Research Article

Modulation of Breast Cancer Risk Biomarkers by High-Dose Omega-3 Fatty Acids: Phase II Pilot Study in Postmenopausal Women

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Abstract

Associational studies suggest higher intakes/blood levels of the omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) relative to the omega-6 arachidonic acid (AA) are associated with reduced breast cancer risk. We performed a pilot study of high-dose EPA + DHA in postmenopausal women to assess feasibility before initiating a phase IIB prevention trial. Postmenopausal women with cytologic evidence of hyperplasia in their baseline random periareolar fine needle aspiration (RPFNA) took 1,860 mg EPA +1500 mg DHA ethyl esters daily for 6 months. Blood and breast tissue were sampled at baseline and study conclusion for exploratory biomarker assessment, with P values uncorrected for multiple comparisons. Feasibility was predefined as 50% uptake, 80% completion, and 70% compliance. Trial uptake by 35 study entrants from 54 eligible women was 65%, with 97% completion and 97% compliance. Favorable

modulation was suggested for serum adiponectin ($P = 0.0027$), TNFα ($P = 0.016$), HOMA 2B measure of pancreatic β cell function ($P = 0.0048$), and bioavailable estradiol ($P = 0.039$). 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 decreased with favorable effects in a proteomics array for several proteins associated with mitogen signaling and cell-cycle arrest; but no obvious overall effect on proteins downstream of mTOR. Although favorable risk biomarker modulation will need to be confirmed in a placebo-controlled trial, we have demonstrated feasibility for development of high-dose EPA and DHA ethyl esters for primary prevention of breast cancer. *Cancer Prev Res; 8(10); 922–31. ©2015 AACR.*

See related article, p. 912

Introduction

The long chain polyunsaturated fatty acids eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and arachidonic acid (AA) are important components of phospholipid membranes; playing key roles in membrane fluidity, cell signaling, and initiation, sustainment and resolution of the immune response (1). The omega-6 fatty acid AA stored in phospholipid membranes of

macrophages and other immune cells serves as a substrate for cyclooxygenase (COX) and lipoxygenase (LOX) enzymatic production of proinflammatory mediators including prostaglandins, leukotrienes, and thromboxanes collectively referred to as eicosanoids (2). These bioactive compounds result in cytokine production and local increase in tissue macrophage infiltration, proliferation, angiogenesis, and resistance to apoptosis (3). Under normal circumstances, the inflammatory response is terminated with the concomitant production of resolvins and protectins from the action of COX and LOX enzymes on the omega-3 fatty acids EPA and DHA (4). High intakes of omega-6 fatty acids with concomitant low intakes of omega-3 fatty acids lead to an excess of proinflammatory eicosanoids, reduced ability to resolve the inflammatory process, and have been implicated in breast cancer risk (5, 6). The importance of breast adipose macrophage infiltration and chronic inflammation has been appreciated as a mediator of obesity-related breast cancer (7). However, inflammation may also play a role in nonobese women as evidenced by the progressive increase in activated macrophage infiltration in women with atypical hyperplasia, *in situ* and invasive breast cancer (8). Higher systemic cytokine levels commonly observed after menopause may render postmenopausal women particularly vulnerable to an unbalanced omega-3:omega-6 ratio (7, 9).

Reduction in mammary cancer development in animals has been reported with combined EPA and DHA intake in the range of 6.6% to 22% of calories, approximately 60 to 100 times the usual

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human intake of 100 mg per day (10–12). The most concentrated source of EPA and DHA is found in marine fatty fish. A 4-oz serving of wild Atlantic salmon provides approximately 2 g of the combination; and non-prescription fish oil supplements generally provide 300 to 700 mg combined EPA + DHA per 1 g capsule (12, 13) Postmenopausal women currently taking fish oil supplements were reported to have a 32% reduction in breast cancer incidence in the large VITAL prospective cohort study (14). A meta-analysis from 21 prospective associational studies primarily in postmenopausal women suggests decreased incidence of breast cancer with higher intakes of EPA and DHA (15). However, initial studies showed little change in serum inflammatory markers in healthy women taking EPA and DHA supplements (16, 17). This lack of effect may be due to type of measurement (serum assays, rather than *ex vivo* monocyte stimulation) or lower doses (<3 g EPA+DHA per day; refs. 18, 19). In addition to effects on inflammation, EPA and DHA can alter tyrosine kinase receptor activation via disruption of lipid rafts in plasma membranes (20).

In a study (21) of dietary intakes and fatty acid levels in healthy women undergoing random periareolar fine needle aspiration (RPFNA; ref. 22), we observed that low levels of omega-3 fatty acids were associated with evidence of cytologic atypia, a known risk predictor for development of breast cancer. We therefore initiated two parallel single-arm pilot studies (one in premenopausal women (23) and one in postmenopausal women, this report) of 3.4 g per day EPA + DHA ethyl esters (4 g of Lovaza), for 6 months to assess feasibility and explore effects on tissue risk biomarkers for breast cancer before planning a larger placebo-controlled phase IIB trial. The rationale for two separate trials was based on higher systemic levels of many proinflammatory cytokines with aging and estrogen depletion (7, 9) as well as potential differential effects of omega-3 fatty acids on EGF-related signaling depending on progesterone levels (24), which are generally higher in premenopausal women.

