CHARACTERIZATION OF FATTY ACID PROFILE IN BREAST TISSUES FROM MANITOBA BREAST CANCER PATIENTS

By

Nazila Azordegan

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Abstract

Background: Breast cancer is the most prevalent cancer diagnosed in women and the second most common cause of female cancer mortality worldwide. Amongst the several risk factors identified for the development of breast cancer, the amount and type of the dietary fat have been the focus of many recent studies.

Objective: This study was carried out to investigate the fatty acid composition of tumoral, marginal (the tissue surrounding the tumoral tissue) and normal breast tissue in female breast cancer patients. It also examines the correlation between the plasma and red blood cell fatty acid profile and severity of the cancer.

Methods: Patients were recruited from St. Boniface General Hospital (SBGH), signed a consent form and filled out a food questionnaire. On Pre-Operative assessment clinic, blood work was done and plasma and red blood cells were separated. After surgery, sections were obtained from tumoral, marginal and normal breast tissues for histology and biochemical analysis. Data were analyzed by analysis-of-variance (ANOVA) testing followed by posthoc tukey test.

Results: We had fourteen female breast cancer patients in our study. They were mostly (63.4%) postmenopausal with mean age of 61.5 years and BMI of 28.1. Half of the patients rarely or never consumed fast food, 64.3% consumed 2 or fewer servings of fish per month, 85.8% were non-smoker. Extracted lipids from marginal tissue were significantly higher than those in normal or tumoral tissue. The lipid profile in tumoral tissue was significantly different in terms of fatty acid composition compared to normal and marginal tissue with less linoleic and alpha linolenic acid and more long chain polyunsaturated fatty acid (PUFA) of omega-3 and omega-6 series. Marginal tissue showed significantly less alpha linolenic acid compared to normal tissue.

There was an inverse association between plasma free fatty acids and grade of breast cancer. A positive correlation was observed between plasma 20:3 n-6 and 18:1 t4 and breast cancer grade. Red blood cells' level of 20:3 n-6 and 22:5 n-6 were positively correlated with breast cancer grade. An inverse correlation existed between plasma level of 22:6 n-3 and breast cancer stage.

Conclusion: Reviewing the literature shows tumor promoting effect of omega-6 series of PUFA and anti-proliferative effect of omega-3 PUFA. The results of our study also showed that high red blood cell omega-6 PUFA and low plasma omega-3 PUFA were associated with breast cancer of higher grade and stage respectively. Furthermore we found significantly different lipid profile in tumoral tissue compared to normal and marginal tissue. The changes in the fatty acid profile could be reflecting the alteration in homeostasis or a response to tumor proliferation. A comprehensive knowledge of breast cancer biology is crucial to developing new treatment and preventive modalities.

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Dedication

I would like to dedicate my Master's thesis to my wonderful parents for their unconditional love and endless support.

TABLE OF CONTENTS Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

EPA	eicosapentaenoic acid
DHA	docosahexaenoic acid
DPA	docosapentaenoic acid
ALA	alpha linolenic acid
TG	triglyceride
FA	fatty acid
PUFA	polyunsaturated fatty acid
MUFA	monounsaturated fatty acid
CLA	conjugated linoleic acid
SFA	saturated fatty acid
SM	sphingomyelin
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PS	phosphatidylserine
CE	cholesteryl ester
PL	phospholipid
FFA	free fatty acid
PG	prostaglandin
TX	thromboxane
SBGH	St. Boniface General Hospital
PTEN	phosphatase and tensin homolog
ATM	ataxia telangiectasia mutated
EGFR	epidermal growth factor receptor
SCC	squamous cell carcinoma
AMACR	alpha-methylacyl-CoA racemase
TLC	thin layer chromatography
GC	gas chromatography
FFQ	food frequency questionnaire
ANOVA	analysis of varience
BRCA	breast cancer
ER	estrogen receptor
PR	progesterone receptor
HER2	human epidermal growth factor receptor 2
IUPAC	The International Union of Pure and Applied Chemistry
VA	vaccenic acid
RA	rumenic acid
IDC NOS	invasive ductal carcinoma not otherwise specified
SBR	Scarff-Bloom-Richardson
BR	Bloom-Richardson
DCIS	ductal carcinoma insitu
LCIS	lobular carcinoma insitu
RBC	red blood cell

EURAMIC European community multicenter study on antioxidants, myocardlal infarction, and breast cancer

E3N-EPIC French cohort of the European Prospective Investigation into Cancer and Nutrition

1. Introduction

Breast cancer is a major health concern and its burden on public health is significant and on the rise (1). Increasing epidemiologic and biochemical evidence suggest that high fat diet is a significant risk factor for the development of breast cancer. The lipid content of diet can modify the progression and aggressiveness of the tumor via different mechanisms such as influencing the gene expression (2), changing the hormonal status, body mass index, oxidative stress and drug metabolism (3).

Although the effect of different fatty acids on risk of cancer in hormone sensitive organs has been investigated in many experimental studies, the exact protective lipid panel is not yet known. Reviewing the literature shows inconsistent results on the effect of individual fatty acids on breast cancer. A number of studies shows beneficial effects of long chain n-3 polyunsaturated fatty acids (PUFA) on breast cancer by slowing the proliferation of tumor cells, and reducing their metastatic potential (4) and detrimental effects of n-6 PUFA as a result of promoting cell proliferation. In a case-control study on seventy-three women with breast cancer and seventy-four controls, it was shown that at a given level of breast adipose tissue n-6 PUFA, long chain n-3 PUFAs (eicosapentaenoic acid and docosahexaenoic acid) may have a favorable effect on breast cancer risk (5).

