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Change in Blood and Benign Breast Biomarkers in Women Undergoing a Weight-Loss Intervention Randomized to High-Dose ω-3 Fatty Acids versus Placebo

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ABSTRACT

The inflammation-resolving and insulin-sensitizing properties of eicosapentaenoic (EPA) and docosahexaenoic (DHA) fatty acids have potential to augment effects of weight loss on breast cancer risk. In a feasibility study, 46 peri/postmenopausal women at increased risk for breast cancer with a body mass index (BMI) of 28 kg/m^2 or greater were randomized to 3.25 g/day combined EPA and DHA (ω-3-FA) or placebo concomitantly with initiation of a weight-loss intervention. Forty-five women started the intervention. Study discontinuation for women randomized to ω-3-FA and initiating the weight-loss intervention was 9% at 6 months and thus satisfied our main endpoint, which was feasibility. Between baseline and 6 months significant change ($P < 0.05$) was observed in 12 of 25 serum metabolic markers associated with breast cancer risk for women randomized to ω-3-FA, but only four for those randomized to placebo. Weight loss (median of 10% for trial initiators and 12% for the 42 completing 6 months) had a significant impact on biomarker modulation. Median loss was similar for placebo (−11%) and ω-3-FA (−13%). No significant change between ω-3-FA and placebo was observed for individual biomarkers, likely due to sample size and effect of weight loss. Women randomized to ω-3-FA exhibiting more than 10% weight loss at 6 months showed greatest biomarker improvement including 6- and 12-month serum adiponectin, insulin, omentin, and C-reactive protein (CRP), and 12-month tissue adiponectin. Given the importance of a favorable adipokine profile in countering the prooncogenic effects of obesity, further evaluation of high-dose ω-3-FA during a weight-loss intervention in obese high-risk women should be considered.

Prevention Relevance: This study examines biomarkers of response that may be modulated by omega-3 fatty acids when combined with a weight-loss intervention. While focused on obese, postmenopausal women at high risk for development of breast cancer, the findings are applicable to other cancers studied in clinical prevention trials.

Introduction

Maladaptive responses by hypertrophied adipocytes and surrounding stroma result in a proinflammatory adipocytokine profile, insulin resistance, and elevated bioavailable hormones which link obesity with postmenopausal breast cancer (1–6). Decreased adipocyte secretion of adiponectin and omentin along with increased leptin, resistin, lipocalin-2, PAI-1, TNFα and IL6 promote hyperinsulinemia, increased stem cell activity, epithelial proliferation, and angiogenesis (4–7). Low circulating adiponectin and/or ratio of adiponectin to leptin is associated with insulin resistance and increased risk for breast cancer (1, 3, 4, 8, 9). Obesity-related NFκB activation and chronic elevation of proinflammatory cytokines result in eicosanoid-triggered elevations of mammary aromatase activity and tissue estrogen, which along with reduced sex hormone

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Note: Supplementary data for this article are available at Cancer Prevention Research Online (http://cancerprevres.aacrjournals.org/).

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binding globulin (SHBG) promotes estrogen receptor (ER) activation (10–12).

The minimum fat/weight loss required to attenuate obesity-associated metabolic abnormalities is unclear. Our prior study suggests a ≥10% loss is needed for significant improvement in serum adiponectin, insulin, and bioavailable hormones (13) although leptin may be improved by more modest loss (14). A sustained ≥10% loss in obese women has been associated with reduction in breast-cancer risk in observational studies (15, 16).

Combined eicosapentaenoic (EPA) and docosahexaenoic (DHA) preparations supplied at 3% to 35% of calories in animal models have been reported to reduce mammary epithelial proliferation and atypia and augment reduction in mammary cancer from weight loss (17, 18). These favorable results are likely due to an increase in the ω-3: ω-6 FA ratio in phospholipid membranes (19, 20). The increased ω-3: ω-6 ratio in turn enhances PPARγ activity and insulin sensitivity, reduces EGFR and HER-2 neu signaling through lipid raft alteration, and reduces mammary inflammation, aromatase activity, and estrogen production (6, 21–26). Clinical trials of marine ω-3 FA combined with energy restriction have been suggested based on promising preclinical leads (27, 28). The amount of human EPA and DHA intake required to mimic animal results is unclear and is partially dependent on ω-6-FA consumption which is abundant in the western diet in soy, corn oil, meat, eggs, and dairy products (29). A meta-analysis of observational studies has suggested a 5% relative reduction in risk for breast cancer for every 0.1 gm increase in ω-3-FA intake (30). EPA+DHA ethyl esters of 3 to 3.5 g daily approximates 2% of calories; a substantial increase from the 0.1% to 0.2% typically found in western diets (31, 32). We administered 3.4 g per day of EPA and DHA ethyl esters for 6 months to high-risk postmenopausal women without selection for weight and found improved cytologic morphology and favorable change in benign breast tissue proteins associated with mTOR pathway activation (33).

We report here trial feasibility and change in metabolic risk biomarkers for breast cancer with administration of 3.25 g/day DHA + EPA ethyl esters or placebo to overweight and obese peri and postmenopausal women initiating a structured behavioral weight-loss intervention. The primary objective was tolerability of high dose EPA + DHA in women undergoing calorie restriction as assessed by study discontinuation rate in the first 6 months for women randomized to ω-3-FA. Secondary objectives included exploration of effects of ω-3-FA on systemic and breast tissue risk biomarkers for breast cancer. As many of these biomarkers are likely changed in the same direction by both weight loss and ω-3-FA (13, 34, 35), we also explored their dual effects. Since 10% is a common goal for behavioral weight-loss interventions and was the median loss achieved in a prior pilot study we assessed the biomarker impact of loss of less than 10% or more than 10% of baseline weight at 6 months within the two randomized arms (13, 36).