Materials and Methods

Eligibility and recruitment

Potentially eligible women were <65 years old, postmenopausal, with stable hormone status for at least 6 months; and at increased risk for breast cancer by one or more of the following criteria: (i) an affected first-degree or two or more second-degree relatives <60, (ii) a prior breast biopsy showing atypical hyperplasia or lobular carcinoma *in situ*, (iii) 5-year Gail risk >1.67% (25) or 10 year risk of $2\times$ or greater for age group as calculated by the Tyrer-Cuzick (International Breast Cancer Intervention Study) model (26), (iv) >50% estimated mammographic breast density, or (v) evidence of hyperplasia with atypia within the prior 3 years by RPFNA (22). Excluded were women who (i) had a BMI of 40 kg/m^2 or higher, (ii) had breast implants, (iii) required more than 7 NSAIDs tablets per week, (iv) were on anticoagulants, (v) had a history of fish allergies, (vi) had used fish oil or flaxseed supplements within 3 weeks before the fine needle aspiration, (vii) had received tamoxifen within the prior 6 months, or (viii) had been on a clinical prevention study within the prior 6 months. A normal mammogram for women >40 years was required within 6 months before entering study. All participants signed separate consents for IRB-approved protocols for screening, repeat RPFNAs (HSC 4601), and administration of Lovaza (HSC 12350; NCT01252290). Protocols were approved by the University of Kansas Medical Center Human Subjects Committee (Kansas City, KS) and were conducted in accordance with the Declaration of Helsinki. The first subject was enrolled in December 2010 and the last subject completed the study in May 2013.

Breast tissue biomarker eligibility for intervention

Women were required to have cytologic evidence of hyperplasia with atypia or borderline atypia (Masood cytomorphology index score ≥14) in specimens obtained by RPFNA (23, 27). Initially, women were not required to have evidence of Ki-67 expression, but later, selection criteria were tightened such that Ki-67 expression of at least 1% was required (the latter based on assessment of 500 epithelial cells) unless the cytomorphology index score was ≥15.

Bio-specimen acquisition and processing

Fasting blood for adipokines, cytokines, hormones, and growth factors (27) was obtained at the on-study visit and at study conclusion (6 months) within 24 hours of drug discontinuation. An interval of 2 weeks was originally specified between study drug discontinuation and postintervention RPFNA due to concerns about excessive bruising if the aspiration was performed while on or immediately after discontinuation of high-dose EPA and DHA. The first 24 participants had a median 14-day interval (range 4–35 days) between last dose of study drug and RPFNA. For these 24, a second fasting blood sample was also obtained at the time of the RPFNA. When excessive bruising was not observed in initial participants, the study was amended specifying RPFNA within 24 hours of last dose of study drug eliminating an extra trip for a blood draw. Consequently, the last 10 women enrolled had off-study blood obtained only once at the time of RPFNA, within 24 hours of drug discontinuation.

All biomarker assays except cytomorphology and Ki-67 were batch processed with samples stored in aliquots at −80°C so that pre- and postintervention specimens were run together.

For the RPFNA procedure, two sites per breast were aspirated (C.J. Fabian) under local anaesthesia as previously described (22, 27). The first aspiration pass per site was placed in a 2-mL cryovial containing 0.25 mL of PBS and immediately immersed in liquid nitrogen with transfer to a −80°C freezer within 12 hours. The frozen aliquots were utilized for fatty acid analysis, reverse-phase protein array, adipokines and cytokines (Luminex), and RT-qPCR (23, 27). The remaining material from all aspiration sites was pooled in a single 15 cc tube with 9 mL CytoLyt and 1 mL of 10% formalin for Ki-67 and cytomorphology performed as previously described using Thin Prep for slide preparation and MIB1 as the antibody for Ki-67 detection (23, 27).

Anthropometric and dietary variables

At baseline and postintervention, women completed an online Food Frequency Questionnaire (28), had height and weight measured, and a Dual Energy X-ray Absorptiometry (DEXA) scan (Lunar Prodigy, GE Healthcare) was performed for body mass, fat mass, lean body mass, and android fat mass.

Mammographic density

Deidentified files (B.F. Kimler) were made of the left cranial caudal images from digital mammograms for area of increased breast density assessment in batches of 14 using Cumulus software (29). Images from the same subject were paired but readers

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were blinded to image sequence. Breast density values estimated individually by the two readers (C.J. Fabian and W.L. Hensing) were averaged and used for analysis. Because BMI did not change during the study, percent density was not corrected for BMI.

Adverse events and quality of life

Adverse events were assessed by the study coordinator and in the event of a significant symptom by the protocol chair. Overall quality of life was assessed by total BCPT score (23, 27).

High-dose omega-3 intervention

The intervention was required to be initiated within 6 months of the baseline RPFNA. Women took two capsules of Lovaza each containing 465 mg EPA and 375 mg DHA as the ethyl ester twice daily (total dose 3,360 mg) with meals for a planned 6 months.

Fatty acid analyses

Blood samples were collected by venipuncture into 5 mL sodium–EDTA tubes and placed on ice immediately. Plasma and erythrocytes were separated by centrifugation (3,000×g, 10 minutes; 4°C), frozen, and stored under nitrogen at −80°C until analysis. Lipids from plasma, erythrocytes, and breast tissue were isolated, fractionated into phospholipids and triacylglycerides by thin-layer chromatography (30). Fractions were trans-methylated (31) and the resulting fatty acid methyl esters were separated using a Varian 3900 gas chromatograph with an SP-2560 capillary column (100 m, Sigma Aldrich). A Star 6.41 Chromatography Workstation (Agilent Technologies) was used for peak integration and analysis (32).