On the contrary Witt et al indicated no association between the content of total or individual marine n-3 PUFA in adipose tissue and development of breast cancer (6). In an animal study in order to determine the effect of fatty acids on later development of breast cancer, prepubertal rats were fed low (16% energy) or high-fat (39% energy) diet composed mainly of n-6 or n-3 polyunsaturated fatty acids. The carcinogen 7,12-dimethylbenz[a]anthracene was used to induce the cancer. The results showed that prepubertal exposure to a low-fat n-3 PUFA diet reduced

later mammary tumorigenesis, while high levels of n-3 PUFA increased the risk; high fat n-6 PUFA diet had no effect on subsequent risk of breast cancer (7).

The conflicting results emphasize the need to consider interactions between fatty acids when evaluating associations between polyunsaturated fatty acid intakes and breast cancer risk (8).

The purpose of the present study was to investigate the lipid profile of different lipid classes in tumor, its adjacent tissue and normal looking breast tissue in female breast cancer patients. We have also examined the grade and stage of the tumor and the lipid constituents of the plasma and red blood cells.

Tissue composition of fatty acids particularly the essential fatty acids is in part dependant on the dietary intake. Thus tissue lipid profile would represent the actual rather than the self-reported dietary intake (9). It is suggested that the interaction between various types of fatty acids modulates the cell growth and differentiation (10) and can be the cause or the consequence of cancer.

2. Literature Review

2.1.Epidemiology: Each year more than a million women are diagnosed with breast cancer and about 440,000 will die from the disease (11). Although it was once considered a disease of the western society, it has now become a global concern (12). There is a fivefold difference in incidence and mortality of the breast cancer around the world. While the incidence is higher in the developed countries the rate is also increasing by up to 5% in developing countries (13). It is estimated that one in every nine women will develop breast cancer sometime during their lifetime and one out of every 28 women with breast cancer will die from the disease.

The most recent Statistics Canada survey reports 19,900 new cases of breast cancer yearly; Manitoba with 730 new cases per year stands at the first place among other provinces in Canada (population adjusted). It is estimated that 220 women will die from breast cancer yearly in Manitoba (14).

2.2. Risk factors: All of the known risk factors for breast cancer account for only 30% of the cases and the cause for the rest of the cases still remain unidentified. There are a number of risk factors recognized for breast cancer which are classified into two major groups of non-modifiable like genetics and modifiable ones such as lifestyle and environmental factors (15). Gail model presents a method to estimate the risk of breast cancer in an individual based on age at menarche, age at first live birth, number of previous biopsies, and number of first-degree relatives with breast cancer (16). Earlier age of menarche, late menopause, older age at first pregnancy and increased density on mammogram have been shown to increase the risk of breast cancer (17).

2.2.1. Non-modifiable risk factors

2.2.1.1. Genetics: Breast cancer is characterized by various genetic mutations and aberrations (18, 19). Although hereditary factors are estimated to account for 25% of the breast cancer cases, high-risk mutations account for only 5% of all cases (20).

Five known high risk mutations are BRCA1, BRCA2, P53 (as in Li-Fraumeni cancer syndrome), PTEN (Phosphatase and Tensin Homolog in Cowden syndrome) and ATM (ataxia telangiectasia mutated). Studies show cancer clustering in certain families with some genetic mutations which are occasionally associated with specific syndromes (21). It has been shown that many cases of sporadic breast cancer with normal BRCA1 gene have BRCA1 protein displaced from its normal nuclear location to the cytoplasm (22).

2.2.1.2.Age: The incidence of breast cancer increases with age more sharply in the reproductive years and then at a slower pace after menopause (20). High concentration of estrogen during reproductive age can be responsible for increased incidence of breast cancer during this period.

Recent studies show even a more distinct age pattern for breast cancer considering estrogen receptor (ER) expression and/or histopathologic subtypes. Age incidence for all histologic types of breast cancer except medullary carcinoma showed a bimodal pattern with peaks at near ages of 50 and 70 years. Medullary carcinoma showed a single peak around age of 50 years (23).

2.2.2. Modifiable risk factors

Environmental factors consisting of diet and lifestyle are responsible for 25-40% of preventable causes of cancer (24).

2.2.2.1.Alcohol: Alcohol is shown to be an independent risk factor for breast cancer (17). Recent studies show a linear dose-effect relationship between alcohol consumption and breast cancer risk. It shows 7% increase in risk of breast cancer for every ten grams of alcohol consumption

per day (25). Insufficient folate intake can further increase the risk of breast cancer in alcoholic women (26). Two major mechanisms suggested for the effect of alcohol on breast cancer are its interaction with DNA repair (27) and interference with estrogen level (28).

2.2.2.2.Smoking: It is evident that smoking of one cigarette per day for at least three months increases the risk of breast cancer (29). A recent study in Canada on 347 patients with breast cancer and 775 population–based controls show that both passive and active smoking increase the risk of breast cancer. The effect may be differentially modified by N-acetyl transferase 2 (NAT2) phenotype (30).

2.2.2.3.Diet: The results of meta-analysis and review of epidemiological cohort studies indicate that some dietary patterns are linked to breast cancer risk (31). Furthermore breast carcinogenesis and its relationship to dietary fat or any specific fatty acid has been a subject of debate (32). Although a large body of research shows an association between dietary fat and breast carcinogenesis, it is still not well understood and remains a matter of controversy (33).

Schulz et al suggested a food pattern instead of a single-nutrient approach to determine the role of diet in breast cancer. They identified a food pattern characterized by low consumption of bread, and fruit juices, and high consumption of processed meat, fish, butter and other animal fats, and margarine explaining >42 % of total variation in fatty acid intake. High consumption of all the four fatty acid fractions (saturated, monounsaturated, n-3 PUFA, n-6 PUFA) was linked to increased risk of breast cancer (34).

In a case control study of 1119 subjects (565 cases and 554 controls), it was shown that a higher dietary (n-3)/(n-6) PUFA ratio may reduce the risk of breast cancer, especially in premenopausal women (35).

