did not alter levels of TNF $\alpha$  ( $P = 0.71$ ), or other biomarkers including the transcriptome in breast samples. Treatment with DHA was overall well-tolerated. Although compliance was confirmed, we did not observe changes in the levels of prespecified biomarkers in the breast after treatment with DHA when compared with placebo. *Cancer Prev Res*; 11(4); 203–14. ©2018 AACR.

See related editorial by Fabian and Kimler, p. 187

## Introduction

Obesity is a risk factor for the development of several epithelial malignancies, including hormone receptor (HR)-positive and negative postmenopausal breast cancer

(1, 2). Estrogen biosynthesis is catalyzed by aromatase (3). The increased risk of developing HR-positive breast cancer in obese postmenopausal women has been attributed, in part, to increased expression of aromatase in breast adipose tissue (4–6).

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Specific strategies that target obesity-related cancers have been limited because of an incomplete understanding of the mechanisms underlying the connection between obesity and cancer. Recently, white adipose tissue (WAT) inflammation has been suggested to play a significant role in both the development and progression of obesity-related breast cancer (4, 7, 8). Obesity is associated with adipose hypoxia, adipocyte hypertrophy, and endoplasmic reticulum stress leading to cell death (9, 10). This process promotes increased cytokine production from adipocytes and immune cell recruitment resulting in WAT inflammation, manifested as crown-like structures (CLS). CLS are formed when macrophages infiltrate adipose tissue, surround, and phagocytize a dead or dying adipocyte, becoming lipid loaded and ultimately form foam cells (11, 12). Approximately 90% of obese women have CLS of the breast (CLS-B; refs. 4, 5). The presence of CLS, a histologic marker of WAT inflammation, is associated with both activation of NF $\kappa$ B, a transcription factor that induces proinflammatory mediators, and increased levels of aromatase (4, 13). Several studies have reported elevated levels of proinflammatory mediators including TNF $\alpha$  in inflamed fat (9, 14). Moreover, a variety of inflammatory mediators including TNF $\alpha$  can induce aromatase in breast adipose stromal cells (15, 16).

Docosahexaenoic acid (DHA), an omega-3 polyunsaturated fatty acid (PUFA) in fish oil, possesses anti-inflammatory properties. The anti-inflammatory effects of DHA have been attributed to a variety of mechanisms including induction of adiponectin, inhibition of NF $\kappa$ B-mediated induction of proinflammatory mediators, and reduced production of proinflammatory eicosanoids (17, 18). Dietary omega-3 fatty acid supplementation has been shown to suppress adipose tissue inflammation in several animal models of obesity (19, 20). Some studies have suggested that diets rich in fish oil, a source of omega-3 fatty acids, are associated with a reduced risk of breast cancer (21–25), a finding supported by preclinical findings (26, 27).

Dietary supplementation with fish, fish oil, DHA-enriched eggs, or an algal-derived source of DHA all result in increased plasma phospholipid DHA levels (28–30). Algal-derived DHA eliminates the risk of anticoagulant effects of eicosapentaenoic acid (EPA) in fish oil, diminishes the risk of cardiac arrhythmia, as well as concerns over mercury (31, 32) or organochlorine (33) contamination of fish or fish oil. On the basis of these observations, we hypothesized that treatment with algal-derived DHA would decrease the expression of key inflammatory mediators and downregulate aromatase in breast tissue of overweight and obese women, defined as a body mass index (BMI)  $\geq 25$  kg/m<sup>2</sup>. To test this hypothesis, we conducted a randomized, placebo-controlled, double-blind phase II study of DHA for 12 weeks in women with BMI  $\geq 25$  kg/m<sup>2</sup> with a history of stage I–III breast cancer, ductal carcinoma *in situ* (DCIS), lobular

carcinoma *in situ* (LCIS), Paget disease, or proliferative benign breast disease (NCT01849250).

## Materials and Methods

### Study design

We conducted a randomized, phase II, multicenter, placebo-controlled, double-blind trial of DHA for 12 weeks with a goal of a total of 50 evaluable overweight and obese patients with a history of stage I–III invasive breast cancer, DCIS/LCIS, Paget's disease, or proliferative benign breast disease. The primary objective was to determine whether treatment with DHA for 12 weeks at 1,000 mg orally twice daily as compared with placebo reduced breast tissue levels of TNF $\alpha$ . The secondary objective was to investigate effects of DHA on additional tissue biomarkers including COX-2, IL1 $\beta$ , aromatase, and WAT inflammation (CLS-B). As an exploratory endpoint, RNA sequencing (RNA-seq) was performed to investigate the effect of DHA compared with placebo on gene expression. Red blood cell (RBC) fatty acid levels were also measured to assess compliance to study treatment. The dose of DHA was selected because of evidence for excellent safety, good bioavailability, and saturating plasma levels at a dose of 2 grams per day (34).

### Patient eligibility

Women with histologically confirmed stage I–III invasive breast cancer, DCIS, LCIS, Paget's disease, or proliferative benign breast disease were eligible if they had a BMI  $\geq 25$  kg/m<sup>2</sup>, no evidence of current disease, completed all breast cancer-directed therapy ( $\geq 6$  months), and had adequate contralateral breast tissue unaffected by invasive cancer for biopsy. Patients could not have had radiation or an implant in place on the side undergoing biopsy. An abbreviated DHA food frequency questionnaire was administered at screening and patients with a daily DHA consumption  $\geq 200$  mg/day in the month prior to screening were ineligible (35). Additional exclusion criteria included history of daily use of aspirin or nonsteroidal anti-inflammatory drugs in the week preceding study entry, history of autoimmune disorder, or any illness that required therapy with chronic steroids or immunomodulators, and history of therapeutic doses of anticoagulants in the preceding year. The institutional review boards of the participating centers approved this protocol. Each participant provided written informed consent. This study was conducted in compliance with the guidelines set forth in the Belmont Report, Declaration of Helsinki, and the Common Rule.

### Treatment

Each participant self-administered either 2 capsules (500-mg each) of DHA or placebo orally twice daily with food. Both DHA and placebo were supplied by DSM

Nutritional Products and were masked to protect blinding. This protocol used an algal-derived source of DHA, Docosahexaenoic Acid-Rich Single-Cell Oil (DHASCO), produced by the microalga *Cryptocodinium cohnii* according to Good Manufacturing Practices for foods. Patients were treated for a minimum of 12 weeks and could stay on study drug for an additional 2 weeks (12+2) to allow for scheduling of the second posttreatment biopsy. Dose adjustments were not permitted. Adherence was defined as having taken study medication for a minimum of 12 weeks with no more than 3 interruptions and no single interruption >7 days with no more than a total of 7 days off study drug.