Hormones, growth factors, and cytokines

Serum was frozen at −80°C in aliquots to avoid thawing and refreezing. Baseline and off-study samples were run together. Serum estradiol via ultrasensitive RIA, sex hormone-binding globulin (SHBG), and testosterone were measured at the University of Virginia Center for Research Reproduction Ligand Assay and Analysis Core Laboratory (Charlottesville, VA) and bioavailable estradiol and testosterone were calculated (27).

Insulin-like growth factor I (IGFI), insulin-like growth factor-binding protein 3 (IGFBP3), high-sensitivity C-reactive protein (hs-CRP), and high-molecular weight adiponectin were performed by ELISA (23, 27). Insulin was assessed by immunoassay and pro-insulin by immunochemo-luminescent assay in CLIA-approved clinical laboratories at the University of Kansas Hospital and Mayo Medical Laboratories, respectively. Insulin resistance (IR), plus insulin sensitivity (%S) and β cell function (%B) relative to normal young adults, was estimated from fasting glucose and insulin levels using a calculator for the updated Homeostasis Model Assessment (HOMA2) available from the University of Oxford website (33).

Serum and tissue for adipokine and cytokine assay by luminex

Adiponectin, leptin, macrophage chemoattractant protein 1 (MCP-1), tumor necrosis factor alpha (TNFα), plasminogen activator inhibitor-1 (PAI-1), hepatocyte growth factor (HGF), nerve growth factor (NGF), resistin, and insulin were assayed in serum and breast tissue using Luminex technology and Milliplex MAP Human Adipokine Magnetic Bead Panel 1 and Panel 2 kits from Millipore Corporation (23). For tissue assays, results were normalized to total protein content (Bio-Rad Protein Assay, #500-0006, Bio-Rad Laboratories, Inc.).

Tissue for RT-qPCR

Total RNA was extracted from frozen RPFNA samples using TRIzol LS (Life Technologies), amplified using MessageAmpII aRNA amplification kit (Life Technologies) and reverse transcribed to cDNA using SMARTScribe Reverse Transcriptase (Clontech Laboratories, Inc.). Real-time PCR (qPCR) was performed using TaqMan chemistry (25). Levels were expressed as relative to three reference transcripts (*PPIA*, *PPIG*, and *HRPT1*) using the $\Delta\Delta C_t$ method. For statistical analysis of changes (23, 27), 31 paired specimens were included where neither was visually bloody. Genes assessed were *ADIPOQ*, *LEP*, *ALOX15*, *ALOX15B*, *ALOX5*, *ALOX5AP*, *CD44*, *COX2*, *Cyclin B1*, *Cyclin D1*, *ERP44*, *ERα*, *GLUT 4*, *GREB1α*, *HGF*, *HPGD*, *ICAM-1*, *IGFBP-2*, *KISS 1*, *LTA4H*, *LKB1*, *MCM2*, *MCP-1*, *PPAR γ*, *PGR*, *pS2*, *SDF-1a*, *SDF-1b*, *SDC1*, *TXNIP*, *VEGF*, *XIAP. ACTB*, *GUSB*, and *B2M* originally assessed as potential reference transcripts were eliminated when all data were analyzed by Genorm^PLUS software (Biogazelle).

Tissue for reverse phase proteomics

Reverse Phase Protein Array assay was performed on frozen RPFNA aliquots in the CCSG Functional Proteomics Core at the University of Texas MD Anderson Cancer Center (Houston, TX). Specimens were spotted onto a glass slide coated with nitrocellulose with each sample represented on the slide as a serial microdilution. Dilution series were replicated on spatially distant portions of the array. Each slide contained multiple positive and negative controls including quantitative peptide and phosphopeptide controls with results normalized to total protein loading (34). A panel of 163 peptides or phosphopeptides was assessed using paired specimens for 21 women where neither specimen in the pair was visually bloody and adequate specimen aliquots were available. Pre- and postintervention specimens were assessed together. For our primary analysis, we considered only the 110 proteins for which the antibody validation process was complete.

Statistical analysis

The primary endpoint was feasibility defined per protocol as (i) 50% or greater acceptance of the study by eligible women; (ii) at least 85% retention and completion of the 6-month trial; and (iii) 70% of subjects compliant with study agent, defined as taking 70% of the prescribed study drug. The target accrual of 40 subjects was designed to provide paired (pre- and postintervention) specimens for biomarker evaluation of at least 30 subjects.

Secondary endpoints were: (i) change in risk biomarkers including cytomorphology categorical descriptor and index score, Ki-67, mammographic breast density, serum fasting insulin and pro-insulin, assessments of insulin resistance/sensitivity, IGFI: IGFBP3, adiponectin and leptin and their ratio, BMI, change in body fat, % body fat distribution, and lean body mass; (ii) change in fatty acid composition primarily in erythrocyte and breast tissue phospholipid compartments; (iii) change in molecular mechanism of action biomarkers including targeted mRNAs, chemokines, and peptides; and (iv) adverse events and quality of life indices.

Data were summarized using frequencies and percentages for categorical variables, and medians, means and SDs for continuous variables. Given the small sample sizes, nonparametric statistical analysis approaches were used throughout. Change in continuous biomarkers over the course of the intervention was assessed by the Wilcoxon signed rank test. For comparison of paired categorical

variables (e.g., atypia), McNemar test was used. All analyses were conducted using SPSS, version 20 (IBM). Since all biomarker analyses were considered exploratory, P values were not corrected for multiple comparisons. A biomarker change with a P value of <0.05 was considered suggestive of modulation and worthy of study in subsequent trials. The reader is advised to interpret biomarker results conservatively.

