LONG-CHAIN POLYUNSATURATED FATTY ACID INTAKE AND ITS RELATIONSHIP TO RED BLOOD CELL AND PLASMA LONG-CHAIN POLYUNSATURATED FATTY ACIDS IN WOMEN AT HIGH RISK FOR BREAST CANCER

by

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Abstract

A higher ratio of omega-6 (n-6) to omega-3 (n-3) long chain polyunsaturated fatty acids (LCPUFA) in breast tissue triglyceride (TG) has been correlated with increased risk of developing breast cancer. Before dietary recommendations can be made regarding n-3 PUFAs in relation to breast cancer risk reduction, a noninvasive biomarker must be identified so that further research can be done in larger populations.

This pilot study compared LCPUFA intake to red blood cell (RBC) and plasma LCPUFAs in women at high risk for breast cancer. Women were screened (n=260) at the University of Kansas Medical Center Breast Cancer Prevention Center high-risk breast clinic. Eighty-six were eligible and of these 48 (58%) completed and returned the diet history questionnaire (DHQ).

The mean age of the subjects was 47 ± 9.9 years, and the mean body mass index (BMI) was 25 ± 4.4. The mean 5-year Gail risk was 2.7 ± 2.2%. Twenty-two (48%) of the subjects were premenopausal and 24 (52%) were postmenopausal. Fifteen (33%) were taking an n-3 PUFA supplement (fish oil or flaxseed). Mean dietary intakes were 9.94 ± 4.9 g n-6/d and 1.26 ± 0.6 g n-3/d, with an n-6:n-3 ratio of approximately 9:1. Total phospholipid (PL) n-6 in RBC and plasma was 27.74 ± 3.44% and 33.92 ± 3.72%, respectively. Total PL n-3 in RBC and plasma was 5.59 ± 1.7% and 4.06 ± 1.19%, respectively. Plasma TG docasahexaenoic acid (DHA) was highly correlated with n-3 intake ($r=0.53$, $p=<0.05$).

Women at the University of Kansas Medical Center Breast Cancer Prevention Center consume an n-6:n-3 LCPUFA ratio typical of the US population. RBC PL n-3s were significantly correlated to n-3, adding to the validity of both intake and biomarker assessment. Additional analyses will address whether breast tissue TG n-3 or n-6:n-3 ratio reflects a blood biomarker of n-3 or n-3:n-6 ratio.

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Abbreviations

AA: arachidonic acid
ALA: alpha-linolenic acid
BCPC: Breast Cancer Prevention Center
BMI: body mass index
COX-2: cyclooxygenase-2
CSFII: Continuing Survey of Food Intake by Individuals
DHA: docosahexaenoic acid
DHQ: Diet History Questionnaire
EPA: eicosapentaenoic acid
EPIC: European Prospective Investigation in to Cancer and Nutrition
EURAMIC: European Community Multicenter Study on Antioxidants, Myocardial Infarction, and Cancer
FAME: fatty acid methyl esters
GLC: gas liquid chromatography
HETE: hydroxyeicosatetaenoic acid
HRBC: High-risk breast clinic
LCPUFA: long-chain polyunsaturated fatty acids
n-3: omega-3
n-6: omega-6
NCI: National Cancer Institute
NSAID: Non-steroidal anti-inflammatory drugs
PL: phospholipid
RBC: red blood cell
RFMMB: Risk factor monitoring and methods branch
RPFNA: random periareolar fine needle aspiration
TG: triglyceride
WWEIA: What We Eat in America

Chapter 1

Introduction

As of 2006, there are over 2.5 million breast cancer survivors in the United States and according to the National Cancer Institute an estimated 192,370 new cases of breast cancer were diagnosed in 2009. A higher ratio of omega-6 (n-6) to omega-3 (n-3) long chain polyunsaturated fatty acids (LCPUFA) in breast tissue triglycerides (TG) has been correlated with increased risk of developing breast cancer (1, 2). Dietary interventions with n-3 PUFAs could reduce this ratio and potentially decrease the risk for breast cancer. Red blood cell (RBC) phospholipid (PL) and plasma PL and TG are commonly used biomarkers for intake of LCPUFA. In the European Prospective Investigation into Cancer and Nutrition (EPIC) project, usual fish intake correlated with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in serum and low density lipoprotein PL and cholesteryl esters fractions demonstrating that these lipid fractions can be used as biomarkers for n-3 LCPUFA intake (3). Studies are lacking that quantify the usual LCPUFA intake in women at high risk for breast cancer or that compare RBC and plasma PL LCPUFA with fatty acid intake.

Justification for Further Investigation

The Evidence Report on the effects of n-3 PUFAs on cancer, published by the U.S. Department of Health and Human Services in 2005, concluded that a beneficial effect of n-3 LCPUFAs on cancer risk in general does not exist but suggested that further research is indicated to determine if n-3 PUFAs are beneficial for specific types of cancer, such as breast cancer (4). Before dietary recommendations can be made regarding n-3 PUFAs in relation to breast cancer risk reduction, a biomarker must be identified so that further research can be done in larger populations. We conducted a pilot study to compare LCPUFA intake to RBCs and plasma LCPUFAs in women at high risk for breast cancer. The advantages of studying these relationships in women who are at risk for breast cancer are: a) blood samples are available as part of routine screening for cell biomarkers and b) women who know they are at risk for breast cancer might be expected to have a more variable intake of n-3 LCPUFA than the general population of women.