### RBC fatty acid levels

Levels of DHA and other fatty acids were measured in RBCs at baseline and posttreatment. RBC fatty acid levels were analyzed at OmegaQuant. A minimum of 2-mL whole blood sample was obtained pre- and posttreatment on study. Blood was separated into plasma, buffy coat, and erythrocytes by centrifugation at room temperature. One milliliter of erythrocytes was then collected and stored at  $-80^{\circ}\text{C}$  until analysis. RBC fatty acid composition was analyzed by gas chromatography with flame ionization detection (36). Unwashed, packed RBCs were directly methylated with boron trifluoride and hexane at  $100^{\circ}\text{C}$  for 10 minutes. The fatty acid methyl esters generated were analyzed using a GC2010 Gas Chromatograph (Shimadzu Corporation) equipped with an SP2560, fused silica capillary column (Supelco). Fatty acids were identified by comparison with a standard mixture of fatty acids characteristic of RBCs (GLC 727, NuCheck Prep). Fatty acid composition was expressed as a percent of total identified fatty acids. The omega-3 index is defined as the percentage of the sum of 20:5n-3 (EPA) and 22:6n-3 (DHA).

### Tissue collection

Random core needle biopsies of normal breast tissue at baseline and after 12 weeks of study intervention were performed. As outlined in the protocol, only patients who had completed a minimum of 60 days of study therapy, as reported on the pill diary and confirmed by pharmacy pill count, were asked to undergo the posttreatment biopsy. Patients were asked to remain on study drug until the time of the posttreatment biopsy. Up to 7 cores of breast tissue were obtained from the unaffected contralateral breast and were processed and utilized as follows: core #1: formalin fixed, paraffin-embedded (FFPE), histologic evaluation at participating site to assess for malignancy as per standard institutional practice; core #2: FFPE, evaluation of CLS-B (as detailed below); core #3–7: pooled to generate RNA for qPCR and RNA-seq (as detailed below). Because of possible fluctuations in breast histology with menstrual cycles, core biopsies were performed at least 14 days after the start of the most recent menstrual period in all premenopausal women.

### Quantitative real-time PCR

Total RNA was isolated from pooled frozen breast tissue from each subject using the RNeasy Mini Kit (Qiagen). One microgram of RNA was reverse transcribed using murine leukemia virus reverse transcriptase and oligo (dT)<sub>16</sub> primer. The resulting cDNA was used for amplification. GAPDH was used as an endogenous normalization control; the forward and reverse primers were 5'-TTCTTTTGGCGTCGCCAGCCGA-3' and 5'-GTGAC-CAGGCGCCCAATACGA-3'. Additional primers used were: TNF $\alpha$ , forward: 5'-CTGCTGCACTTTGGAGTGAT-3', reverse: 5'-AGATGATCTGACTGCCTGGG-3'; IL1 $\beta$ , forward: 5'-GGACAAGCTGAGGAAGATGC-3'; and reverse, 5'-TCGTTATCCCATGTGTCGAA-3'; COX-2, forward: 5'-CCCTTGGGTGTCAAAGGTAA-3'; and reverse, 5'-GCCCTCGCTTATGATCTGTC-3'; aromatase, forward: 5'-CACATCCTCAATACCAGGTCC-3'; reverse, 5'-CAGAGATCCAGACTCGCATG-3'. Real-time PCR was conducted using 2 $\times$  Fast SYBR Green PCR Master Mix on a 7500 Fast real-time PCR system (Applied Biosystems), with relative expression determined using the  $\Delta\Delta\text{C}_t$  analysis protocol.

### RNA-seq

Total RNA was isolated from 37 paired pre- and post-treatment samples using Qiagen's RNeasy Mini Kit. Sequencing libraries were constructed following the Illumina TrueSeq Stranded Total RNA Library preparation protocol with rRNA depletion. Next-generation sequencing was performed with pair-end 51 bp using the Illumina HiSeq4000 platform (Weill Cornell Medicine). Raw sequenced reads were aligned to the Human reference genome (Version hg19 from UCSC) using STAR (Version 2.4.2) aligner. Aligned reads were quantified against the reference annotation (hg19 from UCSC) to obtain FPKM (Fragments per Kilobase per million) and raw counts using CuffLinks (v 2.2.1) and HTSeq, respectively.

### Detection and assessment of WAT inflammation

Consistent with established methods, breast WAT inflammation was defined by the presence of CLS-B, which is comprised of a dead or dying adipocyte surrounded by CD68-positive macrophages (4, 5, 7, 11). From each patient, a FFPE block was prepared from pre- and post-treatment cores and up to 5 WAT sections were generated (approximately 5- $\mu\text{m}$ -thick cut at 50- $\mu\text{m}$  intervals) consistent with previous human studies (4, 5, 7). All sections were immunostained for CD68, a macrophage marker (mouse monoclonal KP1 antibody; Dako; dilution 1:4,000), as described previously (4, 5, 7). The anti-CD68-stained sections were examined by the study pathologist (D.D. Giri) using light microscopy to detect the presence or absence of CLS-B and the number of CLS-B per slide was recorded (4, 5, 7). Digital photographs of each slide were generated and WAT area was measured with ImageJ Software (NIH, Bethesda, MD). The severity of WAT

inflammation was quantified as number of CLS-B per square centimeter of WAT (CLS-B/cm<sup>2</sup>).

### Statistical analysis

This randomized phase II trial was designed to enroll a total of 64 subjects to ensure a targeted 50 evaluable subjects after accounting for an expected 10% dropout rate and around 10% nonevaluable rate. Subjects were randomized at 1:1 ratio to each study arm using urn randomization stratified by study site. The primary objective was to determine whether treatment with DHA compared with placebo reduced normal breast tissue levels of TNF $\alpha$  in overweight and obese patients meeting the study inclusion criteria. Assuming an expected 0.4 correlation between pre- and posttreatment biomarker levels, the planned sample size had 80% power to detect effect size as small as 0.74 in the adjusted TNF $\alpha$  levels between the two arms at a two-sided 0.05 significance level using ANCOVA (37). This sample size also had 80% power at 0.05 significance level for an effect size as small as 0.58 for the post- minus pretreatment percent change in the biomarker level in the treatment arm using a paired two-sided *t* test.