Results

Eligibility testing and trial entry

Of the 192 potentially medically eligible women screened by RPFNA, 133 were not tissue eligible. Of the 59 who were tissue eligible, 5 were found to not qualify for the drug intervention because of concomitant medications or other medical issues, leaving 54 women who were medically and tissue eligible. Nineteen declined participation of which 8 entered another trial (see Supplementary Fig. S1 for CONSORT diagram). Thus, 35 medically and tissue biomarker eligible women entered the trial for an acceptance rate of 65% which exceeded the first protocol-defined feasibility criterion of 50% uptake.

Baseline demographics and risk factors

All women were postmenopausal with a median age of 54 years (range 33–67). Seventeen of the 35 women (41%) were on hormone replacement therapy at baseline and remained so throughout the trial. All but 2 participants were self-described as non-Hispanic white; 1 identified herself as Hispanic and 1 as Asian. All participants had a high school degree, 66% were college graduates, and 34% had postgraduate degrees. Median BMI was 24.7 kg/m^2 (range 19.5–38.6 kg/m^2), with 8 (23%) classified as obese (BMI 30 kg/m^2 or higher). Thirty-one (89%) had a positive family history of breast cancer, 10 of 35 (29%) had a prior precancerous biopsy showing atypical hyperplasia, LCIS, or DCIS. One had a prior invasive cancer. Twenty percent had an estimated mammographic density of >50% (see Supplementary Table S1).

Retention, compliance, adverse events, and quality of life

Of the 35 women who enrolled, 34 (97%) completed the intervention and had a repeat RPFNA. This exceeded the second feasibility criterion of at least 85% of subjects remaining on study. Thirty-three of the 34 subjects (97%) who completed the study took 70% or more of prescribed drug, based on duration on drug (median 182 days, range 133–220 days) and returned pill counts. The one "non-compliant" subject took study drug as prescribed, but for only 4 of the planned 6 months (thus, <70%) before having her off-study RPFNA before back surgery. The third feasibility criterion of at least 70% of subjects being compliant was also met.

Side-effect profile was favorable. Of the 44 Grade 2 or worse adverse events self-reported in 22 participants, only 8 were possibly or probably due to study agent. The main complaints were gastrointestinal side effects of nausea, bloating, and/or diarrhea. One subject went off study early but this was not due to toxicity. The median BCPT quality-of-life score was 10 at both on-study (range 1–57) and off-study (range 0–36).

Dietary intake of omega-3 and omega-6 fatty acids

Before initiating intervention, the median dietary EPA + DHA intake estimated from the food frequency questionnaire was 75 mg/day with a similar amount for AA. The median ratio of total omega-3: omega-6 fatty acids consumed at baseline was approximately 1:10, largely reflective of the intakes of 1.1 g ALA and 11.1 g LA. There were no changes in dietary intake of EPA, DHA, AA, or total omega-3 or omega-6 fatty acids over the course of the study. However, there were trends for participants to have slightly reduced energy intakes (medians of 1,633—1,422 Kcal; $P = 0.20$), reduced carbohydrate (183–158 g; $P = 0.0054$), and fat (65–55 g, $P = 0.011$) intake, with higher % energy from protein (15.9–16.7%, $P = 0.034$).

Fatty acid profiles in blood and breast aspirates

A fasting blood ample was obtained for fatty acid profile within 24 hours of last dose of EPA + DHA ethyl esters for 33 of the 34 women who completed study and showed a median value of 0.83 for the erythrocyte phospholipid (EPA+DHA):AA ratio (Table 1), which corresponds to a 258% increase. For 24 of 34 participants, RPFNA was delayed a median of 14 days following drug discontinuation because of initial concerns about excessive hematomas should the aspiration be performed immediately after stopping drug. For these 24, a second fasting blood sample for fatty acid measurements was obtained before the RPFNA. Median erythrocyte phospholipid (EPA+DHA):AA ratio at 14 days post discontinuation was 0.66, a 147% increase over baseline values. Because approximately 1 of 3 women had their repeat RPFNA within 24 hours of drug discontinuation and the other 2 of 3 an average of 2 weeks after drug discontinuation, the composite erythrocyte phospholipid (EPA+DHA):AA ratio at the time of RPFNA showed a median increase of 193% from baseline ($P < 0.001$; Table 1). The corresponding change in fatty acid composition in the phospholipid compartment of the breast aspiration specimens paralleled that in erythrocytes and while of a lesser magnitude was significant for the (EPA+DHA):AA ratio ($P = 0.017$; Table 1). Although 32 of the 34 women showed an increase in (EPA+DHA):AA ratio in breast triacylglycerides (Fig. 1), 23 exhibited an increase in breast phospholipids.

Anthropometric variables

At baseline, median BMI was 24.7 kg/m^2, body fat 39%, android fat 43%, waist circumference 85 cm, and waist hip ratio 0.83. Over the course of the study, there was no statistically significant change in any anthropometric variable (Supplementary Table S2).

Serum risk and inflammatory biomarkers

Favorable increases in serum adiponectin ($P = 0.0027$) and HOMA %B (a measure of pancreatic B-cell function; $P = 0.0048$), and a decrease in the serum inflammatory cytokine TNFα ($P = 0.016$) were observed. Borderline decreases were observed for bioavailable estradiol ($P = 0.039$; Table 2). No change was observed for insulin, HOMA %S, HOMA IR, high-sensitivity CRP, leptin or the proinflammatory cytokines HGF, MCP-1, NGF, HGF, PAI-1, and resistin.