Methods

Eligibility for study

To be considered for study, women had to be peri or postmenopausal, less than age 70, body mass index (BMI) ≥ 28 kg/m^2, able to participate in moderate intensity exercise, have had a normal mammogram within past 12 months, reasonably normal liver and renal function, and be considered at increased risk for breast cancer. Increased risk was defined as any one or more of the following: prior biopsy showing atypical hyperplasia or lobular carcinoma *in situ*, prior contralateral breast cancer, known moderate or high penetrance germline gene mutation, Tyrer-Cuzick model risk of ≥ 2X that for age group, or ≥ 50% visually estimated mammographic breast density. Hormone replacement was allowed providing no dose adjustments had occurred within 3 months of performing baseline assessments.

Women were ineligible if they had prior bariatric surgery or required current use of a statin, metformin, or other medications for diabetes, chronic aspirin or nonsteroidal anti-inflammatory agent or use of immunosuppressive therapy. They were also ineligible if had been taking of more than 1 g/day ω-3-FA supplements within 3 months, or had been on tamoxifen, raloxifene, an aromatase inhibitor or had taken an investigational drug as part of a chemoprevention trial within 6 months.

Women must have had at least hyperplasia on baseline (screening), random periareolar fine needle aspiration (RPFNA) and 500 epithelial cells on the slides designated for Ki-67 measurement prior to starting the weight-loss intervention and randomization. Protocols for benign tissue sampling by RPFNA (HSC 4601; NCT00291096) and for the study intervention (Study00000703; NCT02101970) were approved by the University of Kansas Medical Center Human Subjects Committee, the institutional review board (IRB) that ensures that studies are conducted in accordance with the ethical principles of the Belmont Rule and the U.S. Common Rule. Separate consents were utilized for the screening and interventional protocols with written consent obtained from all subjects. Eligible subjects were randomized to placebo or EPA + DHA ω-3-FA according to blinded randomization assignments provided by the biostatistical core only to the investigational pharmacy.

Anthropomorphic measures and body composition

Weight, height, waist circumference [below lowest rib; JLN, anaplasticlymphoma kinase (ALK)], and body composition by dual x-ray absorptiometry (DXA, GE Lunar Prodigy) were measured in a hospital gown at baseline, 6, and 12 months. Coefficient of variation (CV) is more than 1% for total mass and fat, for overweight and obese individuals with the GE Lunar Prodigy DXA.

Behavioral weight-loss intervention

Women were instructed in a reduced calorie diet and use of study-provided monitoring tools (Fitbit activity tracker and

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Lose it mobile app) by the group counselor during two in-person group sessions (CAB, DKS). Weekly group phone calls during the initial 26 weeks were led by a registered dietitian or clinical health psychologist. Calls centered on diet and exercise behavior education, goal setting, and discussion of barriers and solutions. Daily meal recommendations during the active weight loss phase (first 6 months) were 2 prepackaged entrees ≤350 calories/entree, 2 to 3 low-calorie high-protein snacks, and 5 servings of fruits and vegetables/day, all purchased by participants. Diets were designed to provide 1,200 to 1,500 kcal (approximately 20% reduction from requirements) with more than 30% from fat and a minimum of 50 grams of protein/day. Women were asked to take 10,000 steps/day and/or perform at least moderate intensity exercise a minimum of 150 minutes/week.

Participants could elect to discontinue after the weight loss phase. Those wishing to continue were reregistered for maintenance weeks 27 to 52. Maintenance caloric intake recommendations were recalculated on an individual basis and women could reduce or eliminate use of prepackaged entrees.

Benign breast tissue acquisition and processing for cytomorphology and immunochemistry

RPFNA (CJF, KRP) was performed 2 to 4 hours after a standard meal on two sites per breast under local anesthesia. Nonfasting blood was obtained at the time of the RPFNA. The first 2 aspiration passes per site (4 sites total) were pooled in a 2-ml cryovial containing 0.25 ml PBS, immediately immersed in liquid nitrogen and transferred to a −80°C freezer within 12 hours for subsequent fatty acid and Luminex assays. Remaining specimens from both breasts were pooled in a single 15 cc tube containing 9 ml of CytoLyt and 1 ml of 10% neutral buffered formalin. Cells were spun, washed, and resuspended in PreservCyt after at least 24 hours in CytoLyt. Aliquots were processed to slides using a Thin Prep nongyn standard protocol (Hologic LP).

Slides for cytomorphology and immunostaining were first Papanicolaou-stained. All slides were assessed by a single cytopathologist (CMZ) who assigned a categorical assessment of nonproliferative, hyperplasia, borderline hyperplasia with atypia, or hyperplasia with atypia as well as a Masood semi-quantitative index score (33). Proliferation was assessed by Ki-67 IHC staining on slides with ≥500 epithelial cells visible by Papanicolaou-staining. Briefly, after antigen retrieval (10 mmol/L citrate, pH 6, for 2 minutes at 120°C in a decloaking chamber), slides were stained with a monoclonal Ki-67 antibody (Dako #M7240, 1:20) using a Dako autostainer and underwent dual scoring (TAP, TM).

Drug supply and dose alteration

Both placebo and EPA + DHA ω-3-FA were supplied as amber capsules from Ocean Nutrition/DSM. Active agent capsules each contained 420 mg of EPA and 210 mg of DHA ethyl esters. The placebo capsule contained corn oil. Women began study agent at 1 capsule/day with meals and increased by 1 capsule/day until they reached 5 capsules daily or 2,200 mg EPA and 1,050 mg DHA ethyl esters. Women who did not tolerate the drug well were to reduce the dose by half and then gradually escalate to full dose. If symptoms recurred women continued at a reduced dose the remainder of the study.

Fatty acid analyses

Plasma and erythrocytes were isolated from fasting blood samples by centrifugation (3,000 × *g*, 10 minutes; 4°C), frozen, and stored at −80°C until analysis. Lipids from erythrocytes were isolated using a modified Folch method and fractionated by thin-layer chromatography (33). Lipid fractions were trans-methylated with boron trifluoride-methanol. Resulting fatty acid methyl esters were separated using a Varian 3900 gas chromatograph with an SP-2560 capillary column (100m, Sigma Aldrich) and Star 6.41 Chromatography Workstation for peak integration and analysis (SEC) (33).

Systemic biomarkers

Blood was obtained both fasting as well as 2 to 4 hours after a study-provided standard meal given the report that ω-3-FA may have more demonstrable effects on insulin sensitivity and inflammation in the postprandial period (37). Blood was processed to serum and plasma and aliquots frozen at −80°C. All samples from the same individual were assayed in duplicate in the same run in the Breast Cancer Prevention Center Laboratory (TAP). For biomarkers not affected by fasting conditions [CRP, insulin-like growth factor-1 (IGF-1), IGFBP-2 and -3, SHBG, estradiol, testosterone], only nonfasting serum was used. Adiponectin, leptin, omentin, resistin, hepatocyte growth factor (HGF), PAI-1, MCP-1, TNFα, IL6, FGF-21, and FABP4 are reported from fasting serum. Biomarkers, conditions, and assay methods are reported in Supplementary Table S1.

RPFNA tissue biomarkers

Cryovials containing frozen benign RPFNA tissue in PBS were thawed and supplemental protease and phosphatase inhibitors added to 1X final concentration (cOmplete protease inhibitor and PhosSTOP phosphatase inhibitor, Millipore-Sigma #4693124001 & #4906837001). An aliquot of 0.15 ml was transferred to a sterile tube and snap-frozen for adipocytokine assay. Remaining sample was sonicated (3 × 10 seconds at ≤10 W using a Fisher 100 Sonic Dismembrator) and aliquots for fatty acid analysis (0.25 ml) and total protein quantification (0.05 mL) were snap-frozen and stored at −80°C until use.

Milliplex MAP Human Adipocyte Magnetic Bead Panel was used to measure adiponectin, leptin, HGF, resistin, PAI-1, MCP-1, TNFα, IL6, and IL8 in RPFNA tissue samples as shown in Supplementary Table S1 and normalized to total protein for each sample (Bio-Rad Protein Assay 500–0006).

Feasibility assessment

Protocol-defined feasibility for the combined ω-3 and caloric restriction intervention was less than 20% dropout prior to completion of the intervention and 6-month biomarker

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assessment in women randomized to 3.25 g of EPA+ DHA ethyl esters/day.

Statistical analysis

Given the modest number of subjects, nonparametric methods were used for all analyses (SPSS, version 25, IBM). The Wilcoxon signed-rank test for paired samples was used for assessment of change in variables over the course of the intervention within the randomized treatment arms of ω-3-FA or placebo and over time for the four groups as defined by both randomization arm and weight loss less than or more than 10% at 6 months. The Wilcoxon rank-sum (Mann–Whitney *U*) test was used for comparisons of differences in biomarker change over time between women randomized to ω-3-FA or placebo. Associations between relative biomarker change and relative weight change were evaluated by Spearman rank correlation (rho) test. For assessment of differences between the four groups formed by randomization and weight loss, initial analysis was performed by Kruskal–Wallis test, followed by individual comparisons by Mann–Whitney *U* tests. For all analyses, $P < 0.05$ (two-sided) was considered statistically significant. As these comparisons were exploratory, no adjustments were made for multiple comparisons.

Results

Screening and study completion

Eighty-two individuals met menopause, BMI, risk, and medical eligibility and signed consent for eligibility RPFNA. Of these 15 did not meet minimum cytomorphology and/or cellularity requirements. An additional 21 eligible following RPFNA declined the weight-loss intervention and randomization, usually due to either time commitment associated with the weight-loss intervention or concerns about ability to follow a diet with prepackaged entrees (see **Fig. 1** Consort Diagram).

Forty-six eligible women stratified by peri or postmenopausal status were randomized 23 each to ω-3-FA or placebo prior to the start of the structured group weight-loss intervention. Women were instructed to start capsules 2 weeks after the start of caloric restriction. One woman, randomized to ω-3-FA, never started the intervention but 45 began the weight-loss intervention and study drug. Three dropped in the initial 6 months prior to reevaluation (1 randomized to ω-3-FA stopped after 4 months due to time commitment, another discontinued study at week 23 prior to biomarker reevaluation, and 1 randomized to placebo discontinued after 1 month due to acid reflux symptoms). Forty-two women, 22 placebo, and 20 ω-3-FA, completed the caloric restriction phase of the weight-loss intervention and were evaluable for anthropomorphic variables and biomarkers at 6 months. Thus, our primary feasibility objective of tolerability of combined high-dose ω-3-FA and caloric restriction was satisfied with a 13% premature discontinuation by 6 months of all randomized and 9% of those who actually started the weight loss and ω-3-FA intervention. Thirty-nine of 42 who completed the 6 months of weight loss were registered for maintenance during which time they continued with their initial study agent. Three women discontinued study after the 6-month evaluation prior to maintenance start [1 randomized to ω-3-FA had ductal carcinoma *in situ* (DCIS) discovered at 6-month visit; 2 randomized to placebo had conflicting work or family medical issues]. Thirty-five of the 39 women who started maintenance underwent biomarker evaluation at 12 months, 19 had been randomized to placebo and 16 to ω-3-FA. One randomized to ω-3-FA was embarrassed about weight regain and dropped prior to reevaluation, 2 randomized to ω-3-FA had foot injuries and needed surgery, and 1 randomized to placebo discontinued because of her husband's medical problems.

Baseline demographics, risk, body composition

For all 46 randomized women the median age was 53 years, 76% were postmenopausal and 24% perimenopausal. The median 10-year risk estimated by the Tyrer-Cuzick model was 8.4%. Two women were African American and three were Hispanic. Eighty-two percent had at least a college degree and 71% had a median income of more than $60,000/year. Median baseline BMI was 31 kg/m^2 (range 28–42 kg/m^2), fat mass index 14.3 kg/m^2 (10.2–21.2 kg/m^2), and waist circumference 100 cm (80–120 cm). A fat mass index of more than 13 kg/m^2 is considered obese and waist circumference more than 88 cm is considered as evidence for abdominal adiposity (38). There were no significant differences in baseline demographic or risk factors or weight between the two randomization arms (**Table 1**). Women randomized to ω-3-FA had slightly higher median percent body fat ($P = 0.028$) which was no longer significant when assessing only the 42 women evaluable at 6 months ($P = 0.078$).

Adverse events

There was no significant difference in total or grade 2 and 3 adverse events (AE) between women randomized to ω-3-FA or placebo (Supplementary Table S2). For the 45 women initiating the intervention 96% of women randomized to placebo and 91% of those randomized to ω-3-FA competed 6 months of treatment and reevaluation. Eighty-three percent of women randomized to placebo and 73% of those randomized to ω-3-FA completed 12 months (weight loss + maintenance phases). None of the four Grade 3 AEs (anaphylaxis, vomiting, aspiration, oral pain) were thought to be due to study drug (Supplementary Table S2). There were two serious AEs (SAE) in which women were briefly hospitalized: one for treatment of DCIS (at 6 months) and another for invasive lobular carcinoma (at 12 months); both in women randomized to ω-3-FA.

Change in the erythrocyte phospholipid EPA+ DHA: arachidonic acid ratio

The median baseline erythrocyte phospholipid ratio of DHA+EPA: arachidonic acid (AA) was 0.31 for both placebo and ω-3-FA. The erythrocyte phospholipid ratio of DHA+EPA: AA increased over 2.5-fold to a median of 0.76

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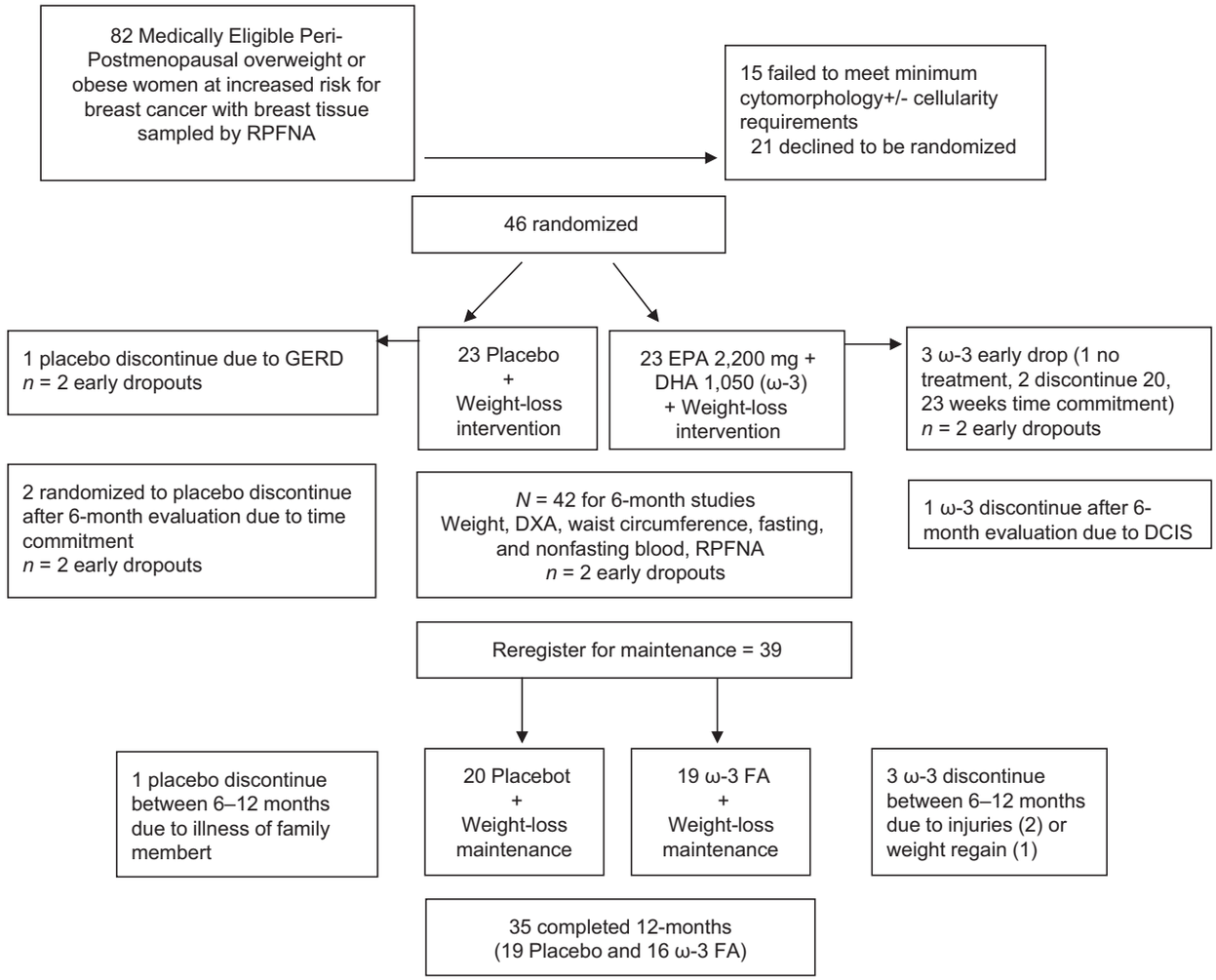

Figure 1.
Consort diagram.

at 6 months for women randomized to ω-3-FA while for those randomized to placebo it remained low at 0.30 (Supplementary Table S3).

Change in weight and body composition measures at 6 months

Considering all 46 women randomized, median relative weight change at 6 months was −10%. This includes the women who dropped out prior to 6 months using their last recorded relative weight change which ranged from −4 to −8%.

The 42 women evaluable for weight and biomarkers at 6 months had a median relative weight change from baseline of −12% with no significant difference between those randomized to placebo (median −11%) or ω-3-FA (median −13%). There was also no difference between women randomized to placebo or ω-3-FA in change in total mass (−10% and −9%), fat mass (−20%), or android fat mass (−11% and −8%) (**Table 2**).

Change in risk biomarkers

Change in 6-month serum risk biomarkers by randomization arm

For the 20 evaluable women randomized to ω-3-FA, significant change ($P < 0.05$) was observed between baseline and 6 months for 12 of 25 assessed biomarkers; adiponectin, leptin, adiponectin:leptin ratio, omentin, insulin, lipocalin-2, resistin, PAI-1, HGF, CRP, SHBG, and free testosterone. In contrast, for the 22 evaluable women randomized to placebo, significant change between baseline and 6 months was observed for only four biomarkers (leptin, adiponectin:leptin ratio, SHBG, and IGFBP2; **Table 3**). Despite the significant changes over time for women randomized to ω-3-FA there was not a significant difference between the randomized arms in 6-month relative change for any biomarker. Patterns at 12 months were similar for the 16 evaluable women remaining on the ω-3-FA arm (Supplementary Table S4). No change was observed between baseline and 6 months for MCP-1, TNFα, IL6, FABP4, FGF21,

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Table 1. Baseline characteristics for 46 women randomized to placebo or ω-3 fatty acid supplementation arms.

Variable	Median (range) or *N* (%) Total *N* = 46	Placebo *N* = 23	ω-3 fatty acids *N* = 23	Comparison between arms *P*[a]
A) Demographic and risk variables				
Age, y	53 (40–67)	52 (40–63)	53 (42–67)	0.61
Tyrer-Cuzick 10-yr risk, %	8.4 (1.9–31.8)	9.0 (3.0–12.7)	7.6 (1.9–31.8)	0.38
Menopausal Peri	11 (24%)	6 (26%)	5 (22%)	1.00
Post	35 (76%)	17 (74%)	18 (78%)	
Genetic test Not tested	36 (78%)	19 (83%)	17 (74%)	0.15
Wildtype	6 (13%)	1 (4%)	5 (22%)	
Del mutation	4 (9%)	3 (13%)	1 (4%)	
Prior precancerous No	36 (78%)	18 (78%)	18 (78%)	1.00
Yes	10 (22%)	5 (22%)	5 (22%)	
On OC/HRT? No	29 (63%)	16 (70%)	13 (57%)	0.54
Yes	17 (37%)	7 (30%)	10 (43%)	
B) Anthropomorphic and body composition variables				
Height, cm	165 (155–175)	163 (155–175)	165 (155–175)	0.15
Weight, kg	84.8 (69.2–115.6)	81.3 (69.2–111.2)	85.8 (75–115.6)	0.081
BMI, kg/m^2	31.0 (27.9–42.4)	30.7 (27.9–39.8)	31.2 (27.9–42.4)	0.28
Waist circumference, cm	100 (80–120)	101 (80–115)	100 (84–120)	0.93
Body fat, percent	49 (37–55)	46 (37–55)	49 (42–53)	0.028[a]
FMI, kg/m^2	14.3 (10.1–21.2)	13.5 (10.1–20.5)	14.7 (11.7–21.2)	0.11

[a]Mann–Whitney nonparametric test, 2-sided. Similar lack of difference between randomized arms was observed if only the 42 women completing the 6-month intervention and evaluable for biomarkers were analyzed for baseline demographic and risk factors. Exceptions were the marginal statistical significance for body fat percent was lost ($P = 0.078$).

IGF-1, IGFBP3, the ratio of IGF-1 to IGFBP2 or IGFBP3, total estradiol, testosterone, or bioavailable estradiol for either ω-3-FA or placebo.

Serum biomarker change by randomization arm and relative weight loss

Weight loss dramatically impacted many of the biomarkers assessed. Change in leptin, adiponectin:leptin ratio, lipocalin-2, insulin, PAI-1, HGF, MCP-1, SHBG, free estradiol, IGFBP2, and IGF1:IGFBP2 ratio was correlated with weight loss but there was not a significant correlation for adiponectin or omentin (Spearman rho $P < 0.05$). We dichotomized weight loss as above or below 10% as it was the median of all women randomized and is generally the goal for weight loss programs. Significant change between baseline and 6 months was observed in 14 of 25 biomarkers for the 21 evaluable women with more than 10% loss but just 4 of the 21 who had less than 10% loss (**Table 3**).

Given the dramatic effect of weight loss, we explored biomarker modulation by both randomization assignment and weight loss less or more than 10% at 6 months forming 4 groups with roughly equal numbers (**Table 4**; **Fig. 2**). The 11 women randomized to placebo with less than 10% relative loss at 6 months (median −6.3%) exhibited significant within-group change in only leptin and the adiponectin:leptin ratio. The 10 women randomized to ω-3-FA with less than 10% relative loss (median −4.8%) showed improvement in four markers (leptin, adiponectin:leptin ratio, PAI-1, and SHBG). The 11 women randomized to placebo with more than 10% loss (median −16%) exhibited change in six serum markers (leptin, adiponectin:leptin ratio, PAI-1, insulin, HGF, and SHBG). The 10 women randomized to ω-3 FA with more than 10% relative loss (median −17%) exhibited change in 14 serum markers (leptin, adiponectin, adiponectin:leptin ratio, PAI-1, insulin, HGF lipocalin-2, resistin, CRP, TNFα, SHBG, and bioavailable estradiol and bioavailable testosterone).

At 12 months we observed similar trends (Supplementary Table S5; **Fig. 2**). Only women randomized to ω-3-FA with more than 10% weight loss showed significant within-group improvement in serum adiponectin, at 6 and 12 months both in fasting and nonfasting blood samples, and significant between group change at 12 months (Supplementary Table S6).

Benign breast tissue biomarkers

Women randomized to ω-3-FA had significant increases in benign breast tissue adiponectin pg/μg protein at 12 months compared with baseline ($P = 0.014$). Women with more than 10% loss had a 77% relative increase in adiponectin at 12 months if randomized to ω-3-FA but only a 27% relative increase for women with more than 10% weight loss who had been randomized to placebo ($P = 0.010$ for between-group change; Supplementary Table S6). No significant change was observed for breast tissue proinflammatory cytokines TNFα, MCP-1, IL6, or IL8 for any group.

There were no significant differences in tissue cytomorphology score or Ki-67 between women randomized to ω-3-FA or placebo or between those with less than 10% or more than 10% weight loss at 6 months (Supplementary Table S7).

Discussion

The primary goal of our study was to determine whether high-dose EPA + DHA ethyl esters were well tolerated during

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Table 2. Changes in weight, BMI, waist circumference, and DXA body composition for 42 women completing 6 months of the weight-loss intervention, randomized to placebo versus ω-3-FA supplementation arms.

		Median values		
		Total	Placebo	ω-3 FA
Variable	Parameter	*N* = 42	*N* = 22	*N* = 20
Weight, kg	Baseline	84.8	81.6	86.3
	6 months	75.0	71.8	76.5
	Change	−9.5	−9.2	−11.3
	Relative change, %	−12	−11	−13
BMI, kg/m^2	Baseline	30.9	30.7	31.2
	6 months	27.5	27.2	28.3
	Change	−3.6	−3.5	−3.8
	Relative change, %	−12	−11	−13
Waist circumference, cm	Baseline	100	102	98.5
	6 months	92	90	92.5
	Change	−8	−8	−7.5
	Relative change, %	−8	−8	−7
DXA total mass, kg	Baseline	84.2	81.4	85.6
	6 months	75.3	73.0	76.3
	Change	−8.2	−8.3	−8.0
	Relative change, %	−10	−10	−9
Total fat mass, kg	Baseline	38.9	35.7	41.6
	6 months	31.1	27.0	32.4
	Change	−8.4	−8.0	−8.7
	Relative change, %	−20	−20	−20
Fat mass index, kg/m^2	Baseline	14.3	13.6	14.6
	6 months	10.8	10.6	12.3
	Change	−3.1	−3.1	−3.4
	Relative change, %	−20	−20	−20
Percent body fat	Baseline	47	46	49
	6 months	41	40	45
	Change	−4	−5	−4
	Relative change, %	−9	−11	−9

Note: Significant ($P \le 0.01$ by nonparametric Wilcoxon signed rank test) within-group change from baseline to 6 months for all body composition variables except: lean mass (total, $P = 0.059$; placebo, $P = 0.47$; omega-3, $P = 0.014$); total body percent fat (omega-3, $P = 0.017$).

the caloric restriction phase of a behavioral weight-loss intervention. Only 9% of women randomized to ω-3-FA + caloric restriction who initiated the intervention failed to complete 6 months and thus the intervention is feasible. An additional 18% failed to complete maintenance and biomarker reassessment at 12 months. Study withdrawal was not due to drug-related AEs and there was no significant difference in study dropout between arms. Early withdrawals for combined ω-3-FA plus caloric restriction in our trial compares well with caloric restriction alone in breast cancer survivors in the LEAN study (19% at 6 months and an additional 23% by 12 months; ref. 39).

Exploration of risk biomarker modulation in preparation for a possible future phase II B trial was a secondary goal. We focused on blood and tissue adipocytokines likely to be modulated by ω-3-FA and implicated in breast cancer risk. Adiponectin is modulated by ω-3-FA in the absence of weight loss (1–5, 19, 21, 33) and it has been estimated for each 1 μg/mL increase in adiponectin there is a 12% relative reduction in risk for breast cancer (40, 41). The protective effect of adiponectin may be due in part to its favorable effects on insulin sensitivity and blunting of the oncogenic effects of leptin (5, 14). Women randomized to ω-3-FA had a 20% increase in 6-month serum adiponectin versus only 3% for placebo despite similar weight loss in both. Our inability to detect a decrease in the proliferative marker Ki-67 as we did in prior trials of ω-3-FA is likely due to the low median baseline level of only 0.7%. In our pilot of ω-3-FA alone in more average weight women the median baseline Ki-67 was 1.7% (33).

The combination of EPA + DHA in high doses is thought to resolve inflammation by decreasing formation of proinflammatory eicosanoids and increasing production of resolvins and protectins. This ultimately leads to reduction in inflammatory cytokines and macrophage infiltration. We did observe a reduction in CRP but it is unclear how much of this change was due to weight loss versus ω-3-FA (42). In contrast to animal studies where EPA and DHA generally made up a larger proportion of daily caloric intake, reduction of inflammatory cytokines in human trials has been inconsistent (43–45).

The significant within-group change over time in a number of biomarkers within pathways thought to be modulated by ω-3-FA and associated with breast cancer risk is encouraging. The lack of significant difference in the relative change between ω-3-FA and placebo is likely due to the combination of small sample size plus the substantial effect of weight and fat loss. As such, we explored biomarker change both by randomization arm and weight loss above and below the median for all randomized women. Those randomized to ω-3-FA with more than 10% weight loss at 6 months exhibited significant within-group change for twice the number of serum biomarkers as those randomized to placebo with more than 10% weight loss. Women initially randomized to ω-3-FA with more than 10% weight loss at 6 months showed significant within-group improvement in serum adiponectin at both 6 and 12 months. Change in breast tissue adiponectin was better for ω-3-FA with more than 10% weight loss at 12 months than for placebo and more than 10% weight loss.

Our study has multiple limitations. Biomarker modulation was designed to be explorative; not definitive. As such, we did not correct for multiple comparisons. We enrolled mainly educated Caucasian women. Feasibility and biomarker modulation may not be generalizable to a more diverse population. Additional risk biomarkers could have been investigated including volumetric mammographic density (46), fatty acid synthase (47), eicosanoids, resolvins, and protectins (20) or those implicated in stem cell turnover (48). The VITAL trial using one fourth of the dose of combined EPA + DHA esters we employed did not show evidence of prevention of cancer or cardiovascular disease (49). However, the dose in the VITAL trial likely produced only a fraction of systemic levels obtained in animal studies where cancer-preventive effects were observed (17, 18, 23). Dose, cohort, and trial design are

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Table 3. Median and range for baseline values and relative difference (percent) between baseline and 6 months for 16 serum biomarkers (of a total of 25 assessed), which exhibited a statistically significant change over time[a] either for the total cohort of 42 women completing the 6-month intervention or for groups defined by randomization or by dichotomization at 10% weight loss achieved at 6 months.

	Baseline values	Relative difference (%)				
	Total cohort		Placebo	ω-3 FA	Wt loss <10%	Wt loss >10%
Analyte or ratio	*N* = 42		*N* = 22	*N* = 20	*N* = 21	*N* = 21
Adiponectin, μg/mL	29 (10–90)	**15%**[b]	3%	**20%**[b]	3%	**30%**[b]
Leptin, ng/mL	30 (6–109)	**−43%**[d]	**−42%**[d]	**−49%**[d]	**−25%**[c]	**−71%**[d]
Adiponectin:Leptin Ratio[e]	1.0 (0.2–16)	**115%**[d]	**115%**[d]	**133%**[d]	**58%**[c]	**274%**[d]
Lipocalin-2, ng/mL	255 (122–441)	−11%	8%	**−23%**[b]	10%	**−23%**[b]
Resistin, ng/mL	38 (12–73)	**−8%**[b]	−6%	**−11%**[b]	−6%	**−12%**[b]
PAI-1, ng/mL	83 (53–161)	**−12%**[d]	−12%	**−11%**[c]	−10%	**−14%**[c]
HGF, pg/mL	304 (36–833)	**−12%**[b]	−10%	**−13%**[b]	0%	**−18%**[c]
Omentin, ng/mL	2.3 (1.4–48.3)	5%	0%	**9%**[b]	1%	16%
Insulin, pg/mL	259 (28–1805)	**−23%**[c]	−20%	**−42%**[b]	−16%	**−54%**[c]
CRP, μg/mL	2.8 (0.2–20.3)	**−18%**[b]	−7%	**−23%**[b]	−13%	**−44%**[b]
IL6, pg/mL	1.8 (0.4–296)	−10%	−5%	−17%	**−19%**[b]	13%
SHBG, nmol/L	61 (23–160)	**24%**[d]	**17%**[c]	**36%**[d]	**13%**[c]	**31%**[d]
Free estradiol, pmol/L	5.0 (0.9–52.8)	**−13%**[b]	−11%	−13%	−7%	**−20%**[b]
Free testosterone, pmol/L	44 (1–2201)	**−17%**[c]	−15%	**−24%**[b]	−6%	**−41%**[b]
IGFBP2, nmol/L (*n* = 18)	0.34 (0.06–0.67)	**86%**[c]	**127%**[b]	41%	32%	**140%**[b]
IGF1:IGBP2 ratio (*n* = 18)	19 (5–140)	−35%	−36%	−34%	−18%	**−42%**[b]

Note: There is no adjustment for multiple comparisons so caution is advised in interpretation of the results.
Abbreviation: Wt, weight.
[a]Wilcoxon nonparametric signed-rank test, 2-tailed, for within-group differences between baseline and 6 months.
[b]$P < 0.05$.
[c]$P < 0.005$.
[d]$P < 0.0005$.
[e]Adiponectin: leptin ratio is computed on the basis of adiponectin values in μg/mL and leptin values in ng/mL. Also assessed were TNFα, MCP-1, FGF-21, FABP4, estradiol, testosterone, IGF-1, IGFBP3, and ratio of IGF1: IGFBP3; no statistically significant changes detected.

important as evidenced by the different CVD outcomes in ω-3-FA arms for the ASCEND (0.84 g total DHA + EPA/day) versus REDUCE-IT trials (4 g EPA/day; ref. 50).

Given the excellent tolerance of 3.2 g of EPA + DHA and initial results suggesting favorable effects on adipocytokines, consideration should be given to further evaluation of the two

Table 4. Biomarker change at 6 months by four groups defined by randomization arm (placebo vs. ω-3 FA) and dichotomization by weight loss (<10% or >10%).

	Median relative change and associated *P* value for change over time							
	<10% weight loss		<10% weight loss		>10% weight loss		>10% weight loss	
	Placebo		ω-3 FA		Placebo		ω-3 FA	
Biomarker or ratio	Group 1 *N* = 11		Group 2 *N* = 10		Group 3 *N* = 11		Group 4 *N* = 10	
Adiponectin	2%	0.33	11%	0.24	19%	0.29	30%	**0.037**
Leptin	−26%	**0.022**	−16%	**0.0069**	−78%	**0.0050**	−65%	**0.0051**
Adiponectin:Leptin ratio	35%	**0.016**	63%	**0.017**	390%	**0.0051**	264%	**0.0051**
Lipocalin-2	13%	0.18	−3%	0.65	−8%	0.51	−34%	**0.0093**
Resistin	−6%	0.86	−6%	0.39	−8%	0.33	−17%	**0.017**
PAI-1	−6%	0.66	−10%	**0.037**	−13%	**0.028**	−16%	**0.022**
HGF	−0%	0.93	1%	0.88	−17%	**0.028**	−20%	**0.0093**
Omentin	−10%	0.33	7%	0.28	19%	0.48	11%	**0.041**
CRP	−11%	0.59	−14%	0.51	−6%	0.48	−52%	**0.0069**
TNFα	−2%	0.33	0%	0.67	−13%	0.57	−4%	**0.032**
SHBG	10%	0.051	27%	**0.013**	26%	**0.0044**	68%	**0.0051**
Bioavailable estradiol	−11%	0.13	3%	0.81	−14%	0.26	−29%	**0.032**
Bioavailable testosterone	−13%	0.42	−5%	0.14	−28%	0.29	−47%	**0.017**
Insulin	−14%	0.42	−20%	0.58	−51%	**0.047**	−61%	**0.0051**
Number modulated	**2**		**4**		**6**		**14**	

Note: Biomarkers are grouped into categories of adipokines, cytokines, hormones/growth factors, and insulin. A total of 25 biomarkers or ratios were assessed. There is no adjustment for multiple comparisons so caution is advised in interpretation of the results.
Wilcoxon nonparametric signed-rank test, two-tailed, for within-group differences between baseline and 6 months.

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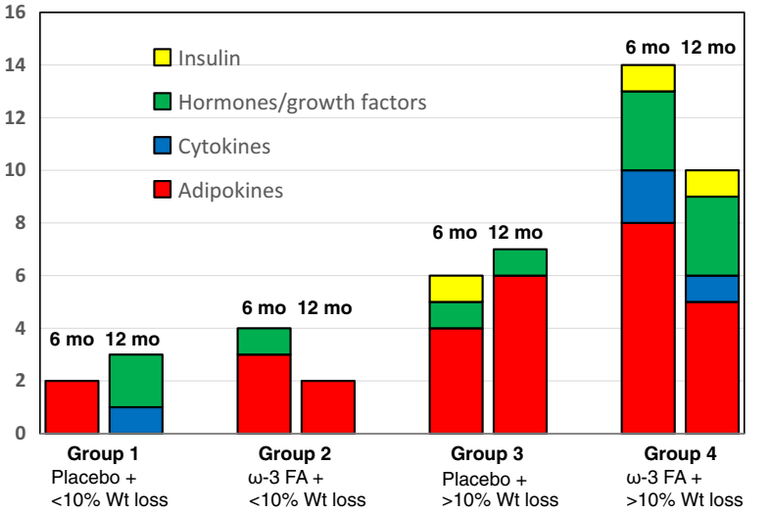

Figure 2.
Number of serum biomarkers modulated at 6 months within each of the four groups; ω-3 versus placebo × weight loss <10% or >10%.

together in a larger phase IIB trial in obese women from diverse racial, ethnic, and socio-economic backgrounds who are at increased risk for breast cancer.

Authors' Disclosures

C.J. Fabian, C.A. Befort, T.A. Phillips, J.L. Nydegger, A.L. Kreutzjans, K.R. Powers, T. Metheny, J.R. Klemp, S.E. Carlson, D.K. Sullivan, J. Hu, and B.F. Kimler report grants to their institution during the conduct of the study from Breast Cancer Research Foundation, NIH, and Morris Family Foundation; non-financial support from DSM Nutritional Products, Inc. No disclosures were reported by the other authors.

Authors' Contributions

C.J. Fabian: Conceptualization, supervision, funding acquisition, investigation, visualization, methodology, writing–original draft, project administration, writing–review and editing. **C.A. Befort:** Supervision, investigation, methodology, writing–review and editing. **T.A. Phillips:** Investigation, visualization, methodology, writing–review and editing. **J.L. Nydegger:** Investigation, methodology. **A.L. Kreutzjans:** Investigation, methodology. **K.R. Powers:** Investigation. **T. Metheny:** Investigation, methodology. **J.R. Klemp:** Conceptualization, supervision, investigation, writing–review and editing. **S.E. Carlson:** Supervision, investigation, methodology, writing–review and editing. **D.K. Sullivan:** Supervision, investigation, methodology, writing–review and editing. **C.M. Zalles:** Investigation, writing–review and editing. **E.D. Giles:** Visualization, writing–review and editing. **S.D. Hursting:** Conceptualization, writing–original draft, writing–review and editing. **J. Hu:** Formal analysis, writing–review and editing. **B.F. Kimler:** Conceptualization, data curation, formal analysis, funding acquisition, methodology, writing–original draft, project administration, writing–review and editing.

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Change in Blood and Benign Breast Biomarkers in Women Undergoing a Weight-Loss Intervention Randomized to High-Dose ω-3 Fatty Acids versus Placebo

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