Statement of Purpose

The present thesis explores the relationship between dietary fatty acid intake and fatty acids in plasma and RBC lipids. The intent was to determine if, at usual intakes of n-3 LCPUFA, the RBC and plasma lipid LCPUFA are related to LCPUFA intake.

Research Questions

Primary Questions:

- What is the usual LCPUFA intake from food and supplements in women at high risk for breast cancer?
- How does LCPUFA intake relate to the fatty acid profile in triglycerides and phospholipids in red blood cells and plasma?

Secondary Questions:

- What is the ratio of n-6/n-3 LCPUFA intake in women at high risk for breast cancer?
- Is n-6/n-3 PUFA intake related to socioeconomic status in women at high risk for breast cancer?

Chapter 2

Review of Literature

Biochemical Studies

Linoleic (18:2n-6) and alpha-linolenic (18:3n-3) acids are essential in the human diet because mammals lack the enzymes found in plants that can insert double bonds 3 (n-3 fatty acids) and 6 (n-6 fatty acids) carbons from the methyl end of the fatty acid. Both linoleic acid and alpha-linolenic acids are found in vegetable oils but linoleic acid is more abundant in the US diet, because of the composition of the vegetable oils usually consumed. Some foods that contain a relatively low ratio of linoleic to alpha-linolenic acid include walnuts, flaxseed and rapeseed (canola) oil. A low ratio of dietary n-6:n-3 PUFAs may protect against cancer (1, 5, 6).

The ratio of n-6 to n-3 PUFA in the Western diet is significantly higher than that of our ancestors who consumed a mostly plant-based diet. Our ancestors consumed an n-6:n-3 PUFA ratio of approximately 1, and the current ratio is approximately 16.7:1 (7), with an average North American per capita intake of docasahexaenoic acid (C22:6 n-3, DHA) + eisocapentaenoic acid (C20:5 n-3, EPA) of 0.1-0.2 grams per day and alpha-linolenic acid (C18:3 n-3, ALA) 1.4 grams per day (8). A high ratio of n-6:n-3 PUFA may contribute to chronic diseases such as inflammation, cardiovascular disease and cancer (4, 5, 9). Studies have suggested that n-3 PUFAs may offer protection against cancer,

while high intake of n-6 PUFA or a high ratio of n-6 to n-3 PUFA may promote cancer (5, 10-12).

Terry, et al. proposed that n-3 PUFA decrease cancer risk by inhibiting conversion of the n-6 LCPUFA, arachidonic acid (AA, 20:4n-6) into eicosanoids via the cyclooxygenase-2 (COX-2) enzyme. Eicosanoids include prostaglandins, hydroxyeicosatetraenoic acids (HETE) and leukotrienes, and eicosanoids may be involved in the carcinogenic process via several mechanisms (5, 10). Eicosanoids are known to modify estrogen metabolism, increase oncogene expression, increase synthesis of cytotoxic cytokines, and modify tumor cell membrane compositions. Tumor cells produce large quantities of prostaglandin E_2 (PGE_2), and PGE_2 production is inhibited by n-3 LCPUFAs (10). It is estimated that the rate of conversion of alpha-linolenic acid (ALA, 18:3n-3) to EPA and DHA is low at 1-10% (13-15). Based on the inefficiency of this conversion, it may be more beneficial to consume dietary EPA and DHA rather than ALA.

N-3 PUFAs have been shown to decrease the risk of breast cancer via several possible mechanisms, including inhibition of angiogenesis (16-22); inhibition of tumorigenesis (23-27); mediation of transcription, mRNA stability and cellular differentiation (9, 28-31); antiproliferative effects (32); and promotion of apoptosis (33-35).

Other studies have looked specifically at the role of DHA in breast cancer. DHA has demonstrated a role in membrane stability, fluidity and

permeability (36). DHA has also been shown to improve the response to cytotoxic agents in breast tumor cells (37) and may also induce cell apoptosis by influencing PPAR-gamma and p53 mechanisms (33, 35).

Animal studies

Rodent studies have also demonstrated a potentially protective effect of n-3 LCPUFAs against breast cancer tumorigenesis (38-43). The mechanism(s) of this association are not entirely understood. The protection may be related to the anti-inflammatory properties of n-3 LCPUFAs (44). Dietary flaxseed was shown to decrease tumor growth and metastasis in nude mice, while also down regulating insulin-like growth factor and epidermal growth factor receptor expression (40). Similar results regarding decreased mammary tumor growth and metastasis were achieved in a study on female BALB/c mice fed high n-3 or high n-6 diets (41). The results of another study showed that dietary DHA intake not only decreased the incidence of mammary tumorgenesis but also increased BRCA1 tumor suppressor protein by 60% (45). Mice fed canola oil (high n-3) compared to corn oil (high n-6) had slower tumor growth (43). Several studies have suggested that n-6 LCPUFAs may promote mammary tumor development via the prostaglandin pathway (41, 46, 47). N-3 PUFAs have also been shown to improve effectiveness of chemotherapy in estrogen-dependent breast cancer and inhibit tumor development, while n-6 PUFAs may promote tumor development in breast cancer in rats and mice (6, 10, 46, 48). One study found that high n-3 fish oil and oleic acid inhibited lung

tumorigenesis and metastasis, but linoleic (18:2 n-6) and linolenic (18:3 n-3) did not (49). This suggests that specific n-3 LCPUFAs may inhibit carcinogenesis, while other n-3 and n-6 LCPUFAs may promote it. The ratio of n-3 to n-6 LCPUFA also appears to be important(1, 5, 38).