Cytomorphology, Ki-67, mammographic density

Ki-67 decreased from a median of 1.7% at baseline to 0.75% off-study, with decreases for 22, increases for 7, and no change for 5 who had 0% at baseline ($P = 0.036$; Fig. 2A). A modest decrease in the cytomorphology index score was observed from a median of 15 at baseline to 14 after intervention ($P = 0.014$; Table 3). A decrease in cytomorphology index score was observed in 19 women, increase in 9, and no change in 6 (Fig. 2B). There was

Table 1. Change in fatty acid content for the 34 subjects who completed the trial

Tissue/lipid compartment fatty acid component	Median values Percent of total fatty acid content				P (Wilcoxon) for baseline to FNA2[d]
	Baseline	6 mo[a]	6.5 mo[b]	At FNA2[c] (relative change)	
Erythrocyte PLs	N = 34	N = 33	N = 24	N = 34	
AA	12.7	10.3	10.0	10.0 (−22%)	<0.001
EPA	0.39	2.66	1.66	1.84 (340%)	<0.001
DHA	2.50	5.13	4.88	4.88 (80%)	<0.001
EPA+DHA:AA ratio	0.22	0.83	0.66	0.69 (193%)	<0.001
Plasma PLs	N = 34	N = 33	N = 24	N = 34	
AA	9.9	8.6	9.2	8.9 (−15%)	<0.001
EPA	0.73	3.40	0.88	0.93 (74%)	<0.001
DHA	2.33	5.05	3.89	4.23 (90%)	<0.001
EPA+DHA:AA ratio	0.26	1.14	0.54	0.62 (118%)	<0.001
Plasma TAGs	N = 34	N = 33	N = 24	N = 34	
AA	1.76	1.46	1.52	1.54 (−18%)	0.0071
EPA	0.26	1.21	0.28	0.43 (71%)	0.0034
DHA	0.37	1.95	0.76	0.94 (151%)	<0.001
EPA+DHA:AA ratio	0.35	1.97	0.77	0.99 (214%)	<0.001
Breast TAGs	N = 34		N = 34	N = 34	
AA	0.41			0.38 (12%)	0.31
EPA	0.033			0.097 (261%)	<0.001
DHA	0.077			0.16 (133%)	<0.001
EPA+DHA:AA ratio	0.24			0.67 (130%)	<0.001
Breast PLs	N = 34		N = 34	N = 34	
AA	1.70			1.24 (−6%)	0.057
EPA	0.040			0.051 (19%)	0.37
DHA	0.14			0.15 (15%)	0.23
EPA+DHA:AA ratio	0.18			0.31 (55%)	0.017

NOTE: Erythrocyte Phosholipids (PLs), Plasma PLs and Triacylglycerides (TAGs), and Breast Aspirate PLs and TAGs.
[a]Fasting blood collected within 24 hours of discontinuation of drug, after a nominal 6 months of intervention.
[b]Second fasting blood specimen collected the same day as RPFNA which was a median of 14 days following drug discontinuation.
[c]The change in values represents change at the time of the RPFNA which for 1/3 of participants was within 24 hours of last dose and for approximately 2/3 was a median of 14 days after last dose.
[d]P-values < 0.05 are indicated in bold.

no significant change in the categorical designation of cytologic atypia. Mammographic breast density (median of 10 months between mammograms) decreased for 24 and increased in 9 women ($P = 0.036$). However, the extent of change was modest with a median decrease of 1.9%.

Breast adipokines and cytokines

Little change was observed in tissue adipokines/cytokines with the exception of a borderline ($P = 0.033$) decrease in breast MCP-1, a chemokine responsible for differentiation of monocytes into tissue-infiltrating macrophages. No change ($P > 0.05$) was observed for breast adiponectin, leptin, adiponectin:leptin ratio, HGF, insulin, NGF, PAI-1, resistin, and TNFα (Supplementary Table S3).

Breast gene expression (mRNA)

For 32 transcripts assessed in 31 pairs of frozen specimens, little change was observed following normalization to reference transcripts, particularly if relative changes of <0.5 or >2 were required for designation of decrease or increase. However, a decrease was suggested for arachidonate 15-lipoxygenease type B ($P = 0.037$), involved in formation of leukotrienes and other eicosanoids from AA, and hydroxyprostaglandin dehydrogenase involved in the catabolism of prostaglandin E2 ($P = 0.033$; Supplementary Table S4).

Tissue PROTEOMICS (RPPA)

Table 4 lists the 17 peptides and phosphopeptides observed to change (uncorrected $P < 0.05$) in breast RPFNA from baseline based on 110 validated antibodies in the Reverse Phase Protein Array panel. A complete listing of all peptides assessed, plus

Breast triacylglyceride compartment
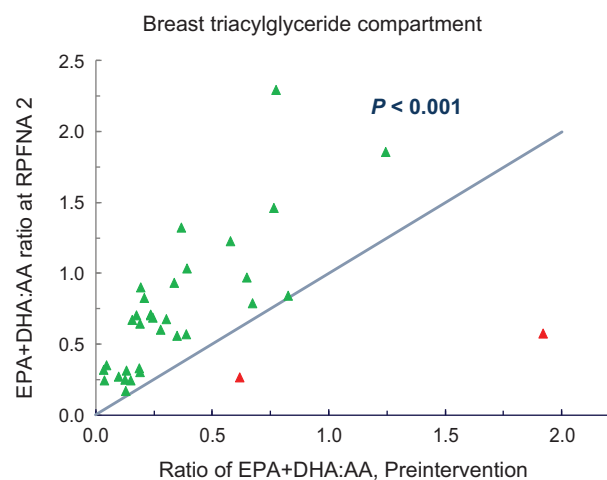
Figure 1.
Changes in the ratio of (EPA+DHA):AA in the breast triacylglyceride compartment. Baseline aspiration values are shown on the x-axis; repeat aspiration on the y-axis. The line represents no change in value; symbols above the line denote an increase and symbols below the line a decrease. P value via Wilcoxon signed rank test.