Lipids are the most important dietary components which can affect the development of cancer. Alteration in fatty acid profile can affect every stage of carcinogenesis; it can influence the angiogenesis and hence the cancerous cell behavior. It has been investigated in different studies that the action of one fatty acid on carcinogenesis depends on its interaction with other lipid components(24); therefore its biological effect on the cellular level depends not only on the total amount of an individual fatty acid but also on its ratio to other fatty acids (36).

2.3. Lipids:

2.3.1.Classification: Lipids play an essential role in cell signaling and subcellular structure and are therefore considered as fundamental biomolecules (10).

The hydrophilic and hydrophobic characteristics of lipids make it very special in context of cell membrane. The main building block of lipids is the fatty acyl, which is a hydrophobic chain of methylene group with a carboxylic group at one end (Figure 1).

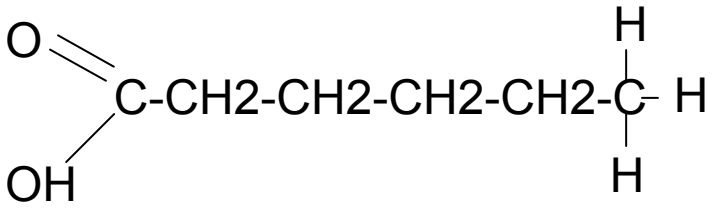


Figure 1- Structure of a fatty acid

2.3.2. Nomenclature of fatty acids

Fatty acids are classified according to their chain length and the degree of saturation. Saturated fatty acids have no double bonds, while monounsaturated has one and polyunsaturated fatty acids have more than one double bond. There are two classification systems for identification of fatty acids considering the number of carbon atoms and presence of double bonds. The systematic name using IUPAC (the International Union of Pure and Applied Chemistry) nomenclature shows the number of carbon atoms followed by the number of double bonds and

the positions of double bond from the carboxyl end as in linolenic acid $18:3^{D\ 9,12,15}$ which indicates the 18 carbon fatty acid with 3 double bonds located at carbons 9, 12 and 15 numbering from the carboxyl group.

The alternative name numbers the carbon atoms from the distal methyl (n or ω) end such as 18:3 (n -3) indicating an 18 carbon atom fatty acid with 3 double bonds in which the first double bond is located at the third carbon atom starting from the ω end.

2.3.3. Trans and Cis configurational isomers

The orientation of the hydrogen atoms with respect to the double bond describes the cis and trans configurational isomers of the fatty acids (Figure 2). In cis isomer (mainly seen in naturally occurring fatty acids) hydrogen atoms are at the same side of the chain, while the hydrogen atoms at trans isomers are on opposite sides of the double bond. In trans isomer the double bond has a larger angle to the chain compared to cis isomer and hence the bond between the acyl chain is tighter (37). Trans fatty acids can be made as a result of hydrogenation of oils to increase its plasticity and chemical stability. They can also be formed in nature in meat and dairy product as a result of anaerobic bacterial fermentation in ruminants (38).

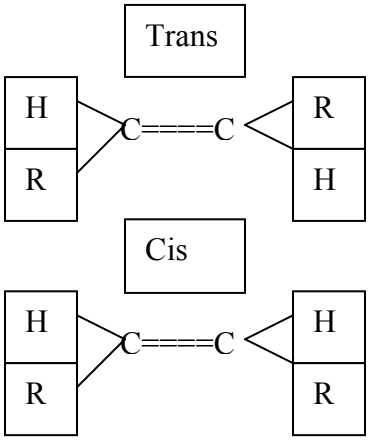


Figure 2- Cis and trans isomers of fatty acids

2.3.4. Essential fatty acids

The majority of fatty acids are endogenously synthesized like monounsaturated and saturated ones; however essential fatty acids (linoleic and alpha linolenic acids) cannot be made in the body (39).

Alpha linolenic acid (18:3 n-3) is metabolized to Eicosapentaenoic acid (EPA) (20:5 n-3) by Δ-6 desaturation -a rate limiting step(39) -and elongation on the endoplasmic reticulum (40); it will further metabolize to Docosahexaenoic acid (DHA) (22:6 n-3) via Δ-5 desaturation and elongation (41).

Alternatively linoleic acid (18:2 n-6) is elongated and desaturated to 22:4 n-6 and 22:5 n-6 in the same pathway of alpha linolenic acid which explains the competitive interaction between the n-3 and n-6 metabolites for enzyme action (Figure 4) (42).

Only a very limited amount of EPA and DHA (<5-10% for EPA and 2-5% for DHA) are synthesized from alpha linolenic acid invivo and are therefore highly dependant on the individual dietary intake (43).

2.3.5. Phospholipids

Phospholipids are the major constituent of the cell membrane. Accordingly incorporation of n-3 PUFA into membrane phospholipids regulates the cellular function. The main phospholipids are sphingomyelin (SM), phosphatidylserine (PS), phosphatidylcholine (PC), lysophosphatidylcholine (LPC), phosphatidylinositol (PI), and phosphatidylethanolamine (PE) (44). Sphingomyelin is mainly found in the cell membrane of the neurons and has a sphingosine base instead of a glycerol structure.

Phosphatidylserine, Phosphatidylinositol and Phosphatidylethanolamine are also mainly incorporated into nervous system and myelin sheath. Phosphatidylcholine is the major

phospholipid of the cell membrane. Lysophosphatidylcholine is the final product of phospholipase enzyme action and hence it lacks fatty acid at the position of the second carbon. The structure of phospholipid is comprised of a glycerol backbone with fatty acids on first (C1) and second carbon (C2) and one phosphate group at the third carbon (C3). Most phospholipids have a saturated fatty acid at C1 and an unsaturated fatty acid at C2. (Figure 3) (44).

