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 docosahexaenoic 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-6:3 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 control studies generally showing 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).

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.
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 found to have a higher risk for developing breast cancer, in that it is biologically related, statistically associated, obtained easily, and, most importantly, reversible (29, 31).

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**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 re aliquoted 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 on 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 using an adapted Masood cytology index (33). Briefly, the number of epithelial cells per slide was estimated and categorized into the following ranges: <10 cells; >10 to 99 cells; 100 to 499 cells; >500 cells; or >5,000 cells. 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.

**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, we measured the fatty acid composition of different types of phospholipids (PLs) and triacylglycerols (TAGs) using a modified Folch and colleagues method (36).
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 RPFNAs 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.
The proliferation rate of proliferation rate or cytology index (Masood score) and several negatively correlated with Masood score ($w_p < 0.04$). 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 23 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 DPAo3-3 was also inversely associated with cytologic atypia and epithelial cell proliferation rate. Higher concentrations of DPAo3-3 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) DPAo3-3 induces 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 ($P < 0.14$).

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 ($1:12$; ref. $39$). Overall, long-chain omega-3 fatty acid consumption was lower than recommended ($39$). There was a difference in the consumption of omega-6 fatty acids LA and AA in breast PLs ($P = 0.05$). The total omega-3:6 ratio was lower in plasma TAGs ($P = 0.02$) and breast TAGs ($P = 0.02$) for women with atypia compared with women without atypia.

Another long-chain omega-3 fatty acid, docosapentaenoic acid (DPAo5-3), was lower among women with atypia in plasma TAGs ($0.12$ vs. $0.26w_p$; $P = 0.04$) as well as in breast TAGs ($0.075vs. 0.13w_p$, $P = 0.036$). All comparisons by RPFNCA 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. Significant correlations with DPAo3 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 ($P = 0.0013$).

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Total fat</th>
<th>Saturated</th>
<th>Monounsaturated</th>
<th>Trans</th>
<th>Omega-3 and omega-6</th>
<th>Omega-6</th>
<th>LA (18:2n6)</th>
<th>AA (20:4n6)</th>
<th>ALA (18:3n3)</th>
<th>EPA (20:5n3)</th>
<th>DHA (22:6n3)</th>
<th>DPA (22:5n3)</th>
<th>EPA + DHA</th>
<th>Total AA</th>
<th>Total DPA</th>
<th>Total EPA</th>
<th>Total DHA</th>
<th>Total EPA + DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median (interquartile range)</td>
<td>50 (34–62)</td>
<td>15 (11–19)</td>
<td>20 (15–25)</td>
<td>2.0 (1.4–2.7)</td>
<td>12 (7.6–15)</td>
<td>11 (6.7–14)</td>
<td>10 (6.6–14)</td>
<td>0.08 (0.05–0.10)</td>
<td>0.98 (0.62–1.2)</td>
<td>0.96 (0.72–1.2)</td>
<td>0.79 (0.62–1.0)</td>
<td>0.13 (0.00–0.03)</td>
<td>0.86 (0.62–1.4)</td>
<td>0.66 (0.49–1.4)</td>
<td>0.92 (0.62–1.4)</td>
<td>0.13 (0.06–0.15)</td>
<td>0.04 (0.02–0.07)</td>
<td>0.11 (0.05–0.33)</td>
</tr>
<tr>
<td>$P$</td>
<td>0.90</td>
<td>0.73</td>
<td>0.98</td>
<td>0.075</td>
<td>0.27</td>
<td>0.25</td>
<td>0.25</td>
<td>0.79</td>
<td>0.15</td>
<td>0.44</td>
<td>0.33</td>
<td>0.35</td>
<td>0.31</td>
<td>0.11</td>
<td>0.11</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

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. Significant correlations with DPAo3 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 ($P = 0.0013$).
mammary gland differentiation (45). The role of DPAA-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 correcting 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
Other (cytopathology consultant): C.M. Zalles

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

<table>
<thead>
<tr>
<th>Lipid compartment</th>
<th>Fatty acid or ratio</th>
<th>Spearman p</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC PL</td>
<td>EPA</td>
<td>–0.26</td>
<td>0.033</td>
</tr>
<tr>
<td>Breast PL</td>
<td>LA</td>
<td>0.32</td>
<td>0.038</td>
</tr>
<tr>
<td>Breast PL</td>
<td>AA</td>
<td>0.34</td>
<td>0.025</td>
</tr>
<tr>
<td>Breast TAG</td>
<td>AA</td>
<td>–0.33</td>
<td>0.038</td>
</tr>
<tr>
<td>Correlations with cytomorphology (Masood score)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RBC PL</td>
<td>20:2w-6</td>
<td>0.27</td>
<td>0.023</td>
</tr>
<tr>
<td>Breast PL</td>
<td>22:2w-6</td>
<td>–0.28</td>
<td>0.024</td>
</tr>
<tr>
<td>Breast PL</td>
<td>DPAA-3</td>
<td>–0.24</td>
<td>0.041</td>
</tr>
<tr>
<td>Breast TAG</td>
<td>AA</td>
<td>–0.28</td>
<td>0.017</td>
</tr>
<tr>
<td>Plasma PL</td>
<td>DPAA-3</td>
<td>–0.25</td>
<td>0.034</td>
</tr>
<tr>
<td>Plasma PL</td>
<td>EPA</td>
<td>–0.24</td>
<td>0.042</td>
</tr>
<tr>
<td>Plasma TAG</td>
<td>EPA</td>
<td>–0.24</td>
<td>0.045</td>
</tr>
<tr>
<td>Breast TAG</td>
<td>LA</td>
<td>0.32</td>
<td>0.045</td>
</tr>
<tr>
<td>Breast TAG</td>
<td>22:4w-6</td>
<td>–0.40</td>
<td>0.011</td>
</tr>
<tr>
<td>Breast TAG</td>
<td>DPAA-3</td>
<td>–0.49</td>
<td>0.0002</td>
</tr>
<tr>
<td>w3-6w-3</td>
<td>–0.49</td>
<td>0.00015</td>
<td></td>
</tr>
</tbody>
</table>

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).
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Cancer Prevention Research

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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