For the primary study endpoint, differences between the active treatment and placebo arms at baseline were compared using Wilcoxon rank-sum test and differences in treatment effects were assessed using ANCOVA where the posttreatment measurements were used as a dependent variable and the pretreatment measurements were included as a covariate. The same analysis approach was used for the continuous secondary endpoints, including the breast tissue levels of COX-2, IL1 $\beta$ , and aromatase. All the relative expression levels were log transformed prior to analysis to ensure the normality assumption of the model was satisfied. For the binary secondary endpoint, CLS-B status, differences between DHA and placebo arms at baseline were compared using Fisher exact test and differences in treatment effects were assessed using logistic regression analysis where the posttreatment measurements were used as a dependent variable and the pretreatment measurements were included as covariates. The outcome analyses were carried out according to the intention-to-treat (ITT) principles among all randomized subjects with biological samples. These analyses were also completed in a preplanned evaluable population defined as those individuals who underwent 2 breast biopsies and were compliant based on the definition above.

Baseline characteristics of study subjects were compared between treatment arms using Fisher exact test for categorical variables and the nonparametric Wilcoxon rank sum test for continuous variables. Post- versus pretreatment difference in the measured RBC fatty acid levels (DHA and omega-3 index) were examined using Wilcoxon signed rank test for each treatment arm. Differences in the post

versus pretreatment change of these variables between the treatment arms were examined using the Wilcoxon rank-sum test.

To evaluate the potential effect of DHA on normal breast transcriptome, RNA-seq gene expression data were analyzed to identify DHA associated differentially expressed genes. The count data were normalized using voom (38) and analyzed using limma (39) with study ID included to properly control for within subject variations. A gene was considered differentially expressed if there was significant post versus pretreatment change in DHA arm and the magnitude of change was significantly greater in the DHA arm compared with the placebo arm. Significance is defined as fold change >1.2, and false discovery rate (FDR) < 0.05. All statistical analyses were carried out using R 3.3.1 (40).

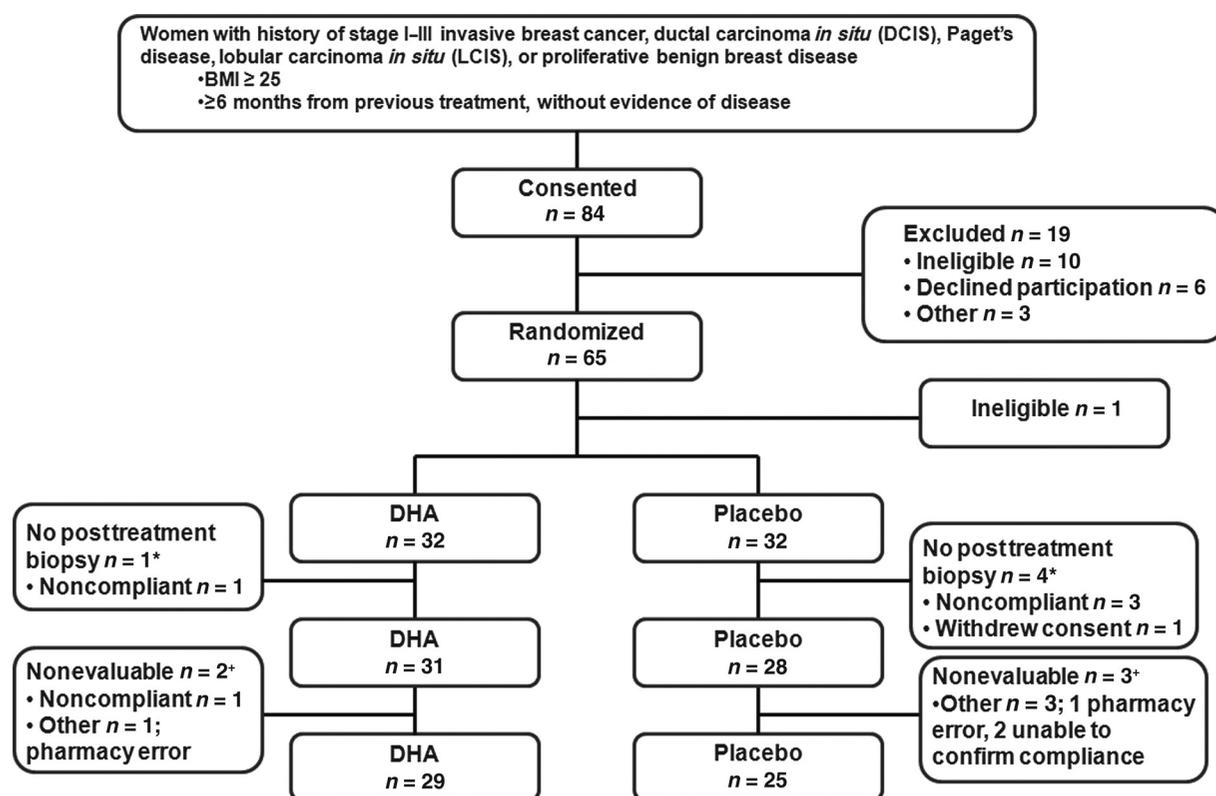
## Results

### Patient demographics

From July 2013 to September 2015, 65 patients (median age 59 years, interquartile range: 52–62) were randomized on study (Fig. 1). One patient did not initiate study treatment despite randomization as she was deemed ineligible secondary to screening laboratory abnormalities. Of the 64 patients who initiated treatment with either DHA or placebo, 54 patients (25 women in the placebo cohort and 29 in the DHA cohort) were considered evaluable at study completion. Participant characteristics of the total study population are described in Table 1. Median BMIs were 29.8 (interquartile range: 27.4–34.0) and 31.2 (interquartile range: 27.5–33.5) in women in the placebo versus DHA arms, respectively ( $P = 0.83$ ). Patients were predominantly postmenopausal (84%) and self-reported as white (86%). Twenty-three percent of patients had a history of a benign lesion, 19% of a premalignant lesion (defined as DCIS, LCIS, or Paget's disease), and 58% of an invasive breast cancer. There were no statistically significant differences in clinicopathologic features noted between the evaluable and unevaluable cohorts.