Table 2. Change in serum biomarkers for 34 subjects who completed the trial

Biomarker	Median prestudy	Median poststudy	Median absolute change	Median relative change	P (Wilcoxon)
ELISA methodology					
High-molecular weight adiponectin, µg/mL	6.4	7.2	0.3	6%	**0.046**
hsCRP, µg/mL	1.9	1.6	−0.2	−5%	0.22
IGF-1, nmol/L	12.3	12.7	0.7	6%	0.064
IGFBP3, nmol/L	105	99	−1	−1%	0.66
IGF-1:IGFBP3 ratio	0.12	0.12	0.01	7%	**0.012[a]**
Ultrasensitive RIA methodology (University of Virginia Center for Center in Reproduction Ligand Assay and Analysis Core)					
SHBG, nmol/L	59.7	68.6	2.5	3%	0.13
Estradiol, pg/mL	24.6	21.8	−2.0	−12%	0.052
Estradiol, pmol/L	90.9	80.7	−7.5	−12%	
Bioavailable estradiol, pmol/L	1.28	1.08	−0.15	−18%	**0.039**
Testosterone, ng/mL	11.4	11.1	0.9	13%	0.14
Testosterone, nmol/L	0.39	0.38	0.03	13%	
Bioavailable testosterone, pmol/L	4.49	5.06	0.35	8%	0.16
Luminex methodology					
Adiponectin, µg/mL	21.7	28.2	2.7	12%	**0.0027**
Leptin, ng/mL	16.9	14.6	1.1	14%	0.26
Adipo:Leptin ratio	1,303	1,624	99	1%	0.45
HGF, pg/mL	343	349	−23	−9%	0.65
Insulin, pg/mL	146	117	3	2%	0.68
MCP-1, pg/mL	278	287	4	1%	0.59
NGF, pg/mL	7.2	6.8	−0.5	−9%	0.23
PAI-1, ng/mL	41.5	46.2	1.9	5%	0.36
Resistin, ng/mL	26.8	24.7	−1.3	−5%	0.24
TNFα, pg/mL	3.7	3.4	−0.2	−8%	**0.016**
Assessment of insulin resistance					
Glucose, mg/dL	93.0	92.0	−2.5	−3%	0.25
Immunoreactive insulin, µIU/mL	5.0	4.6	0.4	13%	0.094
Pro-Insulin, pmol/L	9.8	9.6	0.8	13%	0.18
HOMA2 %B	59	73	6	9%	**0.0048**
HOMA2 %S	157	164	−13	−11%	0.13
HOMA2 IR	0.6	0.6	0.1	3%	**0.045**

[a]While statistically significant, this modest change was not clinically relevant. P-values < 0.05 are indicated in bold.

analysis including bloody specimens, is available in Supplementary Table S5. 5). Of the 17, eight were strongly suggestive of modulation (uncorrected $P < 0.01$). The tyrosine kinase receptors Her-2 and phosphorylated Met (the receptor for hepatocyte growth factor), the Y-box–binding protein 1, which binds to numerous kinase receptors (35), and the regulatory associated protein of mTOR (Raptor), an mTOR scaffolding protein necessary for mTOR activity, were all decreased at the $P < 0.01$ level. Increases at the $P < 0.01$ level were observed in EGFR, p27 phosphorylated at threonine 198 (cell-cycle arrest; ref. 36), Rad50 (DNA repair), and TSC2 phosphorylated at threonine 1462, a condition which is permissive of mTORC1 activity (37).

Discussion

Our feasibility objectives were accomplished by demonstrating 65% uptake of high-dose EPA and DHA fatty acid esters for primary prevention in a postmenopausal high-risk cohort with 97% completion and 97% compliance. We were able to identify risk and mechanistic biomarkers that appear to be modulated and should be assessed in a subsequent placebo-controlled phase IIB trial. As our purpose was to identify biomarkers for potential endpoints in a larger study, we did not correct P values for multiple comparisons. The small sample size, lack of a control group, and the large number of variables examined which increase the chance of a false-positive result are weaknesses and argue for caution in interpreting biomarker results from this pilot study.

The study would also have been strengthened had we been able to measure eicosanoid intermediates downstream of EPA, DHA, and AA.

Similar to our study in premenopausal women, the ratio of omega-3:omega-6 as a percent of total erythrocyte phospholipid fatty acids changed from approximately 1:6 at baseline to a more favorable ratio of 1:2 at the time of study drug discontinuation. Likewise, the ratio of (EPA + DHA) to AA in erythrocyte phospholipid fatty acids increased almost 4-fold from approximately 1:5 at baseline to approximately 4:5 at drug discontinuation and approximately 2:3 at the time of RPFNA. Importantly, the median increase in EPA:DHA/AA ratio of 193% at the time of second RPFNA (where two-thirds of subjects had a median 2 week delay after study drug discontinuation) represents an underestimate of the steady state increases that can be achieved by supplementation. Breast content of omega-3 or omega-6 as a percent of total fatty acid was only about 1 of 10 of that in the erythrocytes but proportional increases in the EPA + DHA:AA ratio were similar. Our data in benign breast tissue are consistent with those of Yee and colleagues who measured change in EPA and DHA as a percent of total (nonfractionated) adipose fatty acids (16).