Epidemiological studies

The uncertainty in cohort studies surrounding the relationship between dietary fat intake and breast cancer risk may be due in part to measurement error (50, 51). Additionally, specific fatty acids are often not specified, and cooking methods (such as frying) which may alter the fat content are often not included in the analyses.

The PUFA intakes in 56,000 French women were determined using validated French diet history questionnaires (52, 53). Breast cancer risk was inversely associated with n-3 LCPUFA (EPA, DPA and DHA) intake in women with the highest intakes of n-6 (LA and AA) LCPUFA. Risk also varied according to food source of alpha-linolenic acid (ALA). For ALA sources of food, risk was inversely correlated with the intake of n-3 PUFA from fruits, vegetables and vegetable oils but was positively correlated with the intake of n-3 PUFA from nut mixes (also highest intake of vitamin E). The authors concluded that it is important to consider the food source of LCPUFA due to potential interactions between ALA and other food components such as antioxidants.

LCPUFA samples were analyzed from the buttock adipose tissue in breast cancer patients during the European Community Multicenter Study on Antioxidants, Myocardial Infarction, and Cancer (EURAMIC) breast cancer study (1991-1992) (53). Neither n-3 nor n-6 PUFA alone was correlated with breast cancer risk; however, the ratio of n-6:n-3 PUFAs was inversely correlated with breast cancer risk in four out of the five centers. The results suggest that the ratio is more important than the individual family of fatty acids. However, not all studies show an inverse relationship with n-3 LCPUFA intake. A study performed in Denmark, where fish consumed tends to be fatty fish (e.g. salmon, tuna, mackerel, sardines, and trout) concluded that higher total fish intake was positively associated with ER+ breast cancer (54, 55). Higher intake of most sea fish is equivalent to higher intake of other things, which could be a confounder.

Most, observational studies suggest that women with relatively lower intakes of n-3 PUFAs have higher risk of breast cancer (52, 53, 56-61). However, the observational studies have many limitations such as lack of characterization of the source and amount of n-3 LCPUFA and an absence of information about individual ratios of n-6:n-3 PUFA, antioxidant status, and single nucleotide polymorphisms (SNPs) of fatty acid desaturase enzymes that appear to influence LCPUFA (5). For example, most observational studies assessed n-3 LCPUFA intake by asking about total fish consumption rather than species consumed and preparation methods even though it is well known that

EPA and DHA levels vary among fish species and some methods of preparation add considerable amounts of trans fatty acids that are associated with lower LCPUFA status (62, 63).

In the EPIC study, total fish intake and types of fish consumed was quite variable between countries as well as among individuals from day to day (55). Such measurement error may account for conflicting epidemiological evidence regarding fat intake and breast cancer risk (64). In the American Association of Retired Persons (AARP) Diet and Health Study dietary fat was correlated with breast cancer risk (65); however, in the Nurses' Health Study it was not (66). Pooled analyses of cohort studies did not relate fat intake to breast cancer risk (10, 67). Other types of fat, such as saturated fat, may be associated with breast cancer (68, 69), again demonstrating that different fatty acids have different roles in carcinogenesis (62, 70, 71).

Questions have been raised regarding the timing of fat intake during the lifespan. Several studies found no association between midlife fat intake and postmenopausal breast cancer (72, 73). However, a nested case-control study within the Nurses' Health Study cohort found that women with higher intakes of eggs, vegetable fat and fiber during adolescence had a lower risk of breast cancer, while butter consumption increased risk (74). Another study found that women with a higher intake of animal fat during premenopausal years had a higher risk of breast cancer (75). It is also noteworthy that breast cancer incidence is much lower in China and Japan than the United States (76), and

dietary factors have been possibly implicated. Groups who have emigrated to the United States from China and Japan have greater breast cancer incidence rates than in their respective countries (77). In a study among Japanese immigrants in Los Angeles County, breast cancer rates were lower in immigrants at a younger age, yet still higher than those in Japan, suggesting that age of dietary change may influence breast cancer risk.

Case-control studies

In general, case-control studies have found an association between saturated and trans-fatty acid intake and n-6 PUFA intake and increased risk of breast cancer (75, 78-80) and between intakes of n-3 PUFA, fish and olive oil and decreased risk of breast cancer (81-86).