### Compliance

Compliance in each of the arms was compared and confirmed by measuring pre- and posttreatment levels of the omega-3 index and DHA. Baseline values for the omega-3 index and DHA levels were similar between the two treatment arms (Fig. 2A and C). More specifically, the mean (standard deviation) and median (interquartile range, IQR) baseline omega-3 index in our ITT population with measureable samples ( $N = 58$ ) were 5.02 (1.39) and 4.88 (4.00, 5.76), respectively. Post- versus pretreatment changes in these two variables are shown in Fig. 2B and D. There was a significant increase in both DHA levels and the omega-3 index in the group randomized to receive DHA ( $P < 0.001$ ). In comparison, a significant change in these values was not seen in the placebo arm. In addition, we



**Figure 1.**

Consort diagram. \*As outlined in the protocol, only patients who had completed a minimum of 60 days of study therapy were asked to undergo the posttreatment biopsy. These patients did not complete the posttreatment biopsy. † A patient will be "evaluable" if they have 2 breast biopsies, take study drug for a minimum of 12 weeks with no more than 3 interruptions and no single interruption >7 days (no more than a total of 7 days off study drug). Two patients in the DHA arm and 3 patients in the placebo arm were deemed to be nonevaluable after study completion.

determined the effects of DHA on the EPA+DHA:AA ratio. At baseline, the median ratios were similar in the DHA and placebo groups [0.28 (0.22–0.36) vs. 0.29 (0.23–0.36);  $P = 0.67$ ]. Posttreatment, we observed a significant increase in the ratio in the DHA-treated group compared with placebo [0.85 (0.79–0.93) vs. 0.28 (0.21–0.32);  $P < 0.001$ ].

### Tissue biomarkers

Tissue biomarker levels were obtained at baseline from breast tissue sampled the day prior to study treatment and after a minimum of 12 weeks of treatment with either placebo or DHA. Baseline levels of TNF $\alpha$ , IL1 $\beta$ , COX-2, and aromatase mRNAs did not differ significantly between the two treatment groups (Table 2). The changes in the levels of TNF $\alpha$  ( $P = 0.50$ ), IL1 $\beta$  ( $P = 0.52$ ), COX-2 ( $P = 0.19$ ), and aromatase ( $P = 0.12$ ) after 12 weeks of treatment did not differ significantly between the two groups in the IIT cohort. Furthermore, in the preplanned evaluable population, there was also no statistically significant difference between the two arms in the change in pre- versus post-treatment levels in the tissue biomarker endpoints (data not shown).

### RNA-seq data

Next, we carried out RNA-seq analysis to determine whether treatment with DHA resulted in changes in gene expression. Adequate RNA was available from 37 (14 patients on the placebo arm; 23 patients on the DHA arm) of the original 64 patients (Supplementary Table S1). In comparison to the placebo group, DHA did not significantly alter the breast transcriptome. In other words, we did not observe a significant treatment effect on gene expression in the DHA versus placebo group defined as (i) a significant difference in levels of gene transcripts in the post- versus pre-DHA group and (ii) a significant difference in the magnitude of post- versus prechange between the two treatment arms (Significance is defined as FDR < 0.05 and fold change >1.2; data not shown).

### White adipose tissue inflammation

Breast WAT inflammation, defined by the presence of CLS-B, was present in 2 patients in the DHA arm ( $N = 31$ ) and 4 patients in the placebo arm ( $N = 28$ ) at baseline. Posttreatment 3 patients in the DHA arm and 3 in the placebo arm were CLS-B positive. Two patients in the

**Table 1.** Clinicopathologic features stratified by treatment arm (total study population)

Variables	All (n = 64)	Placebo (n = 32)	DHA (n = 32)	P
Age at registration, years				
Median (IQR)	59 (52-62)	57 (52-62)	60 (51-63)	0.87
Race, n (%)				
Asian	1 (2%)	1 (3%)	0 (0%)	
Asian-White	1 (2%)	1 (3%)	0 (0%)	
Black or African American	6 (10%)	2 (6%)	4 (12%)	
White	55 (87%)	27 (87%)	28 (88%)	0.67
Not reported	1 (2%)	1 (3%)	0 (0%)	1.00
BMI, kg/m <sup>2</sup>				
Median (IQR)	30.5 (27.5-33.9)	29.8 (27.4-34.0)	31.2 (27.5-33.5)	0.83
Postmenopausal, n (%)				
No	10 (16%)	5 (16%)	5 (16%)	
Yes	54 (84%)	27 (84%)	27 (84%)	1.00
Prior pathology, n (%)				
Benign	15 (23%)	6 (19%)	9 (28%)	
Premalignant <sup>a</sup>	12 (19%)	7 (22%)	5 (16%)	
Invasive	37 (58%)	19 (59%)	18 (56%)	0.62
ER, n (%)				
Negative	20 (31%)	9 (28%)	11 (34%)	
Positive	23 (36%)	14 (44%)	9 (28%)	
Not applicable	21 (33%)	9 (28%)	12 (38%)	0.49
PR, n (%)				
Negative	20 (31%)	10 (31%)	10 (31%)	
Positive	20 (31%)	12 (38%)	8 (25%)	
Not applicable	24 (38%)	10 (31%)	14 (44%)	0.52
Prior chemotherapy, n (%)				
No	32 (50%)	15 (47%)	17 (53%)	
Yes	32 (50%)	17 (53%)	15 (47%)	0.80
Prior AI, n (%)				
No	56 (88%)	28 (88%)	28 (88%)	
Yes	8 (12%)	4 (12%)	4 (12%)	1.00
NSAIDs, n (%)				
No	56 (88%)	28 (88%)	28 (88%)	
Yes	8 (12%)	4 (12%)	4 (12%)	1.00
Statins, n (%)				
No	53 (83%)	28 (88%)	25 (78%)	
Yes	11 (17%)	4 (12%)	7 (22%)	0.51
Oral steroids, n (%)				
No	62 (97%)	30 (94%)	32 (100%)	
Yes	2 (3%)	2 (6%)	0 (0%)	0.49
Immunomodulators, n (%)				
No	60 (94%)	30 (94%)	30 (94%)	
Yes	4 (6%)	2 (6%)	2 (6%)	1.00

Abbreviations: AI, aromatase inhibitor; BMI, body mass index; ER, estrogen receptor; IQR, interquartile range; NSAIDs, nonsteroidal anti-inflammatory drugs; PR, progesterone receptor.

<sup>a</sup>Premalignant defined as ductal carcinoma *in situ* (DCIS), lobular carcinoma *in situ* (LCIS), or Paget disease.

placebo arm were CLS-B positive both pre-and posttreatment. There was no significant change in the presence of CLS-B following treatment with DHA ( $P = 0.90$ ). Given the small number of subjects identified with CLS-B using biopsy samples, no meaningful analysis could be completed with evaluate the change in severity of CLS-B (defined as CLS-B/cm<sup>2</sup>).

