

PHOSPHOLIPID AND TRIACYLGLYCEROL FATTY ACIDS IN
BLOOD and BREAST TISSUE OBTAINED FROM WOMEN AT
INCREASED RISK OF BREAST CANCER

BY

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Abstract

The purpose of this study was to determine the fatty acids composition in breast tissue of women with increased risk of developing breast cancer, and if the fatty acid composition in both phospholipid (PL) and triacylglycerol (TAG) in blood, both plasma and red blood cells (RBC), correlated to the fatty acid composition in breast tissue in the same population. Fifty subjects from clinical patients in a study to develop an assessment of breast cancer risk based on epidemiologic and biologic risk variables enrolled in current study. Blood and tissue samples were taken and dietary history questionnaire, which including usual supplement intake were distributed when the subjects were enrolled. The blood and breast tissue samples were analyzed by gas-liquid chromatography and calculated by the weighed percentage of total areas compared with Supelco 37 mixture as a standard. The means of total n-3 polyunsaturated fatty acids (PUFA) of PL in plasma and RBCs for the subjects were $4.00 \pm 1.09\%$ and $5.47 \pm 1.50\%$, respectively, and in plasma TAG the mean was $2.21 \pm 1.34\%$. The means of total n-3 PUFA of PL and TAG in breast tissue were $0.36 \pm 0.27\%$ and $1.28 \pm 0.44\%$, respectively. The means of total n-6 PUFA of PL in plasma and RBCs for the subjects were $34.04 \pm 3.22\%$ and $28.10 \pm 3.45\%$, respectively, and in plasma TAG was $28.35 \pm 3.97\%$. The means of total n-6 PUFA of PL and TAG in breast tissue were $2.75 \pm 0.99\%$

and $17.53 \pm 2.78\%$. A statistically significant correlation was found in the ratio of total and long chain n-3 (n-3 LCPUFA) to total n-6 PUFA in PL in blood and breast tissue, $r=0.801$ and 0.653 for total n-3 PUFA in RBC and plasma ($p<0.01$), and $r=0.631$ and 0.524 for n-3 LCPUFA in RBC and plasma ($p<0.01$), but no relationship between breast and blood fatty acids in TAG in individual or combined n-3 and n-6 PUFA was found between breast tissue and blood. The ratio of long chain n-3 PUFA to total n-6 PUFA in phospholipid in blood represents the same ratio in breast tissue and could be used as a marker of breast phospholipid n-3 to n-6 ratio though relative amount of n-3 and n-6 fatty acids are very different between red blood cells and breast phospholipids.

The only link found previously between fatty acids in breast tissue and breast cancer is related to the n-3 to n-6 fatty acid ratio in TAG. We need to continue to study their relationships in women who take a supplement of n-3 LCPUFA.

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Chapter 1

Introduction

The relationship between the n-3 long chain polyunsaturated fatty acid (LCPUFA) in lipid classes from fine needle aspirates of the breast and those in the same lipid classes obtained from plasma and red blood cells (RBC) have not been reported. We studied their relationships in women at high risk for breast cancer who were on their usual intake of n-3 LCPUFA.

Fatty acids in the plasma and red blood cell phospholipids are the usual biomarkers of n-3 LCPUFA status. They reflect both immediate (plasma) and long term or usual (RBC) n-3 LCPUFA intake. This is a pilot study for a planned study in the future to provide n-3 LCPUFA as a dietary supplement to women at high risk for breast cancer to measure cytokines and monitor the effect upon cellular biomarkers of cancer risk. The purpose of this study is to determine if breast tissue triacylglycerols (TAG) and phospholipids (PL) n-3 LCPUFA and the ratio of n-3 to n-6 fatty acids in breast tissue relate to the usual biomarkers of n-3 and n-6 LCPUFA status when n-3 LCPUFA intake is in presumed equilibrium.

Some investigators found the lipid profile in TAG found in breast adipose tissue could be used to predict breast cancer risk. However, no previous study has investigated the relationship between fatty acid composition in PL or TAG of breast tissue and blood.

Statement of Purpose

The purpose of this study was to determine the fatty acid composition in breast tissue in women with increased risk of breast cancer and the fatty acid composition in both PL and TAG in blood (both plasma and RBC). The ultimate aim of this study is to determine if blood fatty acids could predict fatty acids in breast tissue aspirates.

Research Questions

1. What are the PL and TAG fatty acid profiles in fine needle aspirates of breast tissue in women at high risk for breast cancer?
2. Do tissue LCPUFA or a combination of n-3 and n-6 fatty acids in phospholipids and triglycerides of fine needle aspirates of breast tissue correlate with plasma and RBC LCPUFA or ratios of n-3 to n-6 fatty acids in PL and plasma TAG, respectively?

Chapter 2

Review of Literature

It is not known how closely n-3 long chain polyunsaturated fatty acid (LCPUFA) in lipid classes from fine needle aspirates of the breast compare to n-3 LCPUFA in the same lipid classes obtained from plasma and red blood cells (RBC) in women at high risk for breast cancer who are on their usual intake of n-3 LCPUFA.

Breast Cancer

Breast cancer forms in tissues of the breast. It usually occurs in the ducts and lobules, which are tubes that carry milk to the nipple and glands that make milk. In rare cases, breast cancer can start in other areas of the breast. Although the incidence of breast cancer is 100 times as frequent among women as among men, the survival rates of breast cancer are equal in both sexes (1).

Breast cancer is the second most common type of cancer after lung cancer. It accounts for 10.4% of all cancer incidences in both sexes combined (2). It is the fifth most common cause of cancer death (3). In 2009, there were approximately 192,370 new diagnoses of breast cancer in females and 1910 diagnoses in male. Deaths in the US for breast cancer were approximately 40,170 in females and 440 in males (1).

Breast cancer is the most frequent cancer and the first cause of death by cancer among women worldwide. Over the course of lifetime, one in eight women will be diagnosed with breast cancer in the United States. The estimated prevalence of breast cancer between 1990 to 2006 and the death rate from breast cancer in the US from 1975 to 2006 was 1.07% and 0.03%, respectively, in female of all ages and races (1).

Risk Factors of Breast Cancer

The primary risk factors for breast cancer include age, gender, family history, personal history of breast abnormalities, genes, race, menstrual cycle, alcohol and tobacco use, childbirth, hormone replacement therapy (HRT), obesity, radiation, and high-fat diet (4).

The risk of developing breast cancer increases in women as they get older, with the majority of breast cancer cases found in women older than 50 years of age (4). As noted earlier, women are about 100 times more likely to be diagnosed with breast cancer than men (1).

A woman's risk of breast cancer is higher if she has one or more first degree relatives, e.g., mother, sister or daughter, who are diagnosed with breast cancer. The higher maternal age at first live birth is associated with higher risk of breast cancer, but this depends on the family history of breast cancer. For women with 0 or 1 affected relatives, risk increases with age at first live birth, but for women with 2 or more first degree relatives, risk

decreases with age at first live birth (5).

Women who experienced early onset of menses (before 12 years old) or who experienced late menopause (after 55 years old) have a slightly higher risk of developing breast cancer (4). Since breast cancer usually occurs in ducts and lobules, abnormalities in these regions of the breast are associated with an increased risk of developing the breast cancer. And diagnose of breast cancer on one breast will increase the risk of breast cancer on the other breast.

Genes are a risk factor for breast cancer. Defects in BRCA1 and BRCA2 make people more prone to developing breast cancer. Women with a defect in one of these genes have up to an 80% chance of developing breast cancer (4). Caucasian women have greater risk of developing breast cancer than African American women and other races, although African American women have a higher death rate in those who diagnosed with breast cancer (4).