In a case-control study assessing n-3 and n-6 PUFAs in breast adipose tissue, samples were taken from the breasts of 241 women with invasive, nonmetastatic breast carcinoma and 88 women with benign breast disease (1). Results showed an inverse relationship between n-3 PUFA levels in the breast adipose tissue and breast cancer risk. In another case-control study, fatty acid content of breast adipose tissue was used as a tissue biomarker n-3 and n-6 LCPUFA status (6). Levels of n-6 PUFAs were significantly higher in the cases than controls, and results once again suggest that a lower ratio of n-6:n-3 PUFAs may decrease the risk of breast cancer.

In a cohort from the Diet, Cancer and Health study, higher total intakes of fish (including fried, boiled, and processed) were correlated with a higher

incidence of ER+ breast cancer (54). Questions have been raised about trans-fatty acids added during cooking, but this variable was not measured in the Danish cohort. In the Multi-Ethnic Study of Atherosclerosis, plasma PL EPA and DHA were associated with intake of non-fried fish when seafood groups were adjusted for; however, when seafood groups were not adjusted for, there was a 67% decrease in the correlation (63).

Human intervention studies

There are currently no published results from human intervention trials regarding intervention with n-3 LCPUFA in breast cancer prevention and treatment. As of April 2010, there are four ongoing clinical trials in this area of research. Much work is yet to be done regarding the safety and efficacy of n-3 LCPUFA for breast cancer risk reduction(4). NCT00114296 is a pilot study looking at the effects of n-3 PUFA supplementation on mammographic breast density in 80 women at high risk for developing breast cancer. Secondary objectives are to determine effects of n-3 PUFA supplementation on atypia and breast cell proliferation, circulating hormone and growth factor levels, expression of estrogen-related proteins, plasma lipid peroxidation levels, and to correlate the modifying effect of lipid peroxidation-related genes with mammographic breast density. The control group of patients receives a placebo for 12 months, and the experimental group of patients receives an unspecified amount of n-3 LCPUFA orally 3 times per day.

NCT00723398 is a randomized, open-label prevention trial investigating the effect of n-3 LCPUFA supplementation alone and in conjunction with antiestrogen therapy in 372 postmenopausal women. There are 5 arms, with the first serving as the control. In the second arm, patients receive 60 milligrams per day of the antiestrogen drug, Raloxifene for two years. In the third arm, patients receive 30 milligrams per day of Raloxifene for two years. In the fourth arm, patients receive 4 grams per day of Lovaza (1860 mg EPA, 1500 mg DHA) for two years. And in the fifth arm, patients receive 30 milligrams per day of Raloxifene plus 4 grams per day of Lovaza. The primary outcomes of the study are the individual and combined effects of the drug (Raloxifene) and n-3 LCPUFA (Lovaza). The primary outcome measure is breast density. Secondary outcomes include markers of oxidative stress, estrogen metabolism, inflammation, and IGF-1 signaling.

NCT00627276 is a phase II randomized, double-blind, placebo control trial looking at the effect of n-3 LCPUFA supplementation in 40 women newly diagnosed with ductal carcinoma in situ and/or atypical ductal hyperplasia. Blood, urine, nipple aspirate fluid, and tissue are analyzed at baseline and completion for genetic markers for breast cancer. Blood samples are analyzed for breast cancer genetic markers by microarray analysis and RBC fatty acids.

NCT00930527 is a phase II trial in which the investigators are studying the safety and efficacy of n-3 supplementation on aromatase inhibitor (AI) induced joint pain in 10 postmenopausal women who had ER+ breast cancer.

The subjects are receiving 4 g of an n-3 supplement per day for 3 weeks. The primary outcome is change in serum free and total estradiol at 3 weeks, and the secondary outcome is change in frequency of analgesics consumed at 12 weeks.

Chapter 3

Methods

Design of Inquiry

Women at high risk for breast cancer were recruited from the University of Kansas Breast Cancer Prevention Center (BCPC) High Risk Breast Clinic (HRBC). Diet History Questionnaires (DHQ) were be administered to obtain LCPUFA intake, and blood samples were obtained for fatty acid analysis.

Inclusion and Exclusion Criteria

The subjects were 48 women seen in the HRBC who had been identified as at high-risk for breast cancer and according to the following criteria:

- First-degree relative (i.e. mother, sister or daughter) who had a breast cancer diagnosis before the age of 60
- Prior diagnosis of atypical hyperplasia carcinoma in situ determined by a breast biopsy
- Previous node-negative breast cancer
- Multiple second-degree relatives who had a breast cancer diagnosis with at least one diagnosis under the age of 50
- Multiple breast biopsies
- Breast density greater than 50%

The following were exclusion criteria:

- Breast implants
- The use of a blood-thinning medication

- Coumadin, Heparin, Plavix, Lovenox

- Taking a chemotherapy or chemopreventative
  - Tamoxifen (Nolvadex), Raloxifene (Evista), Letrozole (Femara) or Anastrozole (Arimidex) for a year or more
- Currently enrolled in another trial

Diet History Questionnaires

The DHQ was obtained from the National Cancer Institute website and distributed to the patients as a hard copy or portable document format (PDF) (see appendix B). The patients were asked to fill out the survey and return it to the investigators during clinic or in a pre-paid envelope. The DHQ responses were entered into the DHQ database and analyzed for nutrient composition. No interviewer was required. The DHQ consists of 124 food items and includes information regarding portion size and dietary supplement usage. It takes approximately one hour to complete the questionnaire.