### Adverse events

The most common, treatment-related toxicities (defined as possibly, probably or definitely attributed to study drug) of any grade were gastrointestinal (Table 3). Reported adverse events were overall similar between the placebo and DHA arms. All adverse events were grade 1 or 2 (Table 3). One patient in the placebo arm withdrew consent and terminated study participation early due to

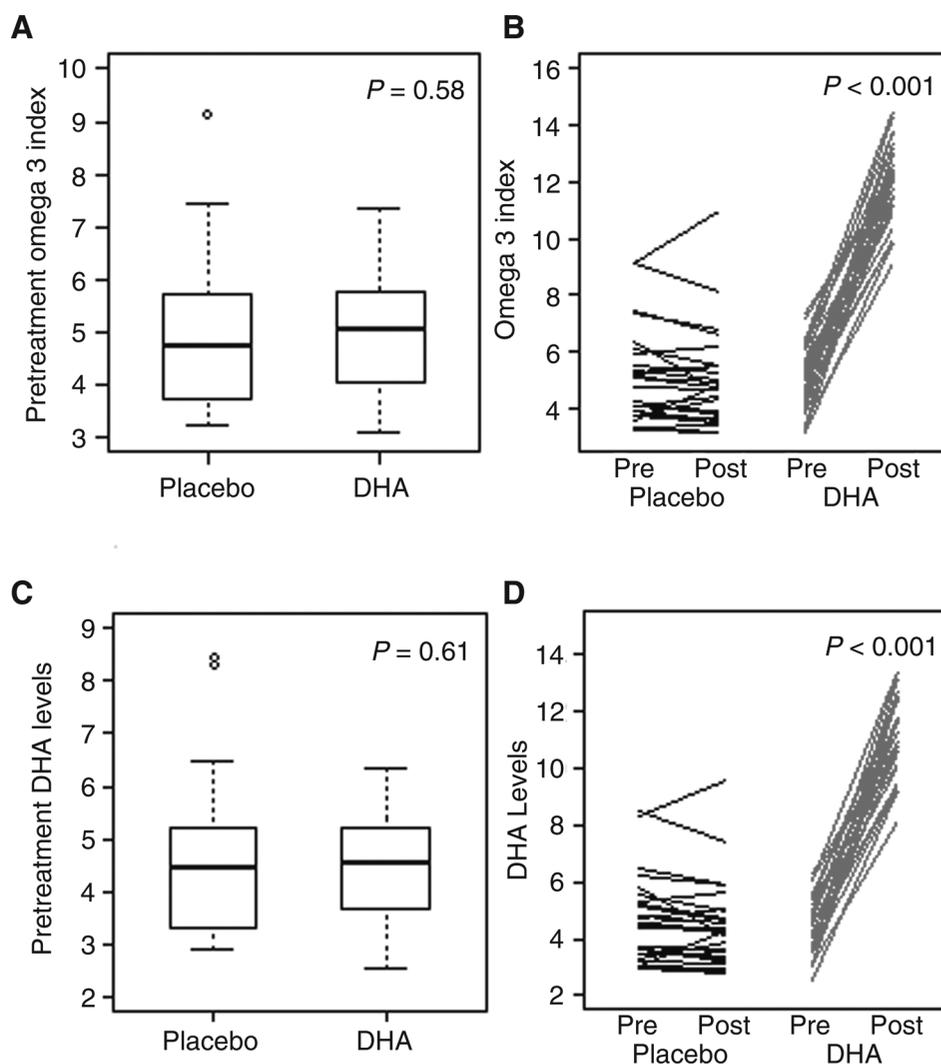
weight gain. There were no  $\geq$  grade 3 treatment-related serious adverse events. One patient reported a grade 3 skin infection requiring intravenous antimicrobial therapy unrelated to study therapy that resolved with appropriate treatment.

### Discussion

Our randomized, phase II study confirms the feasibility of conducting a placebo controlled trial of DHA supplementation in a predominantly survivorship population; approximately, three quarters of patients had a history of either invasive or premalignant lesions at diagnosis. Therapy was overall well-tolerated with high completion (92%) and adherence (84%) rates even with two protocol mandated breast biopsies. Levels of DHA and other fatty acids

**Figure 2.**

Pre- and posttreatment levels of the omega-3 index and DHA ( $N = 64$ ). **A** and **C**, There was no statistically significant difference in baseline values of the omega-3 index ( $P = 0.58$ ) or DHA levels ( $P = 0.61$ ) between the two treatment arms. **B** and **D**, A significant change in post- versus pretreatment values of both the omega-3 index and DHA were not seen in the placebo arm. In comparison there was a significant increase in both variables in the group randomized to receive DHA ( $P$  values  $< 0.001$ ).



were measured in RBCs at baseline and posttreatment. There was no statistically significant difference in baseline values of the omega-3 index or DHA levels between the two treatment arms. The mean and median values for baseline omega-3 index in our ITT population were comparable with published values in other studies (41, 42). As predicted, there was a significant increase in both DHA levels and in the omega-3 index in the group randomized to receive DHA ( $P < 0.001$ ) and no parallel change in these values in the placebo arm confirming compliance with randomization to each arm.

This study was designed to determine whether supplementation with DHA would alter tissues levels of key proinflammatory biomarkers,  $\text{TNF}\alpha$ ,  $\text{IL1}\beta$ , and  $\text{COX-2}$  as well as aromatase. Despite successful enrollment of the target population with low baseline DHA intake, pre-planned randomization versus placebo, and a significant increase in RBC levels of DHA and omega-3 index in the group treated with DHA, we found no changes in tissue levels of the prespecified biomarkers. Several investigators

have examined the effects of omega-3 PUFA supplementation on circulating inflammatory mediators and some but not all studies have shown that consumption of EPA and DHA are associated with lower levels of proinflammatory biomarkers such as  $\text{TNF}\alpha$  (43–45). In comparison, there are less published data regarding tissue level modulation of inflammatory mediators by omega-3 fatty acid supplementation. Fabian and colleagues conducted two parallel pilot studies to evaluate omega-3 supplementation in pre- and postmenopausal women at increased risk for breast cancer; individuals were treated for 6 months with combined EPA and DHA ethyl esters (3.4 g/daily) and underwent pre- and posttreatment biopsies (44, 46). While feasibility was the primary endpoint, secondary endpoints included several blood and tissue-based biomarkers evaluating changes in cytomorphology, Ki-67, circulating inflammatory and adipokine markers, RBC and tissue fatty acid composition, and targeted mRNAs, chemokines, and peptides. Treatment with combined EPA/DHA resulted in favorable modulation in certain tissue and circulating