Environmental factors also contribute to risk of breast cancer. For example, women who consume more than 1 to 2 glasses of alcohol a day may increase the risk of getting breast cancer (4). Women who received HRT to reduce their symptoms of menopause may also have a higher risk for breast cancer (6, 7). Radiation exposure on chest area as a child or young adult will increase the risk of breast cancer (3). Obesity also

increases risk of breast cancer (4). Recently published research indicates that breast tissue density may relate to the risk of breast cancer as well (8). However, the evidence for these factors is not conclusive. More research is needed to validate these risk factors.

Diet and Risk of Breast Cancer

International variations in breast cancer incidence rates and changes in the incidence among migrant populations suggest breast cancer risk is influenced by environmental factors could be amenable to interventions (2).

Diet is one of the environmental factors shown to influence the risk of breast cancer. Few studies have been focused on the cancer incidence in relation to usual diet intake. Diet is thought to be responsible for about 30% to 40% of all cancers. In Asians, lower cancer risk may be due to the frequent consumption of soy products (11). Findings suggest that physical activity, a healthy diet (particularly one low in fat and high in vegetables and fiber), and a healthy weight can reduce the risk of breast cancer (4).

Bosetti et al. (10) reviewed studies conducted in Italy during the early 1990s. He found that some certain unsaturated fats, such as olive oil, reduce the risk of breast cancer, while saturated fat intake was directly related to some common neoplasms, which is a result of the abnormal proliferation of cells. As a result, major characteristics of the Mediterranean diet, which emphasizes high consumption of olive oil, legumes, unrefined

cereals, fruits, and vegetables, moderate consumption of dairy products, fish, wine and low consumption of meat and meat products, may decrease the cancer risk.

Thomson et al. (11) suggested there is a possible benefit of a vegetable-rich, low-fat eating pattern on breast cancer survival. Lower fat intake and higher vegetable and fiber intake may result as well in mild to modest weight loss among overweight and obese women. Weight loss is associated with higher survivability of breast cancer.

Sue et al. (12) found a modest positive association between higher energy intake diet pattern and risk of breast cancer. They found 21% higher risk among women in the highest quartile of energy intake than the lowest quartile. This association is largely independent of Body Mass Index (BMI) and physical activity. Animal studies also provided consistent evidence that energy restriction reduces breast cancer risk.

Consumption of soy products has been associated with lower risk of breast cancer, but this is not conclusive (2). For example, a German study found no relationship between soy products and breast cancer risk (13), while two other studies from the UK found increased breast cancer risk for dietary isoflavone intake (14,15). Two studies based on Asian population reported a decreased breast cancer risk was associated with higher dietary isoflavone intake (16, 17). More research is needed to determine the

effects of soy products on the risk of breast cancer.

In conclusion, few dietary effects have been determined in relation to the risk of breast cancer. Variables studied have not produced convincing evidence of risk or benefit. Additional research is needed to address the importance of diet effect on women at high risk of breast cancer and breast cancer patients. Factors that influence individual recurrence risk of breast cancer, are related to diet need to be examined and established in the future as well.

Dietary Fat May Alter the Outcome of Breast Cancer

The evidence on the relationship between dietary fat and the risk of breast cancer is mixed. Results of animal studies are consistent by showing an increased risk of mammary gland cancer when fat intake is increased (18). Human studies are different because different types of fatty acids are present in different kinds of foods and oils that may influence the risk of breast cancer at different levels.

Several studies have shown an association between fatty acid intake and mammary gland tumor incidence (19-23). After dietary supplementation, several fatty acids, such as alpha-linoleic acid, are selectively incorporated into membrane phospholipids of tumor cells suggests these fatty acids might influence tumor growth through an effect on membrane functions (20-22). Previous studies also found that both

benign and malignant proliferation of breast tissue in women is associated with higher plasma lipids levels, and it is closely related to the progression of breast cancer (24, 25).

Two studies examined the effect of total fat intake on breast cancer risk. One of these studies reported a high fat diet was significantly associated with an increased risk of breast cancer (11). The other study reported a modest increase in the incidence of breast cancer in women who had a higher level of saturated fat intake (26). None of the available cohort studies reported an association between risk of breast cancer and a high fat intake.

Although these studies are not conclusive evidence fat plays a role in the development of breast cancer, the results of previous studies indicate that the relationship between dietary fat intake and risk of breast cancer is worthy of further study.

Biomarker in Breast Cancer

A biomarker is a biological status or substance found and measured in blood, other body fluids, or tissues. It could be used as an indicator of a biological state in the body. It could be objectively measured or evaluated as a sign of normal biological process, pathogenic processes, or pharmacologic responses to therapy. The most common biomarkers used in medicine are disease related biomarkers. When a disease exists already,

biomarkers are considered diagnostic biomarkers. When a biomarker is a reliable indicator for the risk of the disease, individuals identified at high risk of the particular disease should be considered to change any behavior(s) related to the biomarker.

Specific biological markers used for a given cancer have not proven fruitful. Most of the single clinical markers have low accuracy. One marker that is used for diagnosis of breast cancer, CA15.3, has a sensitivity of 23% and specificity of 69%. (27) Instead of single biomarker, a combination of biomarkers may increase the accuracy of prediction. Multiple markers may be very useful in future diagnosis of cancer or cancer risk with higher accuracy.

The fatty acid composition of breast tissue could be used as a biomarker of the exposure to different dietary fatty acids. Yet the individual fatty acid approach may not be appropriate; a combination of fatty acids may need to be estimated due to the interaction between fatty acids. The complex correlation system needs to be simplified to a smaller number of dimensions (28). A review from Bougnoux reports on the lipid profile (lipidome), a composite of fatty acids in breast tissue triacylglycerols (TAG) that appears to constitute a biomarker for the risk of breast cancer (29).

The analysis of lipids is complicated by their unique and diverse chemical and physical properties, especially in complex mixtures. No single

analytical method is available to examine the full lipidome of biological mixtures in a single experiment (27). Michels et al. (30) found that diet assessment methods and other potential factors, such as measurement error and variation of diet, contribute to the difficulty in examining the relationship between dietary fat intake and fat composition in the body. Therefore, a biomarker that could reflect the fatty acids composition in the breast tissue is desired; e.g. ratio of n-3 to n-6 long chain polyunsaturated fatty acid (LCPUFA), for conducting future studies such as the effects of n-3 LCPUFA on the risk of breast cancer.

Chajes et al. (31) reported the fatty acid composition of breast tissue was highly correlated with subcutaneous adipose tissue, suggested that the fatty acid composition of breast may reflect the whole body stores of these fatty acids. The fatty acid composition of white adipose tissue best reflects past dietary fat intake. It is relevant to examine the breast cancer risk and fat intake relationships in relation to fatty acid composition (28). However, a blood based marker that could reflect the fatty acid profile in breast tissue would be useful as breast biopsies are time consuming, cannot be done frequently compared to blood sampling and may be painful or lead to bleeding or bruising. Fine needles aspirates of breast tissue are routinely obtained from women in our clinical population who are at high risk for breast cancer.

Body fluids such as blood are little more trouble to be obtained for analysis of lipid classes than breast tissue, and blood plasma and red blood cells (RBCs) are commonly used as a biomarker because they carry a large number of metabolites (27). King et al. (32) reported that a combination of fatty acids could reflect the consumption of total dietary fat with high sensitivity and specificity. A biological marker of total fat intake in blood related to breast tissue would be useful to reflect the changes of fatty acid composition response to total fat intake.

It is possible to examine the relationship between fatty acids in lipid classes of fine needle aspirates of the breast and plasma and RBC in women at high risk for breast cancer. A study comparing the fatty acid composition of phospholipid (PL) and TAG in plasma or RBC with breast cancer risk were inconclusive (33). Two studies currently have reported fatty acids in breast tissue but fatty acid would be expected to be evaluated by the all types included in the aspirate, neither study reported total, PL or TAG fatty acids in plasma or RBC, as proposed here (23, 34).