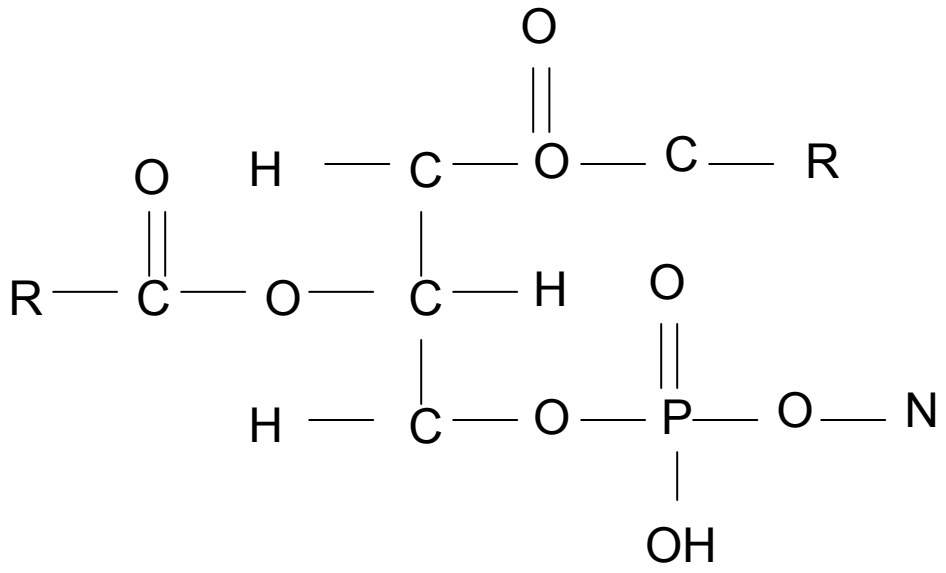


Figure 3. The structure of a phospholipid is shown with a glycerol backbone, fatty acids at C1 and C2 and a phosphate group attached to a nitrogen containing molecule (N) on C3.

2.3.6. Fatty acid metabolites

Various eicosanoids like prostaglandins (PG), thromboxanes (TX), leukotrienes, hydroxyl fatty acids and lipoxins are derived from polyunsaturated fatty acids. These metabolites have opposing effects on the regulatory system. In general eicosanoids (PG and TX) derived from n-6 PUFA are mainly inflammatory, pro-aggregatory and growth promoting, while the majority of the ones derived from n-3 PUFA are anti-inflammatory, anti-aggregatory and anti-proliferative (45).

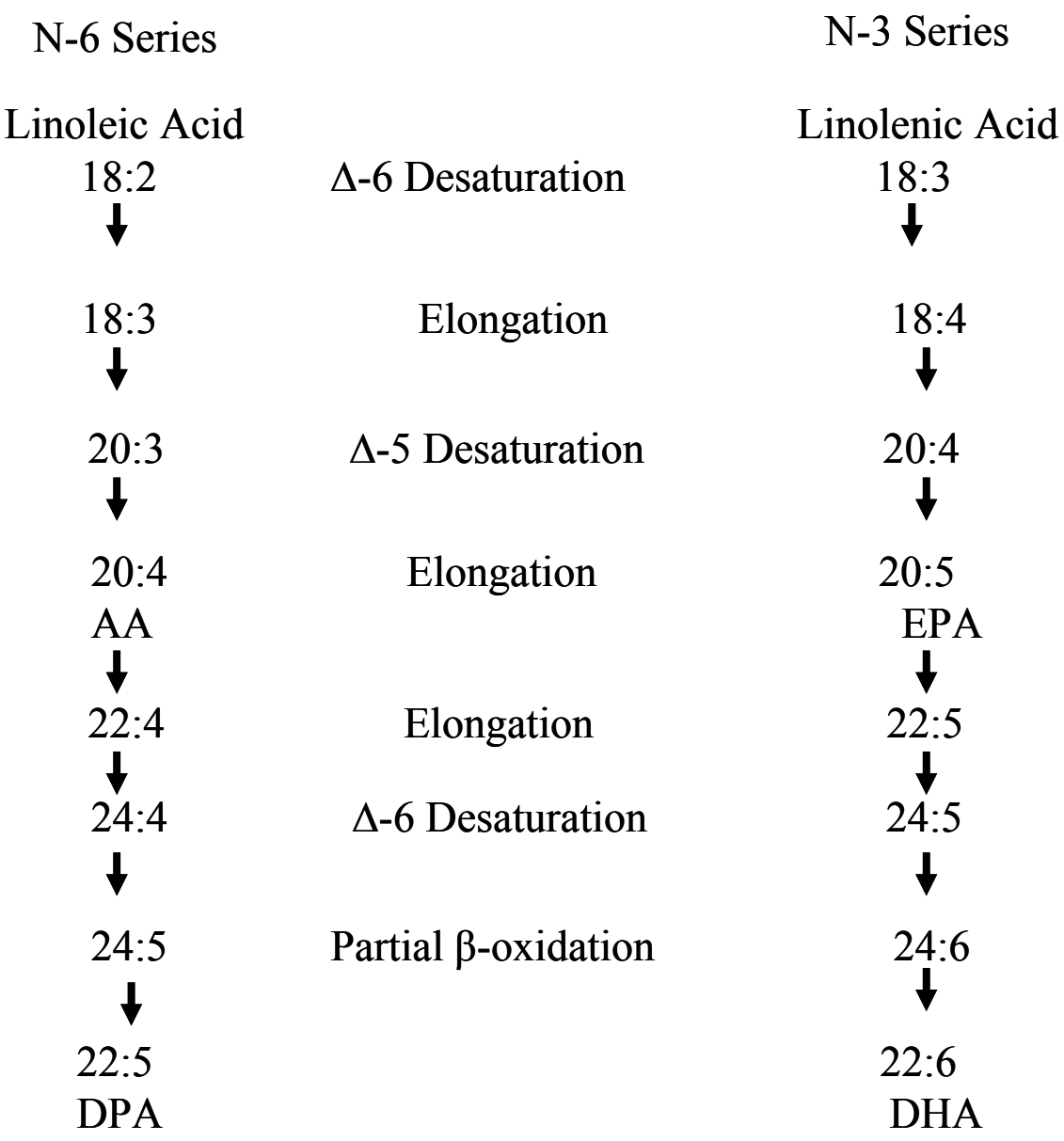


Figure 4. Biosynthesis of n-3 and n-6 PUFA

2.3.7.Major sources of fatty acids

Alpha linolenic acid is found mainly in linseed, green leafy vegetables, flaxseed, canola oil, soybeans and walnuts. Linoleic acid is found in vegetable oils like corn and safflower, while arachidonic acid is found mainly in animal products (43, 46).