The DHQ was developed by the Risk Factor Monitoring and Methods Branch (RFMMB) of the National Cancer Institute (NCI) (http://riskfactor.cancer.gov/DHQ/). It measures usual intakes based on responses concerning foods consumed during the course of the past 12 months. Developers utilized cognitive research to develop a questionnaire that is easy to use. It has been validated to accurately reflect dietary intake by three separate studies (87-89). These studies found response rates from 70-85%, which is not statistically different from shorter questionnaires. The DHQ utilizes an

accompanying database for analysis of nutrients and food groups. The nutrient information is based on the 1994-96 USDA Continuing Survey of Food Intake by Individuals (CSFII), which was used to establish which foods to include in the survey and portion sizes. The DHQ was analyzed using Diet*Calc Software, developed by the NCI.

Blood Samples

Whole blood was drawn into 4 mL tubes containing ethylenediaminetetraacetic acid (EDTA) and was refrigerated for several hours prior to transport to the laboratory where it was analyzed. The tubes were labeled with the patient's HRBC identification number and the study for which it was used. All samples were logged in a book at the HRBC and analysis laboratory.

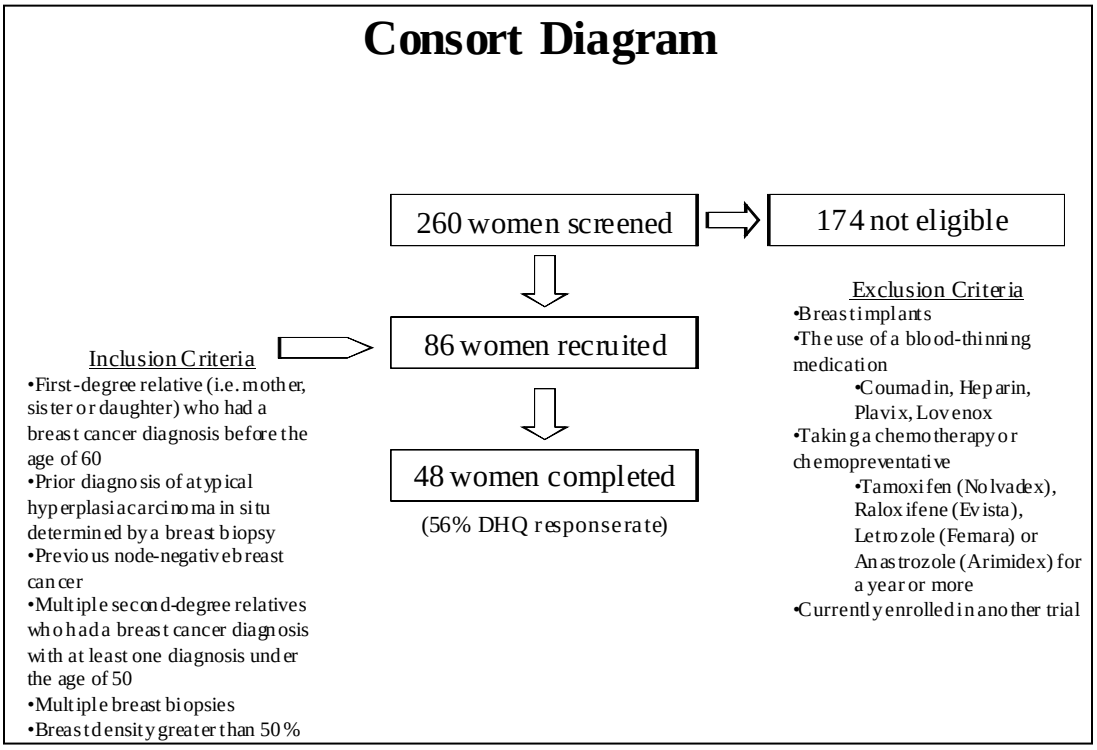
A modification of the method developed by Folch et al. was used to extract total lipids from breast tissue and blood with chloroform: methanol 2:1 (v:v) as part of a parallel study (90). The extract was separated into two phases and washed with KCl. The upper lipid layer was discarded. The lower chloroform layer was evaporated to dryness with nitrogen and redissolved in 100 µL of dichloromethane. Adsorption chromatography (silica gel G. Analtech) was used to separate the TG and PL in 80:20:1 hexane: diethyl ether: acetic acid. The PL band was scraped from the plate and collected in a screw-cap tube containing 1mL BF_3. The TG band was scraped and placed in a separate tube containing 0.25 mL BF_3, 0.2 mL Benzene and 0.55 mL methanol.