**Table 2.** Tissue biomarkers at baseline and after 12 weeks of study treatment, (intention-to-treat population)

Variables	Baseline		Posttreatment		Posttreatment vs. baseline change <sup>a</sup>		Treatment effect <sup>a</sup>	
	DHA (Mean ± SD)	Placebo (Mean ± SD)	DHA (Mean ± SD)	Placebo (Mean ± SD)	DHA (Mean ± SD)	Placebo (Mean ± SD)	Baseline adjusted change (95% CI)	P
TNF- $\alpha$ <sup>b</sup>	0.08 ± 0.78	0.26 ± 0.93	0.16 ± 0.95	0.06 ± 0.92	0.08 ± 0.99	-0.22 ± 1.19	0.17 (-0.32-0.67)	0.50
DHA N = 31 Placebo N = 26								
COX-2	0.31 ± 0.97	0.49 ± 1.35	0.22 ± 1.16	-0.11 ± 1.30	-0.10 ± 1.23	-0.61 ± 1.50	0.41 (-0.21-1.04)	0.19
DHA N = 30 Placebo N = 26								
IL1 $\beta$	0.28 ± 1.50	0.62 ± 2.00	0.19 ± 2.07	0.23 ± 2.06	-0.09 ± 1.84	-0.48 ± 1.61	0.29 (-0.61-1.18)	0.52
DHA N = 31 Placebo N = 26								
Aromatase <sup>c</sup>	0.13 ± 2.38	-0.38 ± 1.93	0.06 ± 1.89	-0.59 ± 2.13	0.05 ± 2.12	-0.41 ± 2.29	0.77 (-0.22-1.76)	0.12
DHA N = 30 Placebo N = 22								

NOTE: Log-transformed relative levels of TNF $\alpha$ , COX2, IL1 $\beta$ , and aromatase were determined by quantitative RT-PCR.

<sup>a</sup>The change between baseline and posttreatment, as well as the treatment effect, was calculated for patients for whom data from both time points were available. Treatment-effect data and P values were obtained from analysis of covariance. N represents the number of patients in each arm with both pre- and post-treatment values for the measured tissue biomarkers.

<sup>b</sup>Of the intention-to-treat population, 2 patients in the placebo arm did not have adequate RNA for analysis, as defined by RNA integrity number >5.

<sup>c</sup>Due to the small volume of tissue obtained from core biopsies, some samples did not yield enough RNA to accurately measure aromatase expression which is known to be expressed at low levels in adipose tissue. Therefore, aromatase measurements are not available in a subset of cases.

markers in both cohorts to varying degrees. In the postmenopausal population, in particular, serum adiponectin ( $P = 0.0027$ ), TNF- $\alpha$  ( $P = 0.016$ ), HOMA 2B ( $P = 0.0048$ ), and bioavailable estradiol ( $P$  equals; 0.039) were modified favorably in response to supplementation. Benign breast tissue Ki-67 ( $P = 0.036$ ), macrophage chemoattractant protein-1 ( $P = 0.033$ ), cytomorphology index score ( $P = 0.014$ ), and percent mammographic density ( $P = 0.036$ ) were all decreased in this cohort. Consistent with our findings, these investigators demonstrated little to no change in breast levels of proinflammatory gene transcripts in response to combined EPA and DHA supplementation (44, 46). In this setting, comparison of the studies by Fabian and colleagues to our study is limited by differences in study population, dose, duration, and formulation of omega-3 supplementation, and study endpoints. We note,

for example, that median baseline BMIs were 24.7 and 25.0 kg/m<sup>2</sup> in the studies by Fabian and colleagues, whereas we selected for overweight and obese women which could, in theory, have led to differences in baseline inflammation. In another recent study, 6 months of high-dose omega-3 fatty acid supplementation raised plasma and adipose tissue omega-3 fatty acid concentrations but had no beneficial effects on adipose tissue markers of inflammation including the number or subtype of macrophages present in the tissue, senescent cells, and the presence of CLS in insulin-resistant overweight and obese adults (47). Similarly, Kratz and colleagues showed that omega-3 fatty acid supplementation over 14 weeks in overweight and obese individuals had no effect on adipose tissue gene expression of inflammatory mediators, including TNF $\alpha$ , or on measured plasma markers of inflammation (48).

We did not observe a significant change in the breast transcriptome in the cohort treated with DHA compared with the placebo group. These data should be interpreted with caution as insufficient tissue was available for RNA-seq analysis in 42% of patients on study (18 patients in the placebo arm and 9 patients in the DHA arm). Moreover, quantitative PCR validation was not carried out due to insufficient amounts of RNA remaining for this exploratory analysis. Furthermore, RNA-seq analysis was performed on random breast core biopsy specimens. Therefore, our data do not exclude the possibility of DHA-mediated effects on gene expression in specific cell types including the epithelium.

Inconsistent dosing and composition of omega-3 supplements utilized across studies, differing sampling methods and assays, and variable patient populations with differences in baseline fat intake and BMI may confound inter-study comparison in this setting and partly explain the mixed results from these studies. The optimal dose and

**Table 3.** Participants experiencing treatment related adverse events; N = 64

Adverse events (Grade 1-2) <sup>a</sup>	DHA N = 32	Placebo N = 32
GI Disorders		
NOS	2	7
Flatulence	2	3
Diarrhea	1	2
Dysgeusia	2	1
Nausea	0	2
Dyspepsia	2	0
Abdominal pain	0	1
Bloating	0	1
GERD	0	1
Breast pain	1	1
Anemia	1	0
Arthralgia	0	1
Headache	1	1
Hot flashes	1	0
Weight gain	0	1

Abbreviations: GERD, gastroesophageal reflux disease; NOS, not otherwise specified.

<sup>a</sup>There were no  $\geq$  grade 3 adverse events related to study drug.

composition of supplemental omega-3 PUFAs required to elicit an anti-inflammatory response in the breast remain unknown. Supplementation with DHA alone at 1,000 mg twice daily may not be adequate to significantly alter the fatty acid composition in the breast resulting in changes in gene expression. Furthermore, elevated BMI has been shown to attenuate increases in DHA and EPA in the serum and DHA in breast adipose tissue in response to oral supplementation suggesting a higher dose of DHA may have been more effective in this cohort with median BMI of 31.2 (45). In addition, a limitation of our study was that we only measured mRNA not protein levels of proinflammatory markers in response to supplemental DHA. Therefore, it is possible, that DHA acts at the post-transcriptional level resulting in changes in protein expression without impacting levels of mRNA (49). Although insufficient tissue was available for us to test this hypothesis, future studies can be done to investigate this question.