The major sources of EPA and DHA are fish, fish oil and algae. The cold water fatty fish like salmon, mackerel, sardine and herring contain highest concentration of EPA and DHA (47).

Elaidic acid a monounsaturated fatty acid (9-trans isomer of oleic acid) can be found in partially hydrogenated oils (Table 1). Vaccenic acid (11-*trans* octadecenoic acid; VA) is a major *trans* fatty acid in the fat of ruminants which is converted in tissues to rumenic acid (9-*cis*, 11-*trans* octadecenoic acid; RA), an isomer of conjugated linoleic acid, by Δ^9-desaturase (48).

Table 1. Major sources of different fatty acids

Fatty Acid	**Major sources**
EPA and DHA	Fatty acid, Fish oil and algae (47)
Alpha linolenic acid	Green leafy vegetables, flaxseed, canola oil, soybeans and walnuts (43)
Linoleic acid	Vegetable oils (corn, safflower, sunflower, soybean) (43)
Arachidonic acid	Animal products (meat, eggs) (43)
Elaidic acid	Sandwiches, pizzas, pastries, crackers, cakes (49)

2.4. Histologic types of breast cancer

Breast cancer is the most common type of cancer in women worldwide (50). It affects the upper outer quadrant of breast in about 50% of the cases. Normal breast parenchyma, a modified sweat gland, is composed of branching ducts and lobules surrounded by fibrotic connective tissue and distributed within adipose tissue.

Histologically the majority of breast cancers are classified as invasive ductal carcinoma not otherwise specified (IDC NOS). The remaining 25% are categorized as special types with different prognosis and treatment choices (51, 52). Breast carcinoma can be either *in situ* when confined to the glandular structures or invasive when a stromal invasion is evident (Figure 5).