Change in established risk biomarkers for breast cancer

We observed favorable changes in several risk biomarkers for breast cancer including serum bioavailable estradiol and adiponectin, mammographic density, and Ki-67 in benign hyperplastic breast tissue (29, 38–40). Ki-67, although present at a low level at

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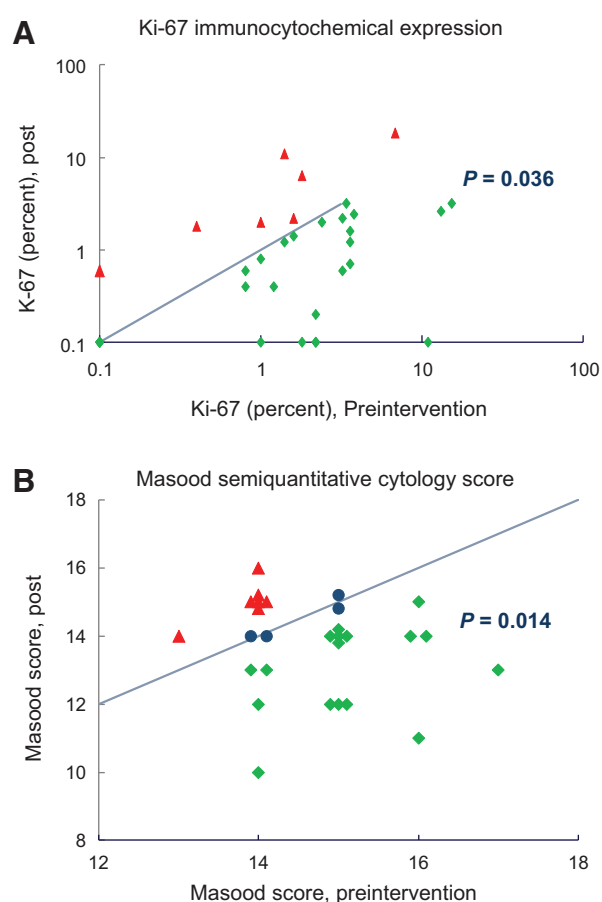
Figure 2.
A, change in Ki-67 expression (percent of cells staining positive) over the course of the intervention. Baseline aspiration values are shown on the *x*-axis; repeat aspiration on the *y*-axis. The line represents no change in value; symbols above the line denote an increase and symbols below the line a decrease. *P* value via Wilcoxon signed rank test. B, change in Masood semiquantitative cytology index score over the course of the intervention. Baseline aspiration values are shown on the *x*-axis; repeat aspiration on the *y*-axis. The line represents no change in value; symbols above the line denote an increase and symbols below the line a decrease. *P* value via Wilcoxon signed rank test.

baseline, was reduced by approximately half, consistent with results in animal model studies including one in which EPA and DHA provided a similar fraction of energy (2%) as in our trial (10, 41). Ki-67 was also reduced in our study of EPA and DHA in

premenopausal women without a change in bioavailable estradiol, testosterone, or IGF-1 (23). We did not observe a change in the designation of cytologic atypia as we did in our premenopausal study; although we did observe a small decrease in the cytology index score (23). The cytology index score has not yet been shown to predict risk of breast cancer as is the case for the categorical designation of cytologic atypia in samples obtained by RPFNA (22). It is possible that observed changes in estradiol, mammographic density, and Ki-67 could be due to the effects of normal aging in this young postmenopausal cohort as the absolute changes were small (42). It would be more difficult to attribute changes in adiponectin, an adipokine positively correlated with insulin sensitivity, to aging over 6- to 10-month interval (43). Supplementation with EPA and DHA has previously been found to increase adiponectin in individuals with coronary artery disease or chronic inflammatory conditions (44). In parallel with increases in adiponectin, we observed an increase in HOMA%B (β cell function) also suggesting improvement in insulin sensitivity (45). EPA and DHA may have different effects on pancreatic B cells; but both work to increase insulin receptor and substrate (IRS) availability and more rapidly clear glucose from the circulation. Preclinical studies generally show an improvement in insulin sensitivity in animals fed a high-fat diet although human studies are mixed (45).

Changes in inflammatory biomarkers

EPA and DHA are thought to inhibit production of cytokines and other pro-inflammatory proteins downstream of NFκB (41, 46–48). The mechanism for inhibition has been suggested to be through binding of G proteins in the plasma membrane and disruption of lipid rafts necessary for translocation and activation of toll-like receptors (49). Our observed decrease in serum TNFα and breast tissue MCP-1 suggests reduction in systemic and breast tissue inflammation. In a dose-escalating study in pre- and post-menopausal women, Yee and colleagues did not observe modulation of pro-inflammatory cytokines (16); but a recent randomized study (47) of several doses of EPA and DHA vs placebo in healthy adults showed a 10% reduction of serum TNFα at the highest dose (1,800 mg) compared with baseline (*P* = 0.08) but no change in serum CRP or IL-6, similar to our results. In healthy populations with low levels of inflammatory cytokines it may be difficult to demonstrate favorable modulation in the absence of ex vivo stimulation of monocytes with lipopolysaccharide (18, 19).