The lipid class bands were heated at 100°C for 10 and 30 minutes, respectively. The tubes were cooled, and fatty acid methyl esters (FAMEs) were extracted into pentane. Pentane was evaporated, and the sample redissolved in dichloromethane. FAMEs were analyzed with one standard run (Supelco 37) in sets of 12 using the Varian 3900 GC and attached autoanalyzer, and composition gas liquid chromatography (GLC) was used to determine composition. FAME was identified by comparing samples to relative retention times of a mixture of weighed standards, and the relative amount (expressed as a weight percentage of total fatty acids) was determined by dividing the area under the curve by the total area for all fatty acids. Each GC run was evaluated and repeated if the sample appeared to be overloaded or have some other analytical problem.

Subjects

A total of 260 women were screened through the University of Kansas Medical Center Breast Cancer Prevention Center high-risk breast clinic. From those screened, 86 were eligible (174 excluded), and 48 completed and returned the DHQ (58% response rate).

Figure 1. Consort Diagram



Statistical Analyses

The DHQ's were analyzed using DietCalc software obtained from the National Cancer Institute (www.riskfactor.cancer.gov/DHQ). Results were downloaded into a Microsoft Excel spreadsheet and merged with all other data. Means and standard deviations were calculated using Microsoft Excel, and correlations were performed using SPSS 17.0 software. Significance was defined as $p = \leq 0.05$.

Chapter 4

Results

Demographics

The mean age of the subjects was 48 ± 10 years and the mean height and weight were 65 ± 2.4 cm and 67.7 ± 16.0 kg, respectively. BMI was 25 ± 4 kg/m^2. Thirty-four percent of subjects were overweight (BMI 25-29.9 kg/m^2) and 15% were obese (BMI≥30 kg/m^2). The mean 5-year Gail risk was 2.7 ± 2.2%(91). The Gail model uses an individual's prior biopsies, menstruation onset age, first live birth age and first-degree relative breast cancer history to estimate 5-year breast cancer risk. The Gail model has been validated for white women.

Twenty-three (48%) of the subjects were premenopausal and 25 (52%) were postmenopausal. Nineteen (40%) were currently using hormonal therapy, 26 (54%) had used hormonal therapy in the past, and 3 (6%) had never used hormonal therapy. Forty-four (92%) of subjects had post-high school education. Thirty-one (65%) subjects had an annual household income >$60,000. Forty-two (88%) of subjects had a family history of breast cancer. Sixteen (33%) were taking a n-3 PUFA supplement (fish oil or flaxseed). See table 1 for socio-demographic characteristics of subjects.

Table 1. Socio-demographic characteristics of subjects.

Characteristics	Subjects (n=48)
Age (years)	48 ± 10*
Height (cm)	65.0 ± 2.4*
Weight (kg)	67.7 ± 16.0*
BMI (kg/m^2)	25 ± 4*
5-year Gail risk (%)	2.7 ± 2.2*
Menopausal status	
Premenopausal	n=23 (48%)
Postmenopausal	n=25 (52%)
Hormonal therapy	
Current	n=19 (40%)
Past	n=26 (54%)
Never	n=3 (6%)
College education	
High school/GED	n=2 (4%)
Vocational/technical school	n=0 (0%)
Associate's degree/some college	n=7 (15%)
College	n=21 (44%)
Graduate/professional school	n=16 (33%)
Other	n=2 (4%)
Household annual income level	
<$20,000	n=0 (0%)
$20-40,000	n=7 (15%)
$40-60,000	n=9 (19%)
>$60,000	n=31 (65%)
Declined to answer	n=1 (2%)
Family history of breast cancer	n=42 (88%)
Taking n-3 fatty acid supplement	n=16 (33%)

*Mean ± standard deviation

Energy and Nutrient Intakes

Energy and nutrient intake was reported as 1415 ± 477 calories/day. Mean carbohydrate intake was 171.11 ± 65.2 g/d, and mean fiber intake was 15.2 ± 7.2 g/d. Mean protein and fat intakes were 59.8 ± 21.9 g/d and 51.2 ±

18.6 g/d, respectively. Distribution of macronutrients as a percentage of calories was as follows: carbohydrate 49 ± 10%, protein 17 ± 3%, and fat 33 ± 7%. Mean saturated and trans fat intakes were 15.46 ± 5.63 g/d and 2.63 ± 1.13 g/d, respectively. Mean MUFA and PUFA intakes were 19.77 ± 7.6 g/d and 12.04 ±5.49 g/d. Mean LA and ARA intakes were 10.78 ±4.97 g/d and 0.08 ± 0.04 g/d, with a total estimated n-6 intake of 10.86 ± 5.0 g/d. Mean ALA, EPA, and DHA intakes were 0.98 ± 0.45 g/d, 0.03 ± 0.03 g/d, and 0.06 ± 0.06 g/d, with a total estimated dietary n-3 intake of 1.07 ± 0.51 g/d. Mean estimated total n-3 intake, including supplements, was 1.27 ± 0.6 g/d. Table 2 depicts the distribution of macronutrient intake.

Table 2 . Distribution of macronutrients as a percentage of total calories.

Macronutrients	Study Subjects	WWEIA 2005-2006 Females Ages 40-49(92)	Acceptable Macronutrient Distribution Ranges (93)
Carbohydrate	49 ± 10*%	48%	45-65%
Protein	17 ± 3*%	17%	10-35%
Fat	33 ± 7*%	34%	20-35%

*Mean ± standard deviation

Table 3 . Mean daily intakes of selected nutrients.