N-3 LCPUFA

N-3 (omega-3) LCPUFA are a family of unsaturated fatty acids that have a final carbon-carbon double bond in the omega-3 position, between the third and fourth carbon from the methyl end of the fatty acid. Important members of n-3 LCPUFA include α -linolenic acid (ALA), eicosapentaenoic

acid (EPA), and docosahexaenoic acid (DHA).

N-3 fatty acids play an important role in cell membrane functions and regulating the body's metabolic processes. Some foods are a good source of omega-3 fatty acids, compounds have a weak estrogen effect and can help reduce the risk of breast cancers depending on estrogen.

Cold-water fish are a good dietary source for n-3 LCPUFA and a few vegetable oils such as flaxseed, walnut, and canola are also high in alpha linolenic acid. In addition to the marine products and oils from special vegetables, whole grains, legumes, nuts and green leafy vegetables are also good sources of n-3 PUFA.

The conversion of n-3 PUFA to n-3 LCPUFA occurs competitively with n-6 PUFA to n-6 LCPUFA, which are derived from another essential fatty acid – linoleic acid. Synthesis of the n-3 LCPUFA from linolenic acid within the body is competitively slowed by the n-6 analogues. Many chronic diseases are related to an imbalance intake in the omega-6 and omega-3 fatty acids (34). As a result, the ratio of n-3 LCPUFA to n-6 LCPUFA becomes important for human health (34).

N-3 LCPUFA and Risk of Breast Cancer

Several studies reported possible anti-cancer effects of n-3 fatty acids, including for breast cancer (35). For breast cancer, neither long-chain nor short-chain fatty acids were consistently associated with breast cancer risk.

Higher levels of DHA, the most abundant n-3 LCPUFA in erythrocyte membranes, was associated with a reduced risk of breast cancer (36).

Maillard et al. (34) found that ALA (18:3n3) was inversely related to the risk of breast cancer in a case-control study. They also found ALA inhibited tumor development and growth in an animal system of N-methylnitrosourea-induced mammary tumors, but this effect is only present in the absence of the antioxidant vitamin E. These results indicate n-3 LCPUFA may inhibit tumor growth are substrates of lipid peroxides, which lead to tumor cell death.

Bougnoux et al. (37) measured and determined the response to anticancer agents for breast cancer in relation to n-3 LCPUFA. They found the storage level of DHA (22:6n3) in lipids of patients was high, the responsive of mammary tumors to induction chemotherapy was increased. They found DHA increased cell lipid peroxidation and generated oxidative stress, which is the mechanism of many anticancer drugs when presented in medium of cultured mammary tumor cell lines. The effect was lost when an antioxidant such as vitamin E was present.

In one animal study, rat mammary tumors were sensitized to epirubicin, an anticancer drug, when fish oil and oxidants were provided in the diet of the rat. The investigators suggested that because lipoperoxidation inhibits tumor growth and this effect is generated during anticancer drug actions,

providing DHA substrate to tumor cells to improve lipoperoxidation would lead to an increased drug efficacy (23).

However, study results based on n-3 LCPUFA and risk of breast cancer are not consistent. There are 2 case-control studies done in North American people that found no protective effect of n-3 LCPUFA on breast cancer (38, 39). In contrast, the other case-control study conducted in Finland found a higher DHA level in breast adipose tissue related to dietary intake was associated with a lower incidence of breast cancer (40).

The ratio of n-3 LCPUFA to n-6 LCPUFA has an important effect on risk of breast cancer. Maillard et al. (34) reported a strong association between ratios of 18:3n3 to 18:2n6 or n-3 LCPUFA to the total n-6 fatty acids and reduction in risk of breast cancer. This indicates the protection of n-3 LCPUFA on breast cancer could depend on the background levels of total n-6 PUFA (34). A decreased risk of breast cancer was found with an increased ratio of n-3 LCPUFA to total n-6 PUFA in a case control study conducted across 5 European countries (41).

As a result, future studies and precise identification on the role of n-3 LCPUFA on breast cancer will be needed to allow them to be used as nutritional targets in breast cancer therapy and prevention.

Chapter 3

Methods

The purpose of this study was to determine if the composition of breast tissue LCPUFA in PL and TAG correlated with blood level LCPUFA, and if the composition of fatty acids in plasma and RBCs could be a validated biomarker for breast tissue fatty acids composition, which may be used in interventions related to breast cancer.

Sample

This study used a sample of convenience. The subjects were clinical patients enrolled in an ongoing study to develop an assessment of breast cancer risk based on epidemiologic and biologic risk variables. As part of the protocol, blood and tissue samples were taken and dietary history questionnaire (DHQ) (including usual supplement intake) were distributed when the subjects were enrolled.

Women at increased risk of breast cancer but without any breast carcinoma were recruited into the study. The increased risk of breast cancer was defined by any of the following: having a mutation in a cancer suppressor gene (BRCA1, BRCA2, p53, etc.); having a first degree relative with breast cancer or two or more second degree relatives with breast cancer; and having had a prior biopsy diagnosed on other breast diseases or T1 or T2 breast cancer in the last 10 years.

Inclusion criteria. Subjects enrolled had to meet the inclusion criteria:

1) the subject should be between the ages of 30 and 55 years old; 2) the subject must be more than six months from ingestion of antihormonal therapy; 3) the subject must be more than 1 year from pregnancy, lactation or chemotherapy; 4) mammograms of the subject had to be read as not suspicious for breast cancer; 5) the subject had to be willing to donate blood and tissue samples and complete personal dietary and health history information.

Exclusion criteria. Subjects were excluded if they met any of following: 1) metastatic malignancy to other organs; 2) on Coumadin or other anticoagulants; 3) bilateral breast implants or tram flap reconstruction; 4) had radiation to both breasts; 5) mammogram or clinical breast exam suspicious for breast cancer and malignancy not ruled out, and 6) who were unable to give an informed consent.

Recruitment

Women who met the inclusion and exclusion criteria were asked to participate in the study at University of Kansas Medical Center (KUMC). Enrollment of subjects started on June 2009 and was completed in January 2010. Consent was explained and signed for every enrolled woman. Fifty-two subjects were enrolled in the study and provided a blood and breast tissue sample and completed the DHQ.

Setting

The subjects were recruited at KUMC, Westwood, Kansas, and the fatty acids were analyzed in Dr. Susan Carlson's lab in Department of Dietetics and Nutrition at KUMC.

Ethical Issues

This study was a pilot study to determine if blood can be used as a biomarker similar to breast tissue for studies to determine n-3 LCPUFA status. Approval for this substudy was under a blanket informed consent obtained by Dr. Carol Fabian, M.D. and her colleagues. This study approved by Human Subjects Committee at KUMC (HSC# 4601).

The dietary history record and clinical interview, such as interview schedules and questionnaire were part of Katherine Harvey's thesis. Her work was used to determine who took n-3 LCPUFA supplements in this study. The fatty acids analyses from the present study were used by her to determine if there was a relationship of dietary intake to tissue fatty acids in either breast or plasma and RBC lipids, in women at high risk of breast cancer.

Procedures

Each subject signed the informed consent document when enrolled in the study. The DHQ was distributed and blood and breast tissue samples were collected. The DHQ was mailed back to KUMC after the participants

completed it. Katherine Harvey R.D. checked and analyzed the mailed back DHQ.

Ten milliliters of venous blood was collected with into a tube containing K₃-EDTA. Blood was not collected after fasting. Plasma and RBC were separated and store at -80°C until analysis. A number of aspirates of breast tissue (approximately 6-8/procedure) were also obtained and kept at -80°C. The aspirates were defined as real breast tissues with liquid around them in this study. Just prior to analysis, the aspirates of breast tissue were combined and a uniform aliquot taken for the PL and TAG analysis (Appendix A).