Our group has standardized the IHC methods for the identification and quantification of CLS in white adipose tissue acquired at the time of surgical intervention, for example, mastectomy. This study is, to our knowledge, the first to evaluate CLS-B in core biopsy specimens. We have previously reported WAT inflammation of the breast, manifest as CLS-B, is present in approximately 50% of women undergoing mastectomy and that the presence of CLS-B is more common among overweight and obese women (4, 5). Thus, in a population enriched for women with BMI  $\geq$  25, we would expect the prevalence of CLS-B to be approximately 70%. The low detection rate for CLS-B in this study, suggests that core biopsies provide insufficient tissue for histologic evaluation of this biomarker.

Although omega-3 fatty acid supplementation has been studied extensively for the prevention and management of multiple chronic illnesses including cancer, data from these studies have yielded mixed results. In particular, conflicting data have prevented a consensus regarding the benefit of omega-3 fatty acid supplementation as a cancer prevention strategy. With this trial, we confirmed the feasibility of conducting a randomized, placebo-controlled trial of DHA supplementation in this study population. However, DHA monotherapy did not decrease the expression of key inflammatory mediators or down regulate aromatase in breast tissue of overweight and obese women. These results diminish enthusiasm for further study of DHA supplementation alone at this dose for breast cancer prevention. Ongoing research evaluating alternative dosing, duration, and formulations of omega-3 supplementation may elucidate the effects of supplementation on tissue specific and circulating biomarkers ultimately allowing for identification of individuals likely to benefit from mechanistically based risk reduction and treatment strategies.

## Disclosure of Potential Conflicts of Interest

J.E. Garber is a consultant/advisory board member for Helix and Novartis. No potential conflicts of interest were disclosed by the other authors.

## Disclaimer

This material should not be interpreted as representing the viewpoint of the US Department of Health and Human Services, the NIH, or the National Cancer Institute.

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## References

- Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011;61:69–90.
- Kerlikowske K, Gard CC, Tice JA, Ziv E, Cummings SR, Miglioretti DL, et al. Risk factors that increase risk of estrogen receptor-positive and -negative breast cancer. *J Natl Cancer Inst* 2017;109:djw276.
- Simpson ER, Mahendroo MS, Means GD, Kilgore MW, Hinshelwood MM, Graham-Lorence S, et al. Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis\*. *Endocr Rev* 1994;15:342–55.
- Morris PG, Hudis CA, Giri D, Morrow M, Falcone DJ, Zhou XK, et al. Inflammation and increased aromatase expression occur in the breast tissue of obese women with breast cancer. *Cancer Prev Res* 2011;4:1021–9.
- Iyengar NM, Morris PG, Zhou XK, Gucalp A, Giri D, Harbus MD, et al. Menopause is a determinant of breast adipose inflammation. *Cancer Prev Res* 2015;8:349–58.
- Brown KA, Iyengar NM, Zhou XK, Gucalp A, Subbaramaiah K, Wang H, et al. Menopause is a determinant of breast aromatase expression and its associations with BMI, inflammation, and systemic markers. *J Clin Endocrinol Metab* 2017;102:1692–701.
- Iyengar NM, Zhou XK, Gucalp A, Morris PG, Howe LR, Giri DD, et al. Systemic correlates of white adipose tissue inflammation in early-stage breast cancer. *Clin Cancer Res* 2016;22:2283–9.
- Iyengar NM, Hudis CA, Dannenberg AJ. Obesity and cancer: local and systemic mechanisms. *Annu Rev Med* 2015;66:297–309.
- Olefsky JM, Glass CK. Macrophages, inflammation, and insulin resistance. *Annu Rev Physiol* 2010;72:219–46.
- Rosen ED, Spiegelman BM. What we talk about when we talk about fat. *Cell* 2014;156:20–44.
- Cinti S, Mitchell G, Barbatelli G, Murano I, Ceresi E, Faloia E, et al. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *J Lipid Res* 2005;46:2347–55.
- Shapiro H, Pecht T, Shaco-Levy R, Harman-Boehm I, Kirshtein B, Kuperman Y, et al. Adipose tissue foam cells are present in human obesity. *J Clin Endocrinol Metab* 2013;98:1173–81.
- Subbaramaiah K, Howe LR, Bhardwaj P, Du B, Gravaghi C, Yantiss RK, et al. Obesity is associated with inflammation and elevated aromatase expression in the mouse mammary gland. *Cancer Prev Res* 2011;4:329–46.
- Shi H, Kokoeva MV, Inouye K, Tzamelis I, Yin H, Flier JS. TLR4 links innate immunity and fatty acid-induced insulin resistance. *J Clin Invest* 2006;116:3015–25.
- Salama SA, Kamel MW, Diaz-Arrastia CR, Xu X, Veenstra TD, Salih S, et al. Effect of tumor necrosis factor- $\alpha$  on estrogen metabolism and endometrial cells: potential physiological and pathological relevance. *J Clin Endocrinol Metab* 2009;94:285–93.
- Samarajeewa NU, Yang F, Docanto MM, Sakurai M, McNamara KM, Sasano H, et al. HIF-1 $\alpha$  stimulates aromatase expression driven by prostaglandin E2 in breast adipose stroma. *Breast Cancer Res* 2013;15:R30.
- Neschen S, Morino K, Rossbacher JC, Pongratz RL, Cline GW, Sono S, et al. Fish oil regulates adiponectin secretion by a peroxisome proliferator-activated receptor- $\gamma$ -dependent mechanism in mice. *Diabetes* 2006;55:924–8.
- Honda KL, Lamon-Fava S, Matthan NR, Wu D, Lichtenstein AH. Docosahexaenoic acid differentially affects TNF $\alpha$  and IL-6 expression in LPS-stimulated RAW 264.7 murine macrophages. *Prostaglandins Leukot Essent Fatty Acids* 2015;97:27–34.
- Kalupahana NS, Claycombe K, Newman SJ, Stewart T, Siriwardhana N, Matthan N, et al. Eicosapentaenoic acid prevents and reverses insulin resistance in high-fat diet-induced obese mice via modulation of adipose tissue inflammation. *J Nutr* 2010;140:1915–22.
- Todoric J, Loffler M, Huber J, Bilban M, Reimers M, Kadl A, et al. Adipose tissue inflammation induced by high-fat diet in obese diabetic mice is prevented by n-3 polyunsaturated fatty acids. *Diabetologia* 2006;49:2109–19.
- Kaizer L, Boyd NF, Kriukov V, Trichler D. Fish consumption and breast cancer risk: an ecological study. *Nutr Cancer* 1989;12:61–8.
- Gago-Dominguez M, Yuan JM, Sun CL, Lee HP, Yu MC. Opposing effects of dietary n-3 and n-6 fatty acids on mammary carcinogenesis: The Singapore Chinese Health Study. *Br J Cancer* 2003;89:1686–92.
- Hirose K, Takezaki T, Hamajima N, Miura S, Tajima K. Dietary factors protective against breast cancer in Japanese premenopausal and postmenopausal women. *Int J Cancer* 2003;107:276–82.
- Brasky TM, Lampe JW, Potter JD, Patterson RE, White E. Specialty supplements and breast cancer risk in the VITamins And Lifestyle (VITAL) Cohort. *Cancer Epidemiol Biomarkers Prev* 2010;19:1696–708.
- Zheng JS, Hu XJ, Zhao YM, Yang J, Li D. Intake of fish and marine n-3 polyunsaturated fatty acids and risk of breast cancer: meta-analysis of data from 21 independent prospective cohort studies. *BMJ* 2013;346:f3706.
- Rose DP, Connolly JM. Omega-3 fatty acids as cancer chemopreventive agents. *Pharmacol Ther* 1999;83:217–44.
- Yee LD, Young DC, Rosol TJ, Vanbuskirk AM, Clinton SK. Dietary (n-3) polyunsaturated fatty acids inhibit HER-2/neu-induced breast cancer in mice independently of the PPAR $\gamma$  ligand rosiglitazone. *J Nutr* 2005;135:983–8.
- Payet M, Esmail MH, Polichetti E, Le Brun G, Adjemout L, Donnarel G, et al. Docosahexaenoic acid-enriched egg consumption induces accretion of arachidonic acid in erythrocytes of elderly patients. *Br J Nutr* 2004;91:789–96.
- Nelson GJ, Schmidt PC, Bartolini GL, Kelley DS, Kyle D. The effect of dietary docosahexaenoic acid on plasma lipoproteins and tissue fatty acid composition in humans. *Lipids* 1997;32:1137–46.
- Stark KD, Holub BJ. Differential eicosapentaenoic acid elevations and altered cardiovascular disease risk factor responses after supplementation with docosahexaenoic acid in postmenopausal women receiving and not receiving hormone replacement therapy. *Am J Clin Nutr* 2004;79:765–73.
- Foran SE, Flood JG, Lewandrowski KB. Measurement of mercury levels in concentrated over-the-counter fish oil preparations: is fish oil healthier than fish? *Arch Pathol Lab Med* 2003;127:1603–5.
- Yoshizawa K, Rimm EB, Morris JS, Spate VL, Hsieh CC, Spiegelman D, et al. Mercury and the risk of coronary heart disease in men. *N Engl J Med* 2002;347:1755–60.
- Melanson SF, Lewandrowski EL, Flood JG, Lewandrowski KB. Measurement of organochlorines in commercial over-the-counter fish oil preparations: implications for dietary and therapeutic recommendations for omega-3 fatty acids and a review of the literature. *Arch Pathol Lab Med* 2005;129:74–7.
- Arterburn LM, Hall EB, Oken H. Distribution, interconversion, and dose response of n-3 fatty acids in humans. *Am J Clin Nutr* 2006;83:1467S–1476S.
- Kuratko C. Food-frequency questionnaire for assessing long-chain omega-3 fatty-acid intake: Re: assessing long-chain omega-3 polyunsaturated fatty acids: a tailored food-frequency questionnaire is better. *Nutrition* 2013;29:807–8.