Nutrients	Mean daily intakes	Range
Energy (kcal)	1415±477	687-2887
Carbohydrate (g)	171.11±65.26	58.20-292.38
Fiber (g)	15.2±7.23	3.76-35.37
Protein (g)	59.83±21.91	25.97-108.96
Fat (g)	51.17±18.64	25.52-95.22
Saturated Fat (g)	15.46±5.63	5.60-30.12
Trans Fat (g)	2.63±1.13	0.66-5.85
MUFA (g)	19.77±7.60	7.58-35.06
PUFA (g)	12.04±5.49	4.73-28.41
LA (g)	10.78±4.97	3.93-25.20
AA (g)	0.08±0.04	0.02-0.18
Estimated total n-6 (g)	10.86±5.00	3.95-25.34
ALA (g)	0.98±0.45	0.38-2.53
EPA (g)	0.03±0.03	0-0.15
DHA (g)	0.06±0.06	0.01-0.34
Dietary n-3 (g)	1.07±0.51	0.43-2.76
Estimated total n-3 (diet + supplements) (g)	1.27±0.60	0.43-3.14

Table 4. Mean n-3 and n-6 levels in breast, plasma, RBCs and diet.

	Plasma		RBC	Intake (g/d)*
Fatty Acid	**PL***	**TG***	**PL***	
EPA	0.72 (0.41)	0.23 (0.23)	0.63 (0.52)	0.03 (0.03)
DHA	3.23 (1.00)	0.43 (0.40)	4.12 (1.14)	0.06 (0.06)
ALA	0.39 (0.52)	0.19 (0.22)	2.54 (0.66)	0.98 (0.45)
Total n-3	4.04 (1.16)	2.23 (1.50)	5.48 (1.52)	1.27 (0.60)
AA	10.36 (1.93)	1.55 (0.50)	13.29 (2.07)	0.08 (0.04)
LA	20.20 (3.34)	20.97 (3.95)	10.13 (1.34)	10.78 (4.97)
Total n-6	34.06 (3.36)	23.64 (3.85)	27.82 (3.43)	10.86 (5.00)
n-6:n-3 Ratio	9.02 (2.59)	12.89 (5.44)	5.42 (1.48)	9.14 (3.35)
Trans Fatty Acids	1.53 (0.46)	1.22 (0.59)	1.99 (0.65)	2.63 (1.13)

*Mean % (SD)

Correlations

Approximately one third of the subjects were taking n-3 supplements (fish oil or flaxseed). Fish oil usage was positively correlated with total estimated n-3 intake (r=0.481, p=0.001) and DHA in RBC PL (r=0.356, p=0.026), plasma PL (r=0.356, p=0.026), and plasma TG (r=0.387, p=0.015). Similarly, fish oil usage was positively correlated with EPA in RBC PL (r=0.514, p=0.001) and plasma PL (r=0.433, p=0.006). Fish oil was negatively correlated with AA in RBC PL (r=-0.510, p=0.001) as well as the n-6:n-3 ratio in RBC PL (r=-0.396, p=0.003) and plasma TG (r=-0.426, p=0.007).

Estimated total n-3 PUFA intake (including both food and supplements) was positively correlated with DHA in RBC PL and plasma TG (see table 5). The best predictor of DHA levels in RBC and plasma was DHA intake (r=0.663, p=<0.001), even though DHA intake was only 0.6 g/d. Plasma TG EPA was also strongly correlated with n-3 intake, while RBC PL AA was negatively correlated with n-3 intake. Other negative correlations of n-3 intake were with the n-6:n-3 ratio in RBC PL, plasma PL, and plasma TG. Dietary n-6 PUFA intake was positively correlated with DHA and EPA in plasma TG (r=0.376, p=0.018; r=0.508, p=0.001, respectively). The same relationship was seen for n-6 LA but not n-6 AA.

Table 5. Correlates with n-3 and n-6 PUFA intake.

	Correlate	**Pearson Correlation** ***(p=<0.05)***
n-3 PUFA Intake	RBC PL DHA	0.348
	Plasma TG DHA	0.532
	Plasma TG EPA	0.511
	RBC PL AA	-0.450
	RBC PL n-6:n-3	-0.453
	Plasma PL n-6:n-3	-0.341
	Plasma TG n-6:n-3	-0.370
n-6 PUFA Intake	Plasma TG DHA	0.376
	Plasma TG EPA	0.508

Chapter 5

Discussion

This observational pilot study provided key analysis of dietary intakes and blood biomarkers of LCPUFA status in women who are at high risk for breast cancer who were consuming very low amounts of n-3 PUFAs. RBC and plasma LCPUFAs were clearly related to n-3 intake, which confirms previous findings (94). These results validate the use of both the DHQ and RBC analysis methods in this population, and will be used in a phase II intervention trial using a high-dose n-3 PUFA supplement as a means for breast cancer risk reduction in a high risk population of women.