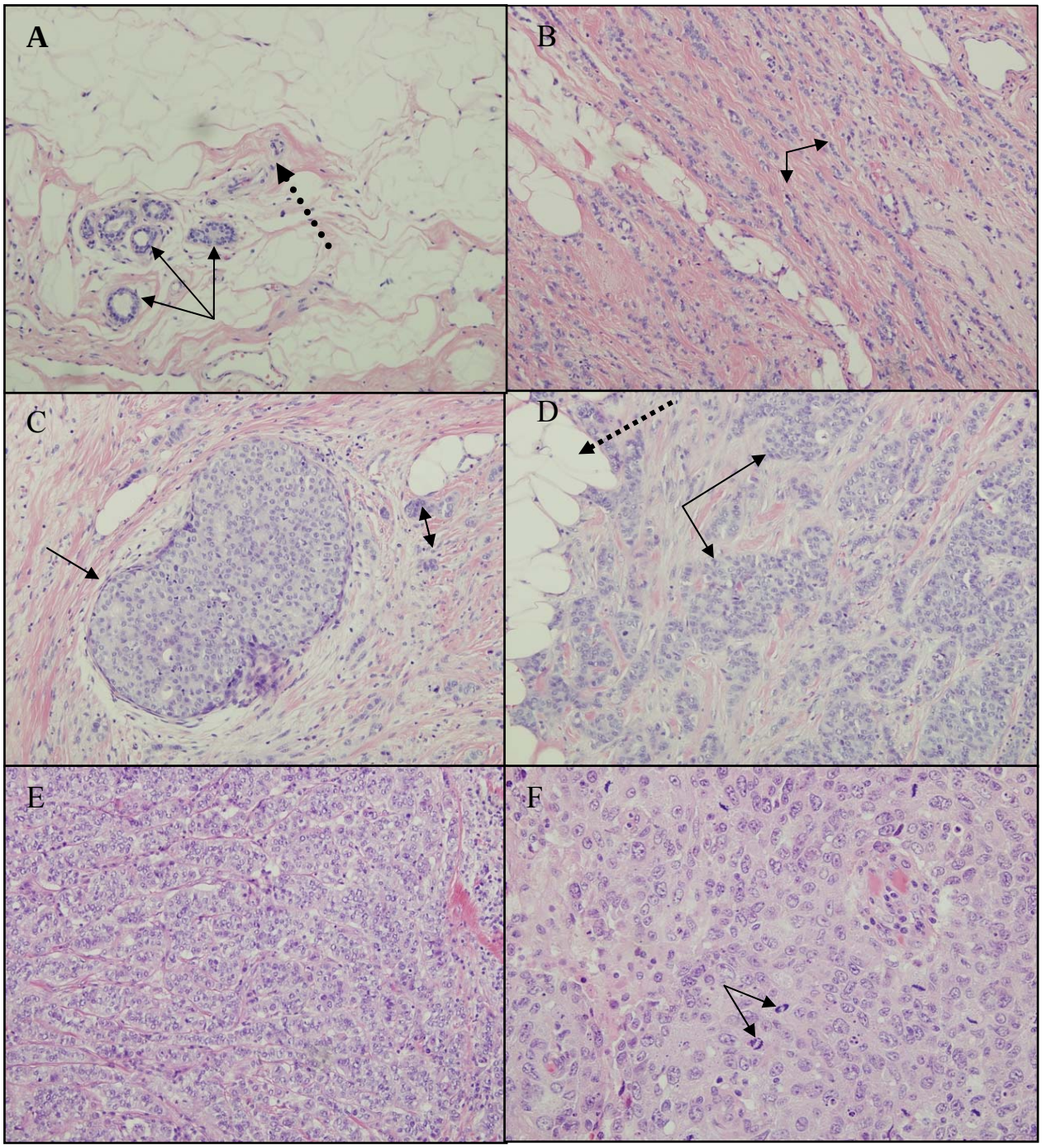

A
B
C
D
E
F

Figure 5. Hematoxylin and Eosin staining histological sections of normal and tumoral breast tissue (A) Section of normal breast tissue, lobules (arrow) are surrounded by connective tissue (dashed arrow) and adipose tissue (x 200) (B) Section of invasive lobular carcinoma, small malignant cells in single files (double arrow) are invading the breast tissue (x 200) (C) Section of a high grade ductal carcnioma with both invasive (double-headed arrow) and *in situ* (arrow) component in a desmoplastic stroma (H& E x 200) (D) Section of a high grade ductal carcinoma, clusters of tumoral cells (double arrow) are surrounded by desmoplastic stroma. The remaining adipose tissue is also evident (arrow) (x 200) (E) Section of an intermediate grade ductal carcinoma, back-to-back clusters of tumoral cells are seen with a delicate stroma in between (H& E x 200) (F) Section of invasive metaplastic carcinoma of breast, squamoid malignant cells with polygonal eosinophilic cytoplasm and multiple mitotic figures are shown (double arrow) H& E x 400).

2.4.1. Prognostic factors

The major determinants of breast cancer prognosis are the grade and the stage of the tumor along with some biomarkers.

2.4.1.1.Grade: Histologic grading of breast cancer is mostly performed by the modified Bloom-Richardson grading system and is based on three morphologic features, the percentage of tubule formation, nuclear pleomorphism and the mitotic activity (53). The final grade of the tumor is defined by summation of the individual scores (54).

2.4.1.2. Stage: The most important predictor of breast cancer prognosis is its stage which is often assessed by American Joint Committee on Cancer (54, 55). It is based on the size of the primary tumor (T), the lymph node status (N) and the presence or absence of metastasis (M).

2.4.2. Biomarkers

Breast cancer is now diagnosed and managed clinicopathologically with identification of various molecular biomarkers which aid in prognostic and treatment modalities.

Among several biomarkers known, hormone receptors (estrogen and progesterone) and human growth factor receptor 2 (HER2) are key biological predictors in breast cancer treatment and management (Figure 6) (56).

A study done on over five hundred patients with breast cancer showed increased local recurrence with peritumoral vascular invasion, multifocality, and absence of estrogen receptors (57). Breast cancer with double hormone positive (ER +, PR +) and negative HER2 has the best prognosis with the least risk of local recurrence and longest survival rate (58).

Pregnant or lactating women, late diagnosis, presence of invasiveness or distant metastasis also decrease the survival rate. Morphologically aside from the SBRS features, presence of tumor necrosis, stromal reaction, microvessel density, and elastosis are associated with more aggressive

type of breast cancer. In addition to hormonal (estrogen, progesterone) and HER2 receptor, there are other biomarkers which relate to prognosis. Accumulation of P53 protein, vimentin positivity and negative or weak Bcl-2 expression are associated with poor prognosis (59).

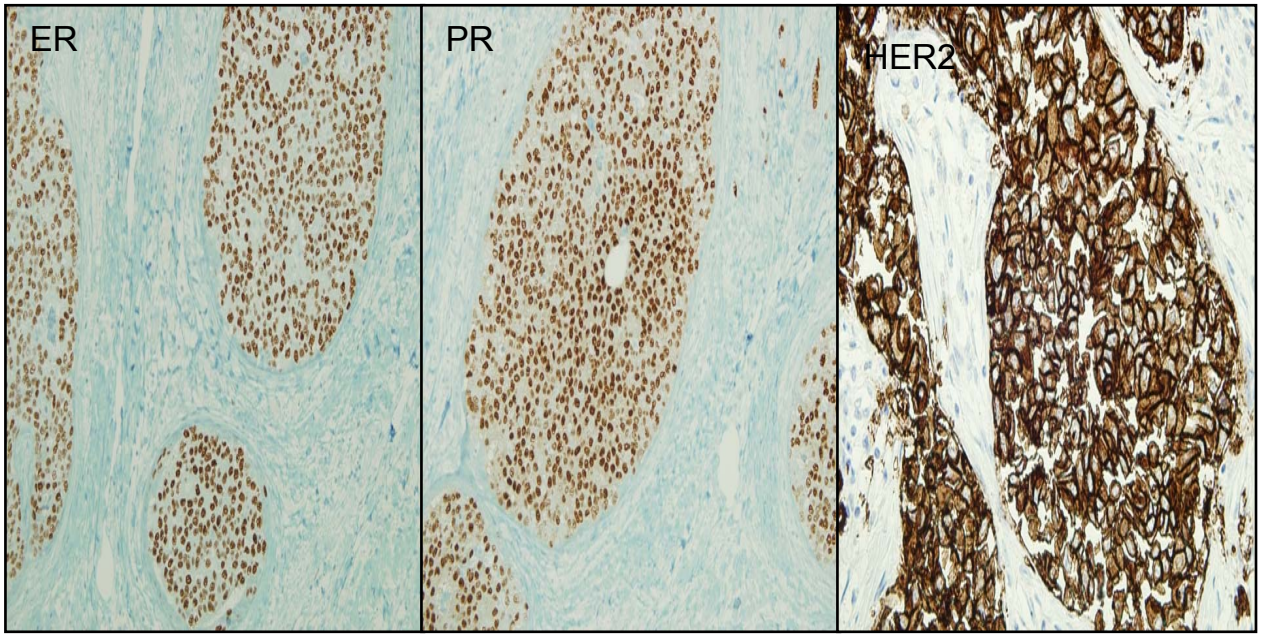


Figure 6. Immunohistochemical staining (x400). Nuclear staining for ER and PR and membranous staining for HER2 have been shown.