36. Harris WS, Pottala JV, Vasan RS, Larson MG, Robins SJ. Changes in erythrocyte membrane trans and marine fatty acids between 1999 and 2006 in older Americans. *J Nutr* 2012;142:1297–303.
37. Borm GF, Fransen J, Lemmens WA. A simple sample size formula for analysis of covariance in randomized clinical trials. *J Clin Epidemiol* 2007;60:1234–8.
38. Law CW, Chen Y, Shi W, Smyth GK. voom: precision weights unlock linear model analysis tools for RNA-seq read counts. *Genome Biol* 2014;15:R29.
39. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* 2015;43:e47.
40. R Core Team. R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2016.
41. Harris WS, Pottala JV, Varvel SA, Borowski JJ, Ward JN, McConnell JP. Erythrocyte omega-3 fatty acids increase and linoleic acid decreases with age: observations from 160,000 patients. *Prostaglandins Leukot Essent Fatty Acids* 2013;88:257–63.
42. Flock MR, Skulas-Ray AC, Harris WS, Etherton TD, Fleming JA, Kris-Etherton PM. Determinants of erythrocyte omega-3 fatty acid content in response to fish oil supplementation: a dose-response randomized controlled trial. *J Am Heart Assoc* 2013;2:e000513.
43. Chapkin RS, Kim W, Lupton JR, McMurray DN. Dietary docosahexaenoic and eicosapentaenoic acid: emerging mediators of inflammation. *Prostaglandins Leukot Essent Fatty Acids* 2009;81:187–91.
44. Fabian CJ, Kimler BF, Phillips TA, Nydegger JL, Kreutzjans AL, Carlson SE, et al. Modulation of breast cancer risk biomarkers by high-dose omega-3 fatty acids: phase II pilot study in postmenopausal women. *Cancer Prev Res* 2015;8:922–31.
45. Yee LD, Lester JL, Cole RM, Richardson JR, Hsu JC, Li Y, et al. Omega-3 fatty acid supplements in women at high risk of breast cancer have dose-dependent effects on breast adipose tissue fatty acid composition. *Am J Clin Nutr* 2010;91:1185–94.
46. Fabian CJ, Kimler BF, Phillips TA, Box JA, Kreutzjans AL, Carlson SE, et al. Modulation of breast cancer risk biomarkers by high-dose omega-3 fatty acids: phase II pilot study in premenopausal women. *Cancer Prev Res* 2015;8:912–21.
47. Hames KC, Morgan-Bathke M, Harteneck DA, Zhou L, Port JD, Lanza IR, et al. Very-long-chain omega-3 fatty acid supplements and adipose tissue functions: a randomized controlled trial. *Am J Clin Nutr* 2017;105:1552–8.
48. Kratz M, Kuzma JN, Hagman DK, van Yserloo B, Matthys CC, Callahan HS, et al. n3 PUFAs do not affect adipose tissue inflammation in overweight to moderately obese men and women. *J Nutr* 2013;143:1340–7.
49. Fluckiger A, Dumont A, Derangere V, Rébé C, de Rosny C, Causse S, et al. Inhibition of colon cancer growth by docosahexaenoic acid involves autocrine production of TNFalpha. *Oncogene* 2016;35:4611–22.