Mean Gail risk of this sample was 2.7 ± 2.2%, which is greater than twice the risk than the average 48 year old woman, who has a 5-year Gail risk of 1.2% (91) . Women who know they are at high risk for breast cancer may choose to modify lifestyle factors, including diet, to decrease risk.

Being overweight is a known risk factor for breast cancer(95). This population of women at high risk for breast cancer had a lower incidence of obesity and higher incidence of overweight than national estimates, with 34% of subjects overweight (BMI 25-29.9) and 15% obese (BMI≥30), compared to CDC statistics from 1999-2000 which classify US adults as overweight and obese at rates of 25% and 23%, respectively (92). BMI was negatively correlated with DHA and seafood intakes, while energy intake was positively

correlated with intakes of n-3 PUFAs, EPA and seafood. It is interesting to note that individuals consuming more energy in conjunction with more n-3 PUFAs had a lower body weight.

Postmenopausal status was correlated with intakes of n-3 and n-6 PUFAs, LA and trans fatty acids. Carbohydrate intake was associated with pro-inflammatory nutrients, including n-6 PUFA, LA, ARA, total PUFA, and trans fatty acids. This may be of concern since inflammation may be associated with chronic diseases such as cancer and heart disease(96, 97), which are concerns in postmenopausal women(98).

Mean energy intake was estimated at 1415 calories per day, which is approximately 24% lower than the NHANES What We Eat in America (WWEIA) 2005-2006 reported food energy for females 40-49 (92). Similarly, mean intakes of macronutrients – carbohydrate, protein and fat – were 23, 21 and 29% lower than WWEIA 2005-2006. However, macronutrient distribution range was similar to WWEIA (see table 2). All macronutrients were within the Accepted Macronutrient Distribution Ranges (see table 2) (93). DHA intake was low in this population, 33% lower than the WWEIA data from 2005-2006 for females 40-49 (92). Total n-3 intake was less related to plasma or RBC total n-3 PUFA than plasma and RBC DHA (data not shown). The strongest biomarkers of n-3 intake were plasma TG DHA (r=0.532) and RBC PL n-6:n-3 (r=0.453). Increasing DHA intake using high-dose supplementation could significantly decrease this ratio, and potentially decrease the risk of breast

cancer. It is still unknown what level of intake is required to influence the n-6:n-3 ratio in breast tissue, which is a known risk factor. It is important to know if and when red blood cell (RBC) phospholipid (PL) and plasma PL and TAG LCPUFA can be used as an indicator of breast tissue PL and TAG LCPUFA.

One limitation of this study is that the subjects were instructed to discontinue use of n-3 PUFA supplements for 3 weeks prior to their clinic visit. It is unknown if and when the subjects stopped taking the supplements, but we were still able to find an association in RBC and plasma with intake. Further, the response rate for the DHQ was only 58%, which is lower than NCI estimates of 70-80% for this survey(88). Another limitation is underreporting associated with the DHQ. Reported energy intakes ranged from 687-2887 kilocalories per day. Women tend to underreport more than men, and underreporting is more common in obese individuals(99). In a review evaluating the validity of reported energy intake, diet history had the least amount of results below the acceptable cut-off point (25% of results), while diet records and diet recalls fell below the cut-off point 64% and 88% of the time, respectively(99). Therefore, the DHQ appears to be more reliable than diet records or recalls when assessing energy intake.

Chapter 6

Summary

The risk of breast cancer is higher in women who have a higher ratio of n-6:n-3 LCPUFAs in breast tissue. Before dietary recommendations can be made regarding n-3 PUFAs in relation to breast cancer risk reduction, a less invasive biomarker than breast tissue must be identified so that further research can be done in larger populations.

This observational pilot study compared LCPUFA intake to RBC and plasma LCPUFAs in women at high risk for breast cancer. Women were recruited from a high risk breast clinic and asked to fill out DHQ's and provide blood samples for fatty acid analyses. The mean age of the subjects was 47 ± 9.9 years, and the mean BMI was 25 ± 4.4. The mean 5-year Gail risk was 2.7 ± 2.2%. Twenty-two (48%) of the subjects were premenopausal and 24 (52%) were postmenopausal. Fifteen (33%) were taking an n-3 PUFA supplement (fish oil or flaxseed). Mean intakes were 9.94 ± 4.9 g n-6/d and 1.26 ± 0.6 g n-3/d, with an n-6:n-3 ratio of approximately 9:1. Total PL n-6 in RBC and plasma was 27.74 ± 3.44% and 33.92 ± 3.72%, respectively. Total PL n-3 in RBC and plasma was 5.59 ± 1.7% and 4.06 ± 1.19%, respectively. RBC PL n-3 was highly correlated with n-3 intake (r=0.42).

Women at the University of Kansas Medical Center Breast Cancer Prevention Center consume an n-6:n-3 LCPUFA ratio typical of the US

population. Even though DHA intake was only 0.6 g/d, it was still highly correlated with plasma TG DHA (r=0.663, p=<.001). At low intakes of n-3 PUFAs, RBC PL n-3s were significantly correlated to dietary n-3, adding to the validity of both intake and biomarker. This study provided key analyses so an n-3 PUFA intervention trial can be performed in women at high risk for breast cancer.

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