Changes in benign breast tissue functional proteomics

Our reverse proteomics assessments provided interesting data which, while exploratory and requiring confirmation in

Table 3. Change in risk and response biomarkers for 34 subjects who completed the trial

Biomarker	Median prestudy	Median poststudy	Median absolute change	Median relative change	*P* Wilcoxon[a] (McNemar)
RPFNA specimens					
Cytomorphology Index Score	15	14	−1	7%	**0.014**
Frequency of atypia, percent	53	41	8 "gain" atypia 12 "lose" atypia		(0.50)
Estimated epithelial cell number per slide	$1–5 \times 10^3$	$1–5 \times 10^3$	16 decrease 6 increase		**0.019**
Ki-67, percent	1.7	0.75	−0.4	−43%	**0.036**
Mammographic breast density					
Percent area of increased density (average of two readers)	24.5	18.7	−1.7	−10%	**0.036**

[a]*P*-values < 0.05 are indicated in bold.

Table 4. Significant ($P < 0.05$) changes in levels of 17 validated peptides and phosphopeptides in RPFNA specimens, assessed by RPPA

Protein (see Supplementary Table for gene name)	Antibody name and specific phosphorylation site	Number of paired specimens where values		P (Wilcoxon[a] 2-sided)
		Decrease	Increase	
4E-BP1	4E-BP1_pS65	6	15	0.010
Akt	Akt_pS473	6	15	0.050
Bad	Bad_pS112	8	13	0.027
Met	c-Met_pY1234_Y1235	16	5	**0.0064**
Epidermal growth factor receptor	EGFR	4	17	**0.0087**
Epidermal growth factor receptor	EGFR_pY1173	13	8	0.013
Her-2	ErbB2/HER2	17	4	**0.0051**
Lymphocyte-specific protein tyrosine kinase	Lck	17	4	0.017
MEK1	MEK1	15	6	0.013
Myosin isoform IIa	Myosin IIa_pS1943	14	7	0.046
p27	p27 KIP 1_pT198	5	16	**0.0015**
Rad50	Rad50	3	18	**0.0078**
Regulatory associated protein of mTOR	Raptor	17	4	**0.0064**
Anti-splicing factor-2	SF2/ASF	15	6	0.013
Smad3	Smad3	14	7	0.025
Tuberin	TSC2 Tuberin_pT1462	2	19	<**0.001**
Y-box–binding protein 1	YB-1	17	4	**0.0051**

[a]P-values < 0.01 are indicated in bold.

subsequent studies, does allow speculation as to possible mechanisms underlying the observed effects on proliferation in our study and preclinical models. Of greatest potential importance is the decrease in proteins associated with tyrosine kinase activity including HER2, phosphorylated Met, and Y-box–binding protein-1 (YB-1). The mechanism is thought to be the displacement of the membrane receptors from their normal lipid raft microenvironment (50). Although an increase was observed in total EGFR, the phosphorylated activated receptor showed a trend toward reduction ($P = 0.013$). A reduction in receptor tyrosine kinase activity, along with increases in p27 phosphorylated at threonine 198 (Thr198) and Rad50 (36) would be expected to result in reduced proliferation as measured by Ki-67.

mTOR signaling, known to be increased by nutrients and growth factors, is important for normal protein synthesis, cell growth, motility, and survival but sustained signaling is often implicated in oncogenesis (51). Activation of kinases and protein assembly downstream of mTOR requires an activated mTORC1 complex which includes mTOR phosphorylated at serine 2448 (Ser2448), PRAS40, and the scaffolding protein raptor which assists in binding the downstream p70S6 kinase and 4E-BP1. Phosphorylation of p70S6 and 4E-BP1 is important in translating growth signals into protein synthesis. Tuberin (TSC2) upstream of mTOR is permissive of mTORC1 activity if phosphorylated at threonine 1462 (Thr1462) or inhibitory if phosphorylated at Thr1345. In animal studies, high dietary omega-3 fatty acids repressed insulin- and nutrient-stimulated mTORC1, as manifest by decreased phosphorylation levels of p70S6 kinase (Thr389) and 4E-BP1 (Ser65; ref. 52). In our study, women were advised to eat a high protein meal before coming for RPFNA. We did not observe any change in p70S6 kinase (Thr389) or mTOR (Ser2448; Supplementary Table S5) and only marginal change in 4E-BP1 (Ser65), thought to be good indicators of mTOR activity (37). The mixed pattern of change in mTOR-associated proteins, increase in TSC2 (Thr1462) but decrease in raptor, likely reflects the non-fasting state in which RPFNAs were performed combined with an omega-3–associated increase in insulin sensitivity (53). This underscores the need for timed, controlled nutrient intake before tissue sampling in future placebo-controlled studies to make adequate assessments of omega-3 effects on the mTOR pathway in benign breast tissue (53).

Conclusion

Our results demonstrate that a primary prevention trial of high-dose EPA and DHA ethyl esters at a dose of 3.4 g/day resulted in good uptake, excellent tolerability, and retention. The increase in the (EPA+DHA):AA ratio in erythrocyte and benign breast tissue phospholipids was accompanied by a favorable modulation of a number of blood and breast tissue risk and mechanistic biomarkers similar to what would be expected from preclinical studies. A phase IIB placebo-controlled primary prevention trial examining change in blood and breast tissue eicosanoid profile and reaffirming markers that appear to be modulated in this single arm pilot study would appear in order.

Disclosure of Potential Conflicts of Interest

S.E. Carlson reports receiving commercial research grant from Mead Johnson Nutrition, has honoraria from speakers bureau from DSM and Mead Johnson Nutrition, and is a consultant/advisory board member for DSM. No potential conflicts of interest were disclosed by the other authors.

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Cancer Prevention Research

Modulation of Breast Cancer Risk Biomarkers by High-Dose Omega-3 Fatty Acids: Phase II Pilot Study in Postmenopausal Women

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