2.4.3. Hormone Receptors

2.4.3.1. Estrogen Receptors

The majority of breast cancers (up to 75%) are positive for estrogen receptors. Although this category of breast cancer is generally well differentiated, less aggressive and benefit from hormonal therapy, long term prognosis of ER + and ER – tumors show almost the same survival rates. Hormonal therapy can aim to either inhibit the activity of estrogen (selective estrogen receptor modulators) or block its production from androgen (aromatase inhibitors) **(56)**.

2.4.3.2. Progesterone Receptors

Progesterone synthesis is regulated through the action of estrogen. Therefore it is not expected to see progesterone receptor positive cell in an estrogen receptor negative cell. However, this hormonal combination do occur which might be explained by either an error in the assay or the silent expression of estrogen receptor gene.

Some studies show that progesterone receptor positive breast cancer exhibit better prognosis than progesterone receptor negative; however, its prognostic value still remains unanswered **(56)**.

2.4.3.3. Combinational hormone assessment

It has been shown that the use of a combination of hormonal assays can be more helpful in clinical management of the breast cancer. Consequently different combination of estrogen and progesterone receptor status identifies different types of breast cancer. These subtypes are ER+/PR+, ER+/PR-, ER-/PR- and ER-/PR+ and each shows a subtype of breast cancer with different biologic characteristics.

The double positive group (ER+/PR+) mostly respond to anti-hormonal therapy and show less invasive behavior, while double negative subtype (ER-/PR-) do not generally respond to

anti-hormonal therapy and have shorter survival rate. Chemotherapy is the option of choice for patients with double negative hormone receptors (60).

2.4.3.4. Human epidermal growth factor receptor 2 (HER2):

HER2 is an epidermal growth factor receptor which has been used in assessment of breast cancer since 1987. Generally it has an inverse relationship to hormonal receptor status; positive HER2 receptor is mostly seen in hormone negative tumors. The positivity of HER2 receptor defines a group of breast cancer which responds to anti-HER2 receptor drugs (Herceptin). Although HER2 positive breast cancer usually defines a group of poorly differentiated and aggressive tumors, there is a complete difference in behavior depending on the status of lymph node.

2.5.Experimental Studies

Cancer is the clinical presentation of the genetic alterations inside the cell. Studies show that dietary intake particularly the lipid content of the diet can influence the development of cancer (24). With the high prevalence of breast cancer, many experimental *in vivo* and *in vitro* studies have tried to investigate its possible preventable causes. It has been shown that the amount and types of the lipids alter the cell growth and behavior. Studies suggest that specific fatty acids can reduce the risk of breast tumorigenesis (61), however there is inconsistency in reported data.

This discrepancy could be in part due to different study designs, methods and models used and partly because it is not the fatty acid in isolation that matters but rather the ratio and its proportion to other fatty acids. Therefore the balance between the fatty acids is of utmost importance in cell regulatory system (62).

2.5.1.Animal Studies

The effect of various diets on fatty acid composition of different lipid fractions has been studied in various animal studies. Fatty acid composition of hepatocyte cell membrane was shown to be modified feeding rats with different dietary fats. This chemical change in cell membrane can subsequently alter the cellular function (63). Therefore any change in dietary lipid component is expected to affect the tissue composition. A study on normal and diabetic rats investigated the effects of dietary fats on phospholipid fatty acid composition and enzyme activity of hepatic microsomes. Rats were fed three different diets with menhaden oil, saturated fat or normal rat chow diet. Rats on menhaden oil diet showed reduced Δ5 desaturase activity and increased incorporation of DHA into hepatic microsomes of diabetic rats (64).

Studies in rodent models of breast carcinogenesis have considered potential effects of amount and type of the dietary fat. A large body of evidence from observational animal studies show that high fat diets and high amount of polyunsaturated fatty acids of the omega-6 class can promote the growth of mammary tumors while consumption of omega-3 rich low fat diet can inhibit its growth (65).

Mice overexpressing HER2/neu in mammary glands demonstrated a tumor promoting effect of high fat diet (45% of calories) compared to low fat diet (10% of calories) (65).

Extra-virgin olive oil showed chemoprotective effect on dimethylbenz(alpha)anthracene-induced breast cancer rats while high corn oil had a stimulating effect (66).

Studies show the protective effect of lignan rich diet in breast cancer. Flaxseed, which is rich in phytoestrogen lignans, reduced the growth of breast cancer cells in athymic mice with low plasma estrogen levels (67).

2.5.2.Clinical Studies

The study of essential fatty acid composition of plasma (as a short term estimate of dietary consumption), washed red blood cells (as a 120-day estimate) and adipose tissue (very long-term biomarker of 2.5 to 3 years) can provide a reflection of dietary intake (68-70). The adipose tissue due to its low lipid turnover (71) is an insensitive marker of recent dietary intake(24). It will reflect an actual rather than reported dietary consumption.

Analytical epidemiology has investigated differential plasma PUFA levels and plasma fatty acid composition in men and women. Ogura et al concluded no significant difference between PUFA levels of plasma and RBC phospholipid fractions and adipose triglyceride among men and women. They showed a positive correlation between age and plasma levels of n-3 PUFA and inverse correlation between age and n-6 PUFA in both men and women. They suggested that although adipose tissue PUFA level is the best indicator of long term dietary PUFA, it is an invasive method and therefore the fatty acid composition of plasma and RBC phospholipid may be a better biomarker of PUFA consumption compared to adipose tissue triglyceride (72).

In contrast to the previous study, the results of another study indicate a difference in relative proportion of n-3 long chain PUFA in serum lipids between men and women. Women had lower EPA and docosapentanoic acid and higher DHA compared to men (73).

Human studies investigating the effect of diet on fatty acid composition of breast cancer tissue are limited (33). However, recent studies demonstrate a change in lipid metabolism and lipid profile in patients with breast cancer compared with disease-free individuals.

Bagga et al performed a 3-month study on twenty-five high-risk localized breast cancer patients. They showed that consumption of low fat diet and daily fish supplement can increase the ratio of polyunsaturated fatty acid of omega-3 to omega-6 class considerably both in tissue and plasma.

The increase in the ratio of omega-3 to omega-6 was more pronounced in breast tissue compared to buttock tissue. This differential dietary effect indicates that dietary modification of omega-3/omega-6 polyunsaturated fatty acid ratios in patients with breast cancer might have some beneficial effect on breast cancer (74).

Data from the EURAMIC (European countries in the European Community Multicenter Study on antioxidants, myocardial infarction and cancer) study also reported a positive association between the low ratio of n-3 PUFA to n-6 PUFA in adipose tissue and risk of breast cancer (62). Reviewing the literature shows that it is not simply the amount of an individual fatty acid that alters the risk of the breast cancer but rather the proportion and relative amount of that fatty acid to other fatty acids. Investigation on 322 women with breast cancer and 1030 matched controls showed a direct association between breast cancer risk and serum levels of palmitic, γ linolenic, palmitoleic and vaccenic acids and inverse association with total n-3 fatty acids, EPA and the saturation index of palmitic to palmitoleic acids (75).

Punnonen et al showed a difference in fatty acid composition of phospholipids in breast cancerous tissue and normal breast tissue. The relative amount of unsaturated fatty acids was increased in all the subclasses of phospholipids in tumor compared to normal tissue. They reported significantly more phosphatidylcholine and phosphatidylethanol-amine and less sphingomyelin in cancerous tissue. Fatty acid composition of red blood cells of their subjects was comparable to that in the reference population (76).

One hundred sixty one patients with breast cancer in T1N0 stage had subcutaneous adipose tissue aspiration (breast and abdominal) at the time of surgery. Although the overall survival Long term follow up of these patients showed a higher risk of positive lymph nodes in patients

with greater proportion of oleic acid, total saturated acids and lower trans fatty acids in subcutaneous adipose tissue (32).

The E3N-EPIC Study examined the association between the risk of breast cancer and dietary factors. It demonstrated a positive association between serum trans-monounsaturated fatty acids and risk of invasive breast cancer in women (49). Elevated levels of trans-palmitoelic acid and elaidic acid were shown to be mostly related to the intake of manufactured foods like biscuits and chocolate bars. Nevertheless a mini-review study on trans fat shows inconsistence evidence of its effect on breast cancer (77).

The breast tissue level of DHA in phospholipids in breast cancer patients was shown to be significantly lower compared to patients with benign breast lesions. However the alterations of fatty acid composition of phospholipids were not associated with the stage of the disease (78).

Recent studies demonstrate that breast cancer induces changes in plasma and tissue levels of fatty acids of individuals. Takata et al examined 130 postmenopausal breast cancer patients and 257 matched controls. They showed no significant difference in fatty acid composition of serum phospholipids for total saturated, monounsaturated and n-3, n-6 or trans-fatty acids, however they reported an inverse relationship between breast cancer risk and levels of trans linoleic acid (18:2n6tt) (79).

Simonsen found out that the levels of tissue omega-3 and omega-6 in isolation do not show any association with breast cancer, albeit the higher the ratio of omega-3 to omega-6 the lesser the risk of breast cancer (62). Likewise Kuriki et al demonstrated an inverse relationship between breast cancer risk and red blood cell membrane composition of n-3 highly unsaturated fatty acid derived from fish intake (80).

2.5.3.Cell culture

In vitro studies show increased plasma levels of plasminogen activator inhibitor-1 (PAI-1) in breast cancer patients. It is shown that free fatty acids especially linoleic acid induce the activity of PAI-1 which facilitate the migration of tumoral cells and hence the progression of cancer (81). The growth promoting effect of estrogen E2 on ERα (+) breast cancer cells was diminished by supplementing the cell culture with conjugated linoleic acid (CLA). CLA which is a naturally produced fatty acid in ruminants has several isomers, among them c9, t11-CLA and t10, c12-CLA are known to have anti-proliferative effect (82). Its growth inhibitory effect is mediated by an increase in the level of P53 tumor suppressive protein and a decrease in Bcl-2 protein expression (83). Although the results from animal and *in vitro* studies show protective effect of conjugated linoleic acids on breast cancer, there is not yet any proven evidence in humans (84).

Erickson et al observed that PyV-mt mammary tumor cells could form tumorspheres in cultures. EPA and DHA inhibited the formation of tumorspheres through blocking the cyclooxygenase pathway. Alternatively adding PGE2 to DHA and EPA could restore the tumorsphere formation by 70% (61).

Differential expression of enzymes in cancer and normal cells has been shown in a number of studies. The fatty acid-activating enzyme, long-chain fatty acyl-CoA synthetase 4 (ACSL4), is overexpressed in cancer cells and its ablation in MDA-MB-231 cell culture makes the tumoral cells 3 times more sensitive to the cytotoxic effects of triacsin C (85).

2.5.4. Treatment modalities

Although experimental evidence for the potential beneficial role of select fatty acids in preventing the risk of breast cancer or enhancing the effectiveness of various drugs in rodent models and *in vitro* studies is increasing, there are only a few human trial studies.

Allen et al showed that high fat diet with increased concentration of phytanic acid can enhance the activity of alpha-methyacyl-coenzymeA racemase (AMACR) and induce carcinogenesis. Phytanic acid is a C20 branched chain fatty acid which is found in high amount in dairy products and red meat (86).

A large body of evidence shows chemoprotective and chemotherapeutic effects of n-3 PUFA in different types of cancer. Its beneficial effect on cancer treatment has been explained in different *in vivo* and *in vitro* studies and is mainly related to the production of reactive oxygen species. Oxidative byproducts of omega-3 PUFA can affect the cell structure by modifying the cell fluidity /permeability and the metabolism of cell membrane protein. They can also interfere with growth cell cycle and induce apoptosis. PUFA can also exert their anti-proliferative effect via changing the lipid raft and fluidity of cell membrane and altering the subsequent EGFR (epidermal growth factor receptor) signal conