

Omega-3 and Omega-6 Fatty Acids in Blood and Breast Tissue of High-Risk Women and Association with Atypical Cytomorphology

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Abstract

The ratio of omega-3 to omega-6 fatty acids, especially the long-chain eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA) to arachidonic acid (AA) ratio, is inversely associated with breast cancer risk. We measured the association between cytologic atypia, a biomarker for short-term risk of breast cancer development, and omega-3 and omega-6 fatty acid intake and levels in blood and breast tissue. Blood and benign breast tissue, sampled by random periareolar fine-needle aspiration (RPFNA), was obtained from 70 women at elevated risk for breast cancer. Self-reported dietary intake was assessed by the NCI's Food Frequency Questionnaire. The fatty acid composition of five lipid compartments, red blood cell, plasma and breast phospholipids, and plasma and breast triacylglycerides (TAG), was analyzed by

gas chromatography as weight percent. Median daily intakes of EPA+DHA and total omega-3 fatty acids were 80 mg and 1.1 g, respectively. The median total omega-3:6 intake ratio was 1:10. Compared with women without atypia, those with cytologic atypia had lower total omega-3 fatty acids in red blood cell and plasma phospholipids and lower omega-3:6 ratios in plasma TAGs and breast TAGs ($P < 0.05$). The EPA+DHA:AA ratio in plasma TAGs was also lower among women with atypia. This is the first report of associations between tissue levels of omega-3 and omega-6 fatty acids and a reversible tissue biomarker of breast cancer risk. RPFNA cytomorphology could serve as a surrogate endpoint for breast cancer prevention trials of omega-3 fatty acid supplementation. *Cancer Prev Res*; 8(5); 359–64. ©2015 AACR.

Introduction

Omega-3 and omega-6 fatty acids are essential nutrients, because they cannot be made endogenously. Humans lack enzymes to convert omega-6 fatty acids to omega-3 fatty acids and vice versa. Therefore, the dietary ratio translates into differences in blood and breast tissue (1). The majority of omega-3 and omega-6 fatty acids consumed are the 18-carbon essential fatty acids, alpha-linolenic acid (ALA), and linoleic acid (LA). ALA and LA share elongases and desaturases that convert them into long-chain (20- and 22-carbon) fatty acids (see Fig. 1). Arachidonic acid (AA), the predominant long-chain omega-6 fatty acid, is a key component of plasma membrane phospholipids (PL), from where it can be released and converted into potent proinflammatory eicosanoids by cyclooxygenase (COX) and lipoxygenase (LOX) enzymes (5, 6). Eicosapentaenoic acid (EPA) and doco-

sahexaenoic acid (DHA), the primary long-chain omega-3 fatty acids, decrease inflammation by (i) competing with AA as enzymatic substrates and (ii) serving as precursors of resolvins and protectins, which terminate inflammatory processes (7, 8).

Over the past century, consumption in the United States of omega-6 (n-6) fatty acids has increased dramatically, mostly from corn and soybean oil, whereas omega-3 fatty acid intake has remained stable (9). This high omega-6 intake relative to omega-3 fatty acids may contribute to breast cancer risk, because a low omega-3:6 ratio in tissue can create a proinflammatory milieu (5, 6) and thereby promote tumor formation and progression (10). A review of 81 rodent studies concluded that dietary omega-6 fatty acids dose-dependently accelerate mammary tumorigenesis (11). Conversely, dietary EPA and DHA prevent mammary tumor development in animals (12–15). The effect of omega-3 and omega-6 intake on breast cancer risk in humans has not been determined. Assessing this relationship is complicated by problems assessing omega-3 and omega-6 intake from food frequency questionnaires (FFQ; 16) and inconsistent results among different populations (17, 18).

Case-control studies generally find an inverse association between dietary intake and/or red blood cell (RBC) fatty acid content of EPA and DHA and breast cancer risk (19–21). Prospective studies also have generally shown an inverse association between consumption of omega-3 fatty acids from fish and/or fish oil supplements and breast cancer incidence (18). However, relatively small nested case-control studies within prospective cohorts, measuring fatty acid levels in the PL compartment of RBCs and serum, have largely failed to find a significant association between EPA, DHA, or total omega-3 fatty acid levels and breast cancer risk (22–28).

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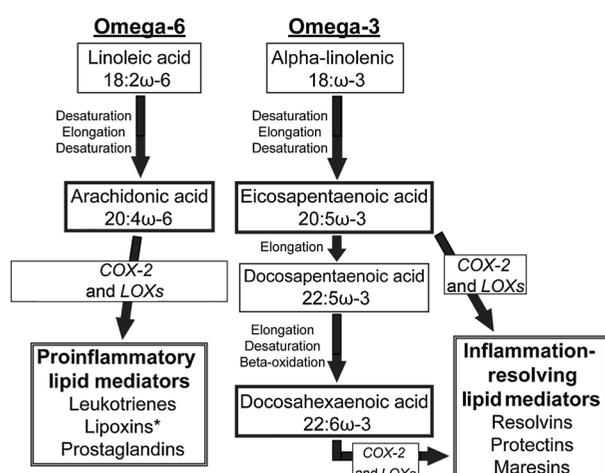
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**Figure 1.**

Omega-3 and omega-6 fatty acid metabolism. The diagram shows the conversion of short-chain omega-3 and omega-6 fatty acids to their long-chain counterparts. EPA, DHA, and AA are the preferred substrate of inflammatory enzymes (*italicized*). Leukotrienes include LTA4, LTB4, and LTC4; lipoxins LXA4, AT-LXA4, 15S-HETE, and 15R-HETE; prostaglandins PGD2, PGE2, and PGH2; resolvins RvE1, RvE2, RvD1, RvD2, 18R-HEPE, 5Hp-18R-HEPE, 17S-HpDHA, and 17S-HDHA; protectins PD1 and PD2; maresins MaR1 and 7S,14S-diHDHA. *AA-derived lipoxins tend to resolve inflammation. The lipid mediators presented are based on the work of Serhan et al. (2-4).

In this cross-sectional study, we measured the association between fatty acid content in blood and benign breast tissue samples and a breast tissue risk biomarker for subsequent development of breast cancer, i.e., cytologic atypia in breast epithelial cells obtained by random periareolar fine-needle aspiration (RPFNA; ref. 29). We have previously reported that, independent of the Gail risk model (30), women whose RPFNA specimens exhibit hyperplasia with atypia were five times more likely to develop ductal carcinoma *in situ* (DCIS) or invasive breast cancer within 4 years, compared with women without evidence of atypia (29). RPFNA atypia fulfills the criteria of a good risk biomarker for breast cancer, in that it is biologically related, statistically associated, obtained easily, and, most importantly, reversible (29, 31). We hypothesized that women with lower intakes and levels of omega-3 relative to omega-6 fatty acids in their blood and breast tissue would be more likely to exhibit RPFNA atypia.

Materials and Methods

Eligibility

Between June 2009 and February 2010, all women undergoing RPFNA were invited to provide a blood sample and information about dietary intake. All receiving RPFNA were participants in a prospective study to evaluate biomarkers and follow women at higher than normal risk for developing breast cancer (HSC #4601). The study was done at the University of Kansas Medical Center's (KUMC) Breast Cancer Prevention Center (BCPC). Women were eligible for RPFNA on the basis of any of the following: (i) an affected close relative under the age of 60; (ii) a prior breast biopsy revealing atypical hyperplasia, lobular carcinoma *in situ*, DCIS, or prior invasive breast cancer (if there is a history of DCIS or invasive cancer, only the contralateral breast is sampled); (iii) multiple breast biopsies; (iv) atypia found on a previous

RPFNA; or (v) >50% radiographic breast density. Participants were excluded if they had breast implants, were using warfarin or other potent anticoagulants, had taken chemotherapy or endocrine therapy within 12 months, or were currently enrolled in an interventional clinical trial.

Food frequency questionnaire

We estimated dietary fat intake using the diet history questionnaire (DHQ-I), an FFQ developed by the NCI (32). The DHQ-I is a 145-item, validated questionnaire that asks women to reflect on their normal diet over the previous 30 days. The DHQ-I queries the usual frequency of consumption and portion size of foods and drinks, as well as dietary supplements.

Specimen acquisition and processing

RPFNA was performed during the follicular phase of the menstrual cycle (days 1-10) for premenopausal women. Women were asked not to take any omega-3 fatty acid supplements or nonsteroidal anti-inflammatory drugs for 3 weeks before the procedure to reduce the risk of bleeding and bruising. Each breast was sampled at two sites under local anesthesia. The first 1 to 2 nonbloody samples from each aspirated site were placed in a 2 mL cryovial containing 0.5 mL PBS, immediately frozen in liquid nitrogen, and transferred to a -80°C freezer within 12 hours. Frozen tissue was thawed, mixed in an ice bath, and reallocated into four cryovials, with one designated for fatty acid analysis, such that only one further thaw would be necessary.

Blood was drawn after a 12-hour fast and separated into plasma, buffy coat, and RBC. Samples were placed on ice until they were processed and then stored at -80°C until fatty acid analysis. Blood samples were not necessarily obtained the same day as the RPFNA, as RPFNA is not performed fasting.

Cytology and proliferation

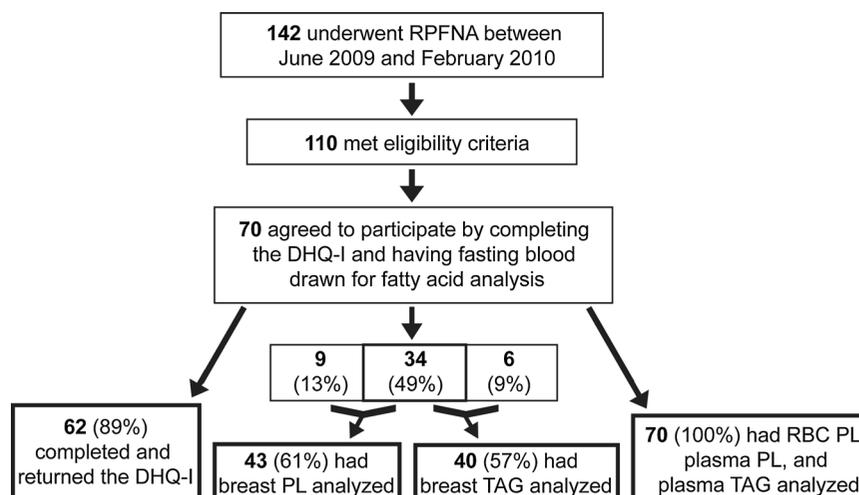
The remaining RPFNA material (after cryovial storage) was pooled in a single 15-mL tube with 9 mL Cytolyt and 1 mL of 10% buffered formalin and processed to slides for pap staining for cytomorphology and Ki-67 immunocytochemistry (33). Briefly, the number of epithelial cells per slide was estimated and categorized into the following ranges: <10, 10-99, 100-499, 500-999, 1,000-5,000, or >5,000. Cell clusters containing the highest proportion of cells staining positive for Ki-67 were preferentially evaluated. The number of positive-staining nuclei out of 500 was recorded by two independent readers. In case of a difference between the two readers, the scores were averaged. Ki-67 was not performed on specimens with <500 cells per slide; instead, Ki-67 was imputed as zero. A single cytopathologist, who did not have access to results from diet and tissue fatty acid measurements, scored specimens using categorical descriptions of (i) normal nonproliferative, (ii) hyperplasia, and (iii) hyperplasia with atypia. Specimens were also scored using a semiquantitative, adapted Masood cytology index (34), in which scores of 11 to 14 correspond to hyperplasia, scores of 15 to 18 hyperplasia with atypia, and 19 to 24 as suspicious for malignancy (34, 35).

Fatty acids

We measured the fatty acid composition of RBC PLs, plasma PLs, plasma triacylglycerides (TAG), breast PLs, and breast TAGs using a modified Folch and colleagues method (36). Briefly,

Figure 2.

Consort diagram of participation and data availability. The boxes with bolded borders represent the data that were included in the final analysis.



hydrophobic extracts of plasma and breast tissue were separated into PLs and TAGs by solid-phase chromatography (37). After transmethylation with boron trifluoride-methanol, fatty acid methyl esters were isolated and analyzed by gas chromatography using a Varian 3900 (Agilent Technologies) with helium as the carrier gas and reported as weight percent of total fatty acids as previously described (38).

Statistical analysis

Analyses were performed using JMP version 9.0.2 (SAS Institute Inc.). Medians, interquartile ranges, and *P* values are presented. Nonparametric analyses were used to control for small sample size. All tests were set at a two-sided false discovery rate of less than 5%. Given the exploratory nature of this analysis, no correction for multiple comparisons has been made. Rather, uncorrected *P* values are provided, and the reader is advised to interpret results conservatively.

Results

Study participation

Between June 2009 and February 2010, 142 women underwent RPFNA at the BCPC, of which 110 met the criteria for eligibility. Seventy ($n = 70$) agreed to complete the DHQ-I and to have additional fasting blood obtained for fatty acid analysis. Sixty-two of 70 women (89%) completed and returned the DHQ-I. About half of the RPFNA samples contained adequate breast tissue for fatty acid analysis: breast PLs ($n = 43$), breast TAGs ($n = 40$), breast PLs or breast TAGs ($n = 49$), both breast PLs and breast TAGs ($n = 34$; Fig. 2).

The majority of the 70 women (96%) were Caucasian, 50% had a college degree, and 70% reported an income of >\$60,000 per year. Fifty-one percent were premenopausal and 83% of those took oral contraceptives. Most of the postmenopausal women (91%) used some type of hormonal supplementation. Twenty-three percent took fish oil supplements until discontinuing the use at least 3 weeks before their RPFNA. The median 5-year Gail risk was 2.3%, and 90% of participants had a family history of breast cancer. Median body mass index (BMI) was 25 kg/m². Thirty-seven percent of the RPFNAs exhibited hyperplasia with atypia, and the overall median Ki-67 was 0.9%.

Characteristics of women with and without cytologic atypia

Table 1 compares demographic and risk characteristics between women with and without atypia. As expected, women with cytologic atypia had higher cytomorphology index scores ($P < 0.0001$) and rates of proliferation ($P = 0.0004$). Women with cytologic atypia were taller ($P = 0.014$) than those without atypia, but both groups had similar BMI.

Atypia and fatty acid dietary intake

The median intake of EPA+DHA estimated by FFQ was 80 mg per day. Although there was no statistically significant difference between women with and without atypia for intake of the individual fatty acids EPA, DHA, and AA, the intake ratio of EPA+DHA:AA was significantly lower among women with atypia (Table 2).

Atypia and fatty acids in blood and breast tissue

The omega-3 and omega-6 fatty acid content in blood from women with and without atypia is presented in Fig. 3. EPA and

Table 1. Participant characteristics by evidence of atypia in RPFNA

	Overall (<i>N</i> = 70)	No atypia (<i>n</i> = 44)	Atypia (<i>n</i> = 26)	<i>P</i>
	Median (interquartile range) or number (percent)			
Age, y	48 (43–56)	48 (43–56)	45 (42–55)	0.48
Height (cm)	164 (161–169)	164 (160–167)	166 (163–173)	0.014
Weight (kg)	67 (62–77)	66 (62–77)	67 (61–78)	0.60
BMI (kg/m ²)	25 (22–28)	25 (22–29)	24 (22–27)	0.57
5-y Gail risk (%)	2.3 (1.2–3.5)	2.4 (1.3–3.3)	1.8 (1.1–3.7)	0.96
Masood score	14 (13–15)	13 (12–14)	16 (15–16)	<0.0001
^a Ki-67 (%)	0.9 (0.0–3.3)	0.4 (0.0–2.4)	2.7 (0.9–5.6)	0.0004
Premenopausal	36 (51%)	21 (48%)	15 (58%)	0.47
OC use	30 (83%)	18 (86%)	12 (52%)	0.68
Postmenopausal	34 (49%)	23 (52%)	11 (42%)	0.47
HRT use	31 (91%)	20 (87%)	11 (48%)	0.54
Family history (+)	63 (90%)	38 (86%)	25 (96%)	0.25
^b Fish oil	14 (23%)	11 (28%)	3 (14%)	0.21

NOTE: *P* values were calculated by the Pearson χ^2 and the Mann-Whitney *U* tests. Significant *P*-values (<0.05) are shown in bold.

Abbreviations: Oral contraceptive (OC), hormone replacement therapy (HRT).

^aFor the 12 women whose RPFNA contained <500 cells on the slide, Ki-67 was not performed and a value of 0% was imputed.

^bFish oil use was measured by the DHQ, thus $n = 62$.

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Table 2. Estimated fat intake (grams/day) by evidence of atypia in RPFNA

	No atypia (n = 40)	Atypia (n = 22)	P
	Median (interquartile range)		
Total fat	50 (34-62)	51 (35-60)	0.90
Saturated	15 (11-19)	15 (12-20)	0.73
Monounsaturated	20 (13-25)	19 (14-23)	0.98
Trans	2.0 (1.4-2.7)	2.4 (2.0-3.3)	0.075
Omega-3 and omega-6	12 (7.6-15)	9.9 (7.2-13)	0.27
Omega-6	11 (6.7-14)	8.9 (6.5-12)	0.25
LA (18:2n6)	10 (6.6-14)	8.8 (6.5-12)	0.25
AA (20:4n6)	0.08 (0.05-0.10)	0.08 (0.06-0.13)	0.79
Dietary omega-3	1.06 (0.80-1.3)	0.84 (0.62-1.2)	0.13
ALA (18:3n3)	0.98 (0.72-1.2)	0.76 (0.61-1.0)	0.14
EPA (20:5n3)	0.02 (0.01-0.04)	0.01 (0.00-0.03)	0.24
DHA (22:6n3)	0.04 (0.02-0.09)	0.03 (0.02-0.08)	0.33
EPA + DHA	0.06 (0.03-0.13)	0.04 (0.02-0.10)	0.31
^a Total omega-3	1.2 (0.94-1.5)	0.86 (0.62-1.4)	0.15
Total EPA	0.04 (0.01-0.21)	0.02 (0.00-0.07)	0.092
Total DHA	0.08 (0.03-0.15)	0.04 (0.02-0.11)	0.13
Total EPA+DHA	0.12 (0.05-0.33)	0.06 (0.02-0.19)	0.11
Ratios			
Dietary (EPA+DHA)/AA	0.80 (0.46-1.2)	0.57 (0.26-1.1)	0.14
Total (EPA+DHA)/AA	1.4 (0.75-3.8)	0.70 (0.33-1.9)	0.032
Dietary omega-3:6	0.10 (0.090-0.12)	0.10 (0.092-0.10)	0.52
Total omega-3:6	0.11 (0.092-0.13)	0.10 (0.092-0.12)	0.30

NOTE: P values were calculated by the Mann-Whitney U test. Significant P-value (<0.05) is shown in bold.

^aTotal omega-3, EPA, and DHA includes fish oil supplementation.

DHA were both significantly lower among women with atypia in multiple blood lipid compartments ($P < 0.05$). The EPA+DHA:AA ratio was lower in the plasma TAGs of women with atypia than in those without atypia ($P = 0.050$). The total omega-3:6 ratio was lower in both plasma TAGs ($P = 0.012$) and breast TAGs ($P = 0.026$) for women with atypia compared with women without atypia.

Another long-chain omega-3 fatty acid, docosapentaenoic acid (DPA ω 3), was lower among women with atypia in plasma TAGs (0.12 vs. 0.26%, $P = 0.048$) as well as in breast TAGs (0.075 vs. 0.13%, $P = 0.036$). All comparisons by RPFNA atypia status of each measured fatty acid of RBC PLs, plasma PLs, plasma TAGs, breast PLs, and breast TAGs are available in Supplementary Tables S1 to S5.

Breast epithelial cell characteristics and fatty acids

Table 3 lists significant correlations identified between proliferation rate or cytology index (Masood score) and several omega-3 and omega-6 fatty acids. The proliferation rate of breast epithelial cells was positively correlated with levels of the omega-6 fatty acids LA and AA in breast PLs ($P < 0.04$). Proliferation rate was negatively correlated with EPA in RBC PLs and AA in breast TAGs ($P < 0.04$). Significant correlations were found between Masood score and various fatty acids in all lipid compartments, except breast PLs. Negative correlations with DPA ω 3 were found in RBC PLs, plasma PLs, and breast TAGs ($P < 0.05$). The total omega-3:6 ratio in breast TAGs was negatively correlated with Masood score ($P = 0.0013$).

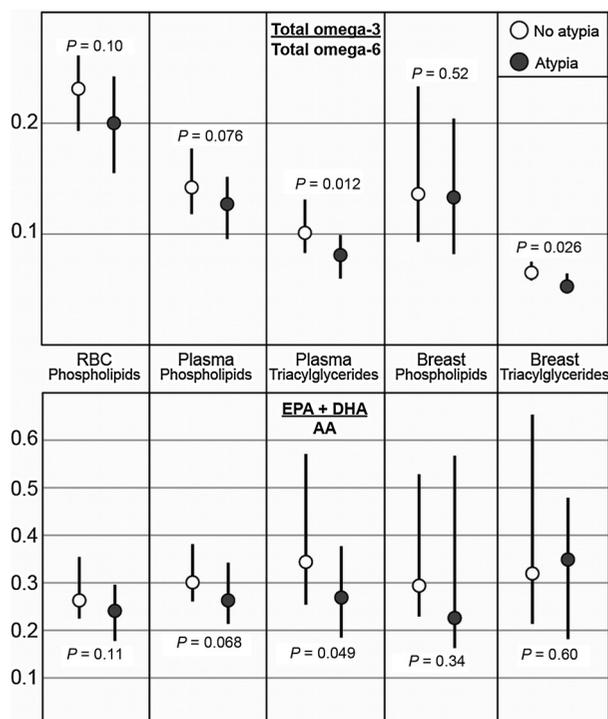
Discussion

In our sample of women at an elevated risk for breast cancer development, the consumption of total omega-3 relative to omega-6 fatty acids was lower (1:10) than that thought to be optimal for health (1:2; ref. 39). Overall, long-chain omega-3 fatty acid consumption was lower than recommended (39). There was

a trend for women with atypia to consume less omega-3 fatty acids; median estimated intake of EPA, DHA, and the (EPA+DHA/AA) ratio for women with atypia was half that of women without atypia ($P < 0.14$). Stronger associations with atypia were found for fatty acid levels in blood and breast tissue lipid compartments.

Our results are consistent with studies that find lower long-chain omega-3 fatty acids and omega-3:6 ratios in RBCs (20, 21) and adipose tissue (40, 41) from women with breast cancer compared with controls. Omega-3 fatty acids are especially prone to oxidation (42), and it is possible that the lack of correlation between omega-3 fatty acids in blood and tissue and breast cancer risk in nested, case-control studies (22-28) is due to the complications of analyzing the fatty acid composition in samples stored for periods ranging from 4 to 25 years (23, 25).

We found several significant associations between lower tissue concentrations of omega-3 fatty acids and biomarkers associated with short-term breast cancer risk. In addition to EPA and DHA, the omega-3 fatty acid DPA ω 3 was also inversely associated with cytologic atypia and epithelial cell proliferation rate. Higher concentrations of DPA ω 3 may reduce breast cancer risk, because (i) it can be converted into anti-inflammatory mediators such as EPA and DHA (43), (ii) higher concentration in RBCs is associated with lower systemic inflammation (44), and (iii) DPA ω 3 induces

**Figure 3.**

Omega-3 and omega-6 fatty acid ratios in blood and breast tissue of women with (solid symbol) and without (open symbol) RPFNA atypia. Medians and interquartile ranges are depicted as circles and error bars, respectively. P values were calculated by the Mann-Whitney U test. $N = 70$ for red blood cell (RBC) phospholipids, plasma phospholipids, and plasma triacylglycerides. $N = 43$ for breast phospholipids and $N = 40$ for breast triacylglycerides. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are long-chain omega-3 fatty acids; arachidonic acid (AA) is the principal long-chain omega-6 fatty acid.

Table 3. Significant correlations between tissue omega-3 and omega-6 fatty acids and breast epithelial proliferation rate and cytomorphology

Lipid compartment	Fatty acid or ratio	Spearman ρ	P
Correlations with proliferation rate (% Ki-67)			
RBC PL	EPA	-0.26	0.033
Breast PL	LA	0.32	0.038
	AA	0.34	0.025
Breast TAG	AA	-0.33	0.038
Correlations with cytomorphology (Masood score)			
RBC PL	20:2 ω -6	0.27	0.023
	22:2 ω -6	-0.28	0.024
	DPA ω -3	-0.24	0.041
	AA	-0.28	0.017
Plasma PL	DPA ω -3	-0.25	0.034
	EPA	-0.24	0.042
Plasma TAG	EPA	-0.24	0.045
	LA	0.32	0.045
Breast TAG	22:4 ω -6	-0.40	0.011
	DPA ω -3	-0.49	0.0012
	ω -3/ ω -6	-0.49	0.0013

NOTE: Significant correlations ($P < 0.05$ using Spearman rank correlation coefficient) of individual omega-3 and omega-6 fatty acids, and ratios, with proliferation (% Ki-67-positive nuclear staining) and cytomorphology (Masood score).

mammary gland differentiation (45). The role of DPA ω -3 in breast carcinogenesis deserves more investigation.

Our results incite two general conclusions and a hypothesis. First, women consuming their usual diet have a low intake of omega-3 relative to omega-6 fatty acids that is reflected in the omega-3:6 ratio in multiple tissues. Second, we found inverse associations between tissue levels of long-chain omega-3 fatty acids and omega-3:6 ratios and a reversible biomarker of breast cancer risk. In this study, the small sample size limited what could be analyzed (e.g., no corrections for multiple comparisons; no multivariable analysis). Even though many of the differences are of borderline statistical significance, the consistent trends across various lipid compartments (see specifically total omega-3:6 ratio in Fig. 3) strengthen the conclusion of a relationship between fatty

acid levels and detection of cytologic atypia. Supplementation of long-chain omega-3 fatty acids is a safe and effective method of increasing tissue concentration (46); therefore, we hypothesize that supplementation of long-chain omega-3 fatty acids may correct cytologic abnormalities by balancing the ratio of omega-3 and omega-6 fatty acids in tissue. We explored the effects of long-chain omega-3 fatty acid supplementation on breast tissue in parallel pilot studies of 3.4-g EPA+DHA ethyl esters per day for 6 months in premenopausal and postmenopausal women, with cytomorphology and proliferation as response endpoints.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: K.E. Harvey, D.K. Sullivan, B.F. Kimler, C.J. Fabian
Development of methodology: K.E. Harvey, D.K. Sullivan, C.J. Fabian
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Li, K.E. Harvey, C.J. Fabian
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B.H. Hidaka, S. Li, K.E. Harvey, S.E. Carlson, D.K. Sullivan, B.F. Kimler
Writing, review, and/or revision of the manuscript: B.H. Hidaka, S. Li, K.E. Harvey, S.E. Carlson, D.K. Sullivan, B.F. Kimler, C.J. Fabian
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B.H. Hidaka, K.E. Harvey, B.F. Kimler, C.M. Zalles
Study supervision: S.E. Carlson, B.F. Kimler, C.J. Fabian
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