Total lipids were extracted from all specimens with chloroform: methanol 2:1 (v:v) by using a modification of the method of Folch et al. (42) (Appendix B). The extract was washed with KCl and separated into two phases. The lower chloroform layer was transferred to a tube and evaporated to dryness under nitrogen. The lipid extract was dissolved in 100 µl dichloromethane. TAG and PL were separated by adsorption chromatography on silica plates in 80:20:1 hexane: diethyl ether: acetic acid. The PL band was scraped from the plate and collected in a screw-cap tube with 1mL boron-trifluoride (BF₃). The TAG band was collected in another tube with 0.25 ml BF₃, 0.2 ml Benzene and 0.55 ml methanol.

Fatty acids methyl esters (FAMES) were analyzed in groups of 12 with one weighted standard run (Supelco 37 mixture) using the Varian 3900 GLC and attached CP 8400 autoanalyzer. Samples were identified solely by a code number within a batch. FAMES composition was determined by gas-liquid chromatography (GLC). Identification of FAMES was determined by comparison of their relative retention times against those of pure standard mixtures. The relative amount of each fatty acid was quantified by integration of the peak at baseline and dividing the results by the total area for all fatty acids. Each GLC run was analyzed for quality and repeated if there was any indication of oxidation or underloading of the sample.

Statistics

Each fatty acid was expressed as a percentage of total fatty acids. Analysis focused on n-3 and n-6 LCPUFA, individually and combination, and the ratio of n-3 to n-6 LCPUFA. We evaluated specifically the composite group of fatty acids identified by Bougnoux et al. as related to the risk of breast cancer (29). The groups we used in this study were, total n-3 PUFA, long chain n-3 PUFA, total N-6 PUFA, and total trans fatty acids. The combination of total n-6 and total and long chain n-3 was the same as the ones used in the study of Maillard et al (34).

Data for individual fatty acids and groups of fatty acids in each tissue and lipid class were expressed as means with their standard deviations.

The data were assumed to under normal distributions. Comparisons among tissues and lipid classes were expressed as correlation coefficients. Correction for DHA was made because it is our last peak during analyzing. We used the response factor for DHA from Supelco 37, a weighed FAME mixture for the correction. Pearson correlation was calculated to determine the relationships between fatty acids compositions in breast tissue and blood (both plasma and RBCs). A P value of < 0.05 was considered statistically significant. All the data were analyzed by using SPSS 17.0 statistical software.

Chapter 4

Results

This section includes the results for fatty acids composition of blood, including plasma and RBCs, and breast tissue, and the correlations between them.

Sample

Blood samples were collected from 50 women at high risk for developing breast cancer, and breast tissue samples were available from 34 women. All of the samples were analyzed for fatty acid composition. The DHQ from 46 women at high risk of breast cancer enrolled in this study were collected and analyzed by a registered dietitian.

The mean age of the subjects was 48.4 ± 9.8 years. The mean height and weight of the subjects were 65.0 ± 2.4 inches (19.8 ± 0.7 m) and 152.1 ± 27.8 pounds (69.0 ± 12.6 kg), respectively. The mean BMI was 25.1 ± 4.4 kg/m². The percentage of subjects who were premenopausal was 48% (n=22) and the percentage of subjects who were postmenopausal was 52% (n=24). A woman was defined as postmenopausal if she has not menstruated 12 months or more, otherwise, she was defined as premenopausal. The demographic data are shown in Table 1.

Table 1. Demographics of the Subjects

Characteristic	n=50
Mean Age (y)	48.4 ± 9.8
Height (Mean ± SD) (in) (m)	65.0 ± 2.4 (19.8 ± 0.7)
Weight (Mean ± SD) (lb) (kg)	152.1 ± 27.8 (69.0 ± 12.6)
BMI (Mean ± SD) (kg/m ²)	25.1 ± 4.4
Premenopausal (%)	48
Postmenopausal (%)	52

Fatty Acids Compositions in Blood and Breast Tissue

The fatty acid composition of blood samples, including plasma and RBCs, and breast tissues for each subject were analyzed by GLC. Each fatty acid was expressed as a percentage of total fatty acids.

The mean of total n-3 PUFA of PL in plasma and RBCs for the subjects were 4.00 ± 1.09% and 5.47 ± 1.50%, respectively. The mean of total n-3 PUFA of TAG in plasma was 2.21 ± 1.34%. The mean of total n-3 PUFA of PL and TAG in breast tissue were 0.36 ± 0.27% and 1.28 ± 0.44%, respectively.

The means of total n-6 PUFA of PL in plasma and RBCs for the subjects were 34.04 ± 3.22% and 28.10 ± 3.45%, respectively. The mean of total n-6 PUFA of TAG in plasma was 28.35 ± 3.97%. The mean of total n-6

PUFA of PL and TAG in breast tissue were $2.75 \pm 0.99\%$ and $17.53 \pm 2.78\%$.

The mean ratio of n-3 to n-6 PUFA in PL were 0.09 ± 0.03 and 0.12 ± 0.03 in plasma and RBCs, respectively. The mean ratio of n-3 to n-6 PUFA in TAG of plasma was 0.11 ± 0.05 , and the ratios were 0.20 ± 0.07 and 0.07 ± 0.03 of PL and TAG in breast tissue. The ratio of 18:3n3/18:2n6 in plasma and RBC PL were 0.008 ± 0.003 and 0.01 ± 0.003 , respectively, and 0.06 ± 0.02 in TAG of plasma. The mean ratio of 18:3n3/18:2n6 in breast tissue were 0.02 ± 0.01 and 0.05 ± 0.01 in PL and TAG. The mean ratio of n-3 LCPUFA to n-6 total PUFA in plasma were 0.11 ± 0.03 and 0.03 ± 0.02 in PL and TAG and 0.19 ± 0.07 in RBC. In the breast tissue, the mean ratio of long chain n-3 PUFA/n-6 PUFA were 0.08 ± 0.04 and 0.02 ± 0.02 in PL and TAG, respectively.

The percentage of subjects who were taking a n-3 PUFA (either fish oil or flaxseed) were 33% (n=15). The mean intakes were 1.26 ± 0.6 g n-3/d and 9.94 ± 4.9 g n-6/d, and the ratio of n-6/n-3 intake was approximately 9:1.

The PL and TAG compositions of all the fatty acids in blood (plasma and RBCs) were shown in Table 2 and the PL and TAG composition of all the fatty acids in breast tissue were shown in Table 3.

Table 2. Fatty Acids Composition in Blood Samples (n=50)

	Phospholipid (PL)		Triacylglycerol (TAG)
	(Mean ± SD) (%)		(Mean ± SD) (%)
	Plasma	RBCs	Plasma
n-3 PUFA			
18:3n3 (ALA)	0.16 ± 0.07	0.10 ± 0.04	1.18 ± 0.38
20:5n3 (EPA)	0.70 ± 0.40	0.61 ± 0.48	0.24 ± 0.22
22:6n3 (DHA)	3.18 ± 0.96	4.10 ± 1.16	0.42 ± 0.39
Total n-3 ^a	4.00 ± 1.09	5.47 ± 1.50	2.21 ± 1.34
n-6 PUFA			
18:2n6c (LA)	20.37 ± 3.20	10.30 ± 1.40	21.19 ± 4.01
20:4n6 (AA)	10.36 ± 1.93	13.29 ± 2.07	1.55 ± 0.50
Total n-6 ^b	34.04 ± 3.22	28.10 ± 3.45	28.35 ± 3.97
Monounsaturated FA			
18:1n9c (OA)	8.14 ± 1.25	11.21 ± 0.87	31.02 ± 3.94
Trans FA			
Total trans ^c	1.54 ± 0.46	1.98 ± 0.59	1.29 ± 0.62
Ratios n-3/n-6			
18:3n3/18:2n6	0.008 ± 0.003	0.01 ± 0.003	0.06 ± 0.02
Total n-3/n-6	0.09 ± 0.03	0.12 ± 0.03	0.11 ± 0.05
LC n-3 ^d /total n-6	0.11 ± 0.03	0.19 ± 0.07	0.03 ± 0.02

^a Included: 18:3n3, 20:3n3, 20:5n3, 22:5n3, 22:6n3.

^b Included: 18:2n6c, 20:3n6, 20:4n6, 22:4n6, 22:5n6.

^c Included: 18:1n9t, 18:2n6t.

^d Included: 20:5n3, 22:5n3, 22:6n3.

Table 3. Fatty Acids Composition in Breast Tissue Samples (n=34)

	Phospholipid (PL) (Mean ± SD) (%)	Triacylglycerol (TAG) (Mean ± SD) (%)
n-3 PUFA		
18:3n3 (ALA)	0.03 ± 0.04	0.86 ± 0.21
20:5n3 (EPA)	0.05 ± 0.05	0.14 ± 0.34
22:6n3 (DHA)	0.24 ± 0.29	0.10 ± 0.08
Total n-3 ^a	0.36 ± 0.27	1.28 ± 0.44
n-6 PUFA		
18:2n6c (LA)	2.06 ± 2.46	16.66 ± 2.70
20:4n6 (AA)	1.10 ± 1.63	0.38 ± 0.19
Total n-6 ^b	2.75 ± 0.99	17.53 ± 2.78
Monounsaturated FA		
18:1n9c (OA)	12.76 ± 2.07	35.95 ± 3.23
Trans FA		
Total trans ^c	6.72 ± 1.21	2.32 ± 0.68
Ratios n-3/n-6		
18:3n3/18:2n6	0.02 ± 0.01	0.05 ± 0.01
Total n-3/n-6	0.20 ± 0.07	0.07 ± 0.03
LC n-3 ^d / total n-6	0.08 ± 0.04	0.02 ± 0.02

^a Included: 18:3n3, 20:3n3, 20:5n3, 22:5n3, 22:6n3.

^b Included: 18:2n6c, 20:3n6, 20:4n6, 22:4n6, 22:5n6.

^c Included: 18:1n9t, 18:2n6t.

^d Included: 20:5n3, 22:5n3, 22:6n3.

Correlations between Blood and Breast Tissue Fatty Acids

Composition

The relationship between total n-3 PUFA composition in blood (plasma and RBCs) and breast tissue were shown in Table 4. No significant correlation was found in the total n-3 fatty acids profile between blood and the fine needle aspirates of breast tissue.

The correlations of n-6 PUFA profile were shown in Table 5. The plasma total n-6 PUFA composition in TAG was significantly correlated with the total n-6 PUFA composition of breast tissue in both PL and TAG ($p < 0.01$). However, no significant correlation was found between the PL in RBCs or plasma and the lipid profile in breast tissue.

The results of the correlation of the n-3 PUFA to n-6 PUFA ratio between blood species and tissue were shown in Table 6. No significant correlation was found between the ratio of 18:3n3/18:2n6 in the breast tissue and the blood. There was a significant correlation between the ratio of n-3 PUFA to n-6 PUFA in PL of breast tissue and that in the PL of both RBCs and plasma ($p < 0.01$), and TAG of plasma ($p < 0.01$).

The relationship of the ratio of n-3 LCPUFA to n-6 PUFA between blood (plasma and RBCs) and breast tissue was shown in Table 7. No significant correlation was found between the n-3 LCPUFA to n-6 PUFA ratio in breast tissue TAG and those in blood samples, except PL of the

plasma ($p<0.01$). The ratio of n-3 LCPUFA to n-6 PUFA in the PL of breast tissue was significantly correlated with that in the PL of RBC ($p<0.01$) and plasma ($p<0.01$), respectively.

Table 4. Correlations of Total N-3 PUFA Composition between Blood (plasma and RBCs) and Breast Tissue

		Lipid Profile of Fine Needle Aspirates			
		PL		TAG	
		<i>r</i>	<i>P</i> value	<i>r</i>	<i>P</i> value
RBC	PL	0.102	0.612	0.012	0.949
Plasma	PL	-0.001	0.996	0.169	0.355
	TAG	0.016	0.936	0.042	0.821

* Correlation is significant at the 0.05 level (2-tailed)

** Correlation is significant at the 0.01 level (2-tailed)

Table 5. Correlations of Total N-6 PUFA Composition between Blood (plasma and RBCs) and Breast Tissue

		Lipid Profile of Fine Needle Aspirates			
		PL		TAG	
		<i>r</i>	<i>P</i> value	<i>r</i>	<i>P</i> value
RBC	PL	0.046	0.832	-0.088	0.631
Plasma	PL	0.200	0.350	0.027	0.884
	TAG	0.521**	0.009	0.533**	0.002

* Correlation is significant at the 0.05 level (2-tailed)

** Correlation is significant at the 0.01 level (2-tailed)

**Table 6. Correlations of N-3/N-6 PUFA Composition between Blood
(plasma and RBCs) and Breast Tissue**

		Lipid Profile of Fine Needle Aspirates				
		PL	TAG			
		<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	
			value		value	
	RBC	PL	0.096	0.634	0.209	0.259
18:3n3/18:2n6	Plasma	PL	-0.047	0.818	0.126	0.498
		TAG	0.191	0.340	0.083	0.657
	RBC	PL	0.801**	0.000	0.201	0.270
Total n-3/n-6	Plasma	PL	0.653**	0.000	0.025	0.893
		TAG	0.627**	0.000	-0.079	0.666

* Correlation is significant at the 0.05 level (2-tailed)

** Correlation is significant at the 0.01 level (2-tailed)

**Table 7. Correlations of N-3 LCPUFA/N-6 PUFA Composition
between Blood (plasma and RBCs) and Breast Tissue**

		Lipid Profile of Fine Needle Aspirates			
		PL		TAG	
		<i>r</i>	<i>P</i> value	<i>r</i>	<i>P</i> value
RBC	PL	0.631**	0.000	0.276	0.133
Plasma	PL	0.524**	0.003	0.501**	0.004
	TAG	0.313	0.092	0.272	0.138

* Correlation is significant at the 0.05 level (2-tailed)

** Correlation is significant at the 0.01 level (2-tailed)

Chapter 5

Discussion

The purpose of this study was to examine the fatty acids composition in breast tissue of women at increased risk of breast cancer and whether the fatty acids composition in blood relate to that in the breast tissue in women at increased risk of breast cancer.

Fatty Acids Composition in Blood and Breast Tissue in Women at High Risk of Breast Cancer

Maillard et al. (34) reported fatty acids composition of breast adipose tissue in 88 women had benign breast disease. The mean percentage of total n-3 PUFA in their participants was 0.98 (SE=0.027), and the mean percentage of total n-6 PUFA was 14.67 (SE=0.420). The average ratio of 18:3n3/18:2n6 and long chain n-3/n-6 PUFA were 0.039 (SE=0.001) and 0.034 (SE=0.002), respectively. The combination of both total n-3 and n-6 PUFA Maillard et al. (34) used in their research were the same as those in the current study. Mean percentage of total n-3 and n-6 PUFA composition in TAG of breast tissue was higher than mean percentages of total n-3 and n-6 PUFA in previous study by Maillard et al (34). The ratio of n-3 LCPUFA/n-6 PUFA was lower than the previous study (34).

Takata et al. (43) reported the fatty acids composition in plasma phospholipid in 130 women who diagnosed with breast cancer. The mean

percentage of total n-3 PUFA and n-6 PUFA in those participants were 3.80 and 33.8, respectively. Both of them were lower than the results in the current study. The ratio of total n-3 to n-6 PUFA in Takata et al. study was 0.11, which was higher than the result in the present study. The combination used for total n-3 and n-6 PUFA in report of Takata et al. were the same as those in the current study as well.

Both Maillard et al. (34) and Takata et al. (43) subjects were women with breast cancer. The population in our current study was women with high risk for subsequent development of breast cancer but not yet diagnosed with breast cancer. The difference between the characteristics of subjects may cause these variations in those fatty acids compositions. Also, different supplement used may affect the fatty acids composition in the blood and breast tissue.

The compositions of fatty acids in PL and TAG were quite different, regardless of the blood or breast tissue. The mean percentage of oleic acids in the TAG was much higher than that in the PL in both plasma and breast tissue. This result is consistent with the composition of fatty acid in human milk, which suggests that this fatty acid can be assimilated by the breast tissue itself. The mean percentage of arachidonic acid (AA) was lower in the TAG than in the PL, especially in the plasma. The fatty acid compositions in blood and breast tissue were different, especially for the PL

profile, the DHA and EPA much higher in the PL of plasma and RBC than those in the breast tissue, and so did the AA. The major fatty acids of PL in the breast tissue were palmitic acid, stearic acid and oleic acid.

No previous study was found that examined the fatty acid composition in RBC phospholipid or the comparison of the fatty acids composition in both PL and TAG in blood and those in breast tissue using a population with high risk of breast cancer.

Correlation of Fatty Acids Composition in Blood and Breast Tissue

A correlation was found between the ratio of n-3/n-6 PUFA in blood samples and breast tissue in women at high risk of breast cancer. Total n-3/n-6 PUFA in PL in plasma and RBC were significantly correlated to in fine needle aspirates. The ratio of long chain n-3 to total n-6 PUFA in PL in plasma and RBC was found related to that in breast tissue, and the ratio of n-3 LCPUFA/ n-6 PUFA in PL in plasma was significantly correlated to that in TAG in breast tissue, suggesting the ratio of n-3/n-6 PUFA may be a good indicator of the fatty acids composition in breast tissue. However, the combination of n-3 PUFA and n-6 PUFA was not found related to those in breast tissue independently, indicating that a combination of n-3 and n-6 fatty acids together may be better than using single fatty acid as a biomarker.

There were several studies did lipids of breast tissue and risk of breast cancer. A study conducted in France found that the lipidome of breast adipose tissue may predict the risk of breast cancer. The investigators found the level of DHA, and the ratio 18:3n3/18:2n6 and long chain n-3/total n-6 PUFA were inversely associated with breast cancer incidence (34). The European Community Multicenter Study on Antioxidants, Myocardial Infarction, and Cancer (EURAMIC) breast cancer study made a similar observation (44). Another study in North America also found the same results for long chain n-3 PUFA (45). All of the studies concluded that the ratio of total n-3 to n-6 PUFA in breast tissue predict the risk of breast cancer. However, other studies based on a similar design were not conclusive (38, 39).

In the current study, no relationship was found between independent or total n-3 and n-6 PUFA in the blood (both plasma and RBC) and the breast tissue in women at high risk of breast cancer. For n-3 and n-6 PUFA, no correlation was found in the ratio of 18:3n3 to 18:2n6 in the blood and the breast tissue, but there were significant correlation between total and long chain n-3 PUFA to total n-6 PUFA in PL of both plasma and RBC and PL in fine needle aspirates, suggesting that the ratio of total or long chain n-3 PUFA to total n-6 PUFA may be a biomarker of changing of fatty acids composition in breast tissue, which may used in interventions related to

breast cancer. This may allow the population of a high risk of breast cancer to prevent breast cancer with a dietary change.

A significant correlation was found in the ratio of total n-3 to n-6 PUFA in PL between RBC and breast tissue (figure 1). This result suggests that the ratio of total n-3/n-6 PUFA in the PL of RBC may be a convincing biomarker of that ratio in breast tissue. A high ratio of total n-3/n-6 PUFA may reflect a high ratio in the breast tissue. A weak correlation was found in the ratio of long chain n-3 to total n-6 PUFA in the PL of plasma and that in the TAG of breast tissue (figure 2), but the correlation was not as strong as those found in PL of RBC and breast tissue. Although results in plasma PL were highly correlated with those in plasma TAG, no relationship was found in fatty acid composition between PL and TAG in breast tissue.

Cognault et al. (46) found that the action of n-3 PUFA on mammary tumorigenesis depended on their interaction with other components of lipids in a rat model. Bougnoux et al. (28) found fatty acids were not independent variables. These results may explain the reason the independent n-3 and n-6 PUFA in blood do not correlate with those in breast tissue but the ratio did. These results indicated that focusing on single lipid component may not be an appropriate approach because of the complex interactions between all the fatty acids that determine the lipid profile in the body.

No previous studies assessed the correlation of fatty acids composition between the blood (plasma and RBC) and breast tissue as a biomarker. All of the previous studies focus on fatty acids composition and breast cancer risk were based on breast tissue fatty acids levels.

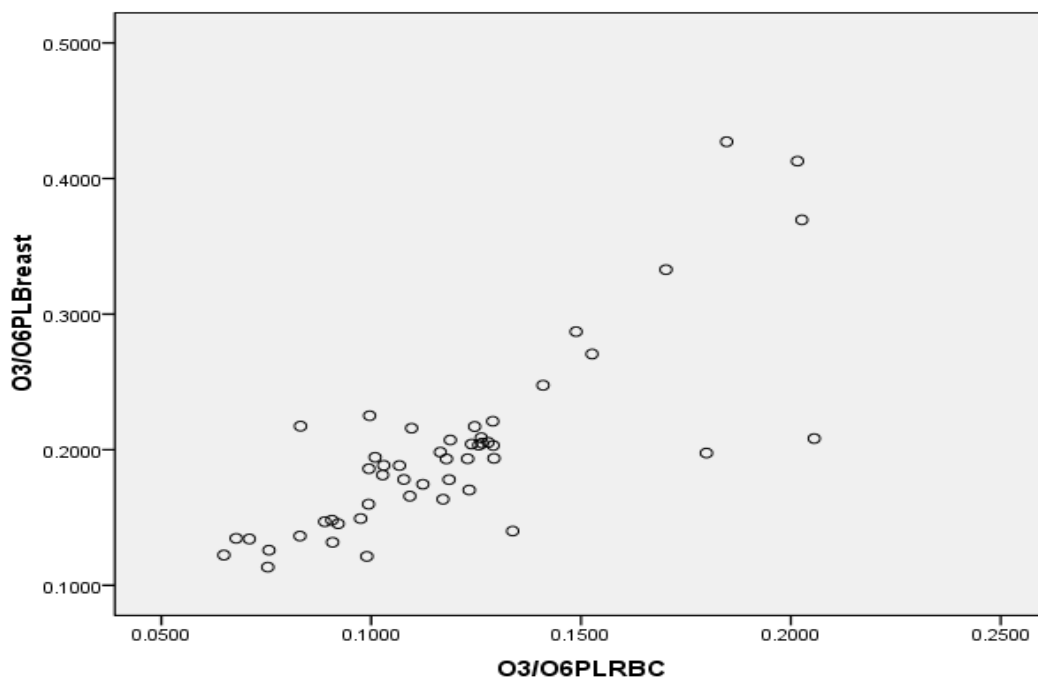


Figure 1. Correlation in the Ratio of Total n-3/n-6 PUFA in PL between RBC and Breast Tissue

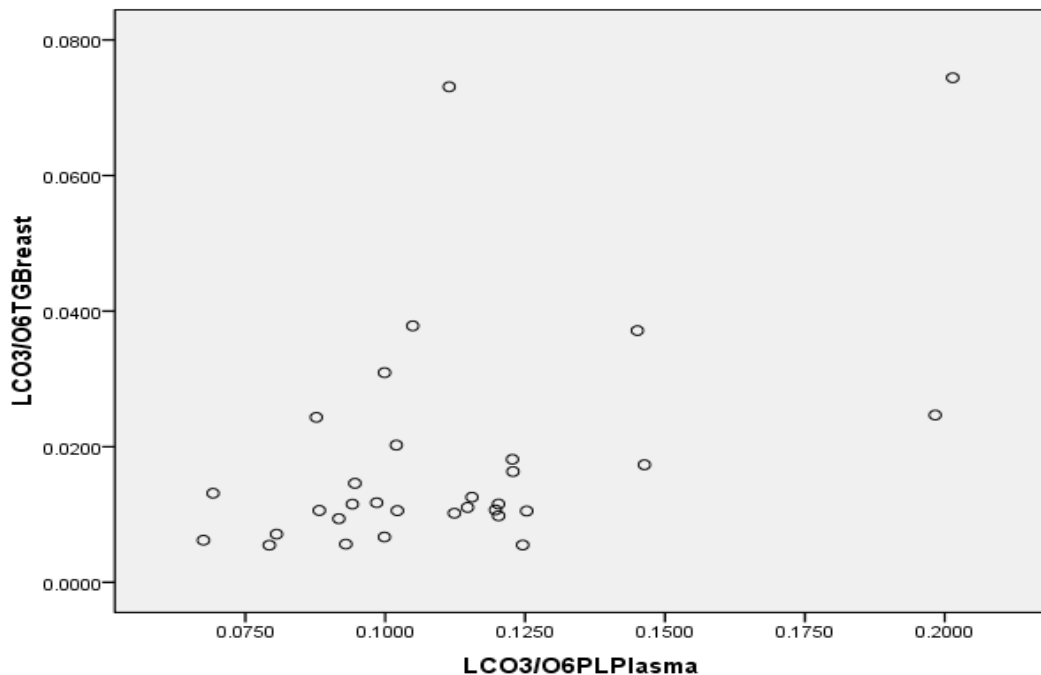


Figure 2. Correlation in the Ratio of LC n-3/total n-6 PUFA between Plasma PL and Breast Tissue TAG

Limitations

The limitations for this study were the sample size and control of confounding factors that influenced the fatty acids composition in blood and breast tissue.

Because the study was done in women at high risk for breast cancer, the women studied were also at high risk of breast cancer. We did not have any basis to estimate study power. A power analysis was not conducted to determine a specific sample size required to obtain statistical significance from the data. Typically, significant correlations between different lipids for

n-3 LCPUFA can be found with a sample of 10. The sample size for this study was 50. We chose this size because this sample size was able to explore different individual and combinations of fatty acids and to compare women who consume n-3 LCPUFA supplements to those who do not – this was occurring with 33% frequency in the cohort. However, the number of women enrolled in this study compared less than other reports in the literature related to fatty acids composition and breast cancer. The previous studies conducted by Maillard et al. (34) and Takata et al. (43) had 88 and 130 subjects, respectively. A larger sample will be needed in the future which will allow us to examine potentially influential variables such as menopausal status, BMI, and total fat intake.

Another limitation of this study was the influence factors. Only one blood sample was obtained from the study participants. One blood sample may not reflect the true fatty acids composition of the blood. Blood sample, especially the plasma, influenced by many outside factors, such as changes of diet, taking or stopping supplements, and et al. Supplements of n-3 PUFA will affect coagulation, participants were required to stop all supplements three weeks before the biopsy. This change in some participants may affect the blood fatty acids composition. Two or more blood samples might have been more representative of true fatty acids composition status.

Implications

In the current study, the ratio of total or long chain n-3 PUFA to total n-6 PUFA in blood was significantly correlated with those in breast tissue. Although other n-3 or n-6 PUFA combination in blood did not correlate with those in breast tissue, the results suggest the possibility of using fatty acids composition in blood as a biomarker to indicate the changing of fatty acids composition in breast tissue, which may be used in future interventions. This biomarker will be useful to reflect the fatty acids profile in breast tissue while avoiding the surgery for obtaining breast biopsies which are time consuming and may be painful or lead to bleeding or bruising.

Future Studies

For the current study, only 50 women were examined for the fatty acids composition relationship between blood and breast tissue, and only 34 breast tissue samples were obtained. A larger sample size will be needed to confirm the results in this study. Adjustment of individual characters, such as BMI, diet fat intake and supplement intake, are also needed in future studies for controlling the confounding factors. It would be beneficial for future researchers to try other combination of fatty acids in blood and breast tissue to see if there are other predictors of breast cancer risk.

Conclusion

In this study, significant correlations were found between the ratio of total and long chain n-3 PUFA to total n-6 PUFA in blood and breast tissue. However, no significant correlations were found between total n-3 PUFA and total n-6 PUFA in blood and breast tissue with the population having a high risk of breast cancer. The results in the study suggested that there may be a relationship between the fatty acids composition in blood and breast tissue.

In conclusion, the ratio of total or long chain n-3 PUFA to total n-6 PUFA might be a good biomarker of fatty acids composition in breast tissue to predict breast cancer risk. However, these results are need further validated in the future. Future larger studies are also need to determine other biomarkers for breast cancer risk.

Chapter 6

Summary

The purpose of this study was to determine the fatty acids composition in breast tissue in women with increased risk of breast cancer and if the fatty acid composition in both PL and TAG in blood (both plasma and RBC) correlated to the fatty acid composition in breast tissue.

Fifty patients were enrolled in a larger study to develop an assessment of breast cancer risk based on epidemiologic and biologic risk variables at University of Kansas Medical Center. Blood and tissue samples were taken and DHQ (including usual supplement intake) were distributed when the subject were enrolled. Blood and breast tissue samples were analyzed by GLC and calculated by their area percentage.

The total n-3 and n-6 PUFA composition in TAG of breast tissue and blood were higher than mean percentages of total n-3 and n-6 PUFA in previous study by Maillard et al. (34) and Takata et al (43). The ratio of n-3 LCPUFA/n-6 PUFA in breast tissue and blood were lower than the previous studies (34, 43) when using a same combination of the fatty acids.

A correlation was found between the ratio of n-3/n-6 PUFA in blood samples and breast tissue in women at high risk of breast cancer. Total n-3/n-6 PUFA in PL in plasma and RBC was significantly correlated to that in fine needle aspirates ($p < 0.01$). The ratio of long chain n-3 to total n-6

PUFA in PL in plasma and RBC was found related to that in breast tissue as well ($p<0.01$), and the ratio of n-3 LCPUFA/ n-6 PUFA in PL in plasma was also significantly correlated to that in TAG in breast tissue ($p<0.01$), suggesting that the ratio of n-3/n-6 PUFA may be a good indicator of the fatty acids composition in breast tissue.

Therefore, the ratio of total or long chain n-3 PUFA to total n-6 PUFA might related to fatty acids composition in breast tissue and the results suggested the possibility of using fatty acids composition in blood as a biomarker to indicate the breast cancer risk. Further studies are still needed to confirm these results.

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APPENDIX A

Frozen FNA Sample Handling

1. FNA samples are collected into 250 µl sterile phosphate-buffered saline (PBS) in sterile 2 ml pp cryo-vials in the clinic. Two vials are collected for each breast. The first 2 sticks from the upper outer quadrant of one breast are collected into 1 vial (the PBS is used to rinse the FNA sample from the syringe needle) and immediately snap-frozen in liquid nitrogen. The first stick from the upper inner quadrant is collected into a second vial and snap-frozen. The procedure is repeated for the other breast into 2 additional vials. The first 1 or 2 sticks from each quadrant are usually not bloody; excessively bloody sticks are not included in the frozen FNA samples.
2. After all samples have been frozen in liquid nitrogen they are transferred to -80°C and transported (with -80°C frozen cold-packs) from the clinic to the research laboratory where they are stored at -80°C. At approximately 2 week intervals Teresa Phillips logs-in samples from the clinic and places the frozen FNA samples in labeled storage boxes at -80°C.
3. Frozen FNA samples from ~10 patients can be handled at one time for thawing, pooling, and aliquotting. Before removing samples from freezer prepare for each patient:
 - a) label 1 sterile pp 12 x 75 mm snap-cap tube for pooling sample,
place on ice

- b) label and prepare 1 sterile nuclease-free 1.5 ml tube for RNA extraction sample by adding 750 μ l TRIzol[®] LS to tube and leave at room temperature
 - c) label 1 sterile 1.5 ml tube for sample for cytokine analysis, place on ice
 - d) label 1 sterile 1.5 ml tube for sample to be transferred to Dr. Carlson's lab for fatty acid analysis, place on ice
 - e) label 1 sterile 1.5 ml tube for sample to be used for total protein assay (for "normalization"), place on ice
4. Remove frozen FNA samples from -80°C to ice. Working one patient at a time, thaw frozen tissue samples in cold water bath (cold tap water) until just thawed, then put vials on ice and keep on ice throughout procedure.
5. Using 1000 μ l pipettor, mix contents of each vial and transfer to 12 x 75 mm sterile pp snap-cap tube, on ice. Measure and record total volume. Mix well by pipetting a few times.
- a) Remove 250 μ l and add to 750 μ l TRIzol[®] LS, mixing well by pipetting. Leave at room temperature at least 5 min (longer is OK) then after all samples collected store at -80°C until ready to isolate RNA.

- b) Remove 250 μ l and transfer to sterile 1.5 ml tube for cytokine analysis (place tube into pulverized dry ice), store at -80°C .
6. Use Fisher 100 Sonic Dismembrator to lyse main sample, 3X setting 5 (≤ 15 W) 10-15 sec each, keeping sample tube submerged in ice.
 7. Transfer 250 μ l of sonicated sample to sterile 1.5 ml tube (place tube into pulverized dry ice), store at -80°C before transport to Dr. Carlson's lab for extraction and analysis.
 8. Transfer remainder of sonicate to tube labeled for total protein assay sample (place tube into pulverized dry ice); after all samples collected store at -80°C until ready to perform total protein assay.

APPENDIX B

Fatty Acid Analysis Protocol

METHOD:

Spinning Blood: RBC and Plasma

1. Before samples can be centrifuged, they must be balanced. To balance you will need an even number of tubes and they must have equal amounts of either sample or water. There are empty 4ml tubes to fill with water on the shelf to the left of the centrifuge.
2. Remove the caps and place in opposite buckets in centrifuge. Turn centrifuge on (switch is on the right side). Set the centrifuge to spin at 3000*g for 10 minutes at 4°C.
3. While blood is being spun, log the samples in KUDOS log book and set out three Pasteur pipettes with bulbs, and two 2 ml microcentrifuge tubes for each sample.
4. Each tube must be labeled with RBC/Plasma, subject ID, subject initials, baseline/ cord blood /postpartum/4 month, date, and initials of who spun the blood.
5. When samples are ready, transfer the top (yellow) layer of plasma to the tube labeled plasma with a Pasteur pipette.
6. After the plasma layer is removed, there is a thin white layer above the RBCs that needs to be removed. This layer may be discarded into the sink.

7. Last, using a clean Pasteur pipette transfer the remaining RBCs to the RBC labeled tube.
8. Layer each tube with Nitrogen and place in appropriate box in -80 freezer. The third shelf contains the tray for the KUDOS study.
9. Discard dirty pipettes in biohazard box on counter. Any contaminated gloves, bulbs, and bags must be thrown away in the biohazard waste.

Extraction:

Before starting the extraction: turn on the N-evaporator water bath, cut TLC plates into 4 plates and place in TempCon Oven, turn on TempCon Oven.

Set out 2 test tube racks and eighteen 15 mL tubes with caps. New tubes are required for the methylation step. Number tubes 1-6 so that there are 3 sets. Also, set up TLC chamber with 80:20:1 Hexane: Ether: Acetic acid. Place a sheet of filter in tank. Chambers last for about 2 days or about 12 samples.

In the first set of 15mL test tubes:

1. Pipette 4mL of methanol into clean extraction tubes.
2. Add 500µL RBC or Plasma, cap, and immediately vortex for a few seconds.
3. Add 100µL internal standard (17:0) and vortex. The 17:0 PE aliquots are kept in the -80 freezer in labeled box on the 3rd shelf.

4. Add 8mL Chloroform and vortex. Use the multi-tube vortexer on the lab counter. 15 minutes for RBC and 10 minutes for Plasma.
5. Transfer contents through a funnel lined with filter paper (Whatman #1) into a clean extraction tube.
6. Add 1.6mL (0.5 Molarity) KCl and vortex for 10 seconds.
7. Centrifuge for 5 minutes at 750 r.p.m. and then using a clean Pasteur pipette discard upper phase into appropriate hazardous waste bottle.
8. Evaporate the lower phase in a water bath at 35°C under nitrogen. This takes about 30-45 minutes. [Add 1-2mL benzene and re-evaporate if small amounts of water remain].

*While waiting for samples to evaporate, pipette 1mL BF₃ into 3rd set of extraction tubes and place on ice. Turn on dry bath.

Separation of total phospholipids:

9. When extract is completely dry, dissolve in 100µL dichloromethane and place immediately on ice until ready to spot plates.
10. When plates have been heated for at least 20 minutes, take out of oven and allow to cool. Then spot all 100µL of a sample on the plate and place immediately in the TLC chamber with 80:20:1 Hexane: Ether: Acetic Acid. (Two spotted plates fit in the chamber at a time).

11. Allow the solvent front to run to the top of the plate and then remove from TLC chamber to dry.

12. Identify phospholipid line (it is the same line as the spotting line).

Remove the gel containing the lipid fraction of interest with a single edge razor blade onto weighing paper. Carefully transfer the gel to the 3rd set of extraction tubes containing 1 ml cold BF₃.

Separation of triacylglycerol:

13. When extract is completely dry, dissolve in 100 μ L dichloromethane and place immediately on ice until ready to spot plates.

14. When plates have been heated for at least 20 minutes, take out of oven and allow to cool. Then spot all 100 μ L of a sample on the plate and place immediately in the TLC chamber with 80:20:1 Hexane: Ether: Acetic Acid. (Two spotted plates fit in the chamber at a time).

15. Allow the solvent front to run to the top of the plate and then remove from TLC chamber to dry. Spray BBOT on the plate
Identify triacylglycerol line under UV reader (it is the middle band on the plate). Remove the gel containing the lipid fraction of interest with a single edge razor blade onto weighing paper. Carefully transfer the gel to the 3rd set of extraction tubes containing 0.25ml cold BF₃, 0.2ml benzene and 0.55ml methanol.

Transmethylation:

16. Layer each tube with Nitrogen and place on ice until all samples are finished spotting.
17. Tighten caps and place in dry bath at 100°C for 10 minutes.
18. After 1-2 min. retighten caps as they tend to loosen during heating.
19. After transmethylation is complete, immediately place tubes on ice.

When tubes are very cool, open and add 1ml of H₂O and 2 mL pentane.
20. Vortex for 1-2 minutes to extract fatty acid methyl esters (FAME) into the pentane phase, then centrifuge for 5 min. at 800 r.p.m.
21. Transfer the upper phase (pentane) with a Pasteur pipette to a Varian 2mL vial. Transmethylation tubes may be thrown away in the glass container when done.
22. Concentrate the FAME under a stream of Nitrogen.
23. When completely dry, add 70µL of dichloromethane to vial. Swirl tube to catch FAME on all sides and then transfer with Hamilton syringe to sleeve. Place sleeve inside of 2 mL vial and cap with Teflon-lined cap.
24. Place sample in autosampler tray to inject or in freezer until it can be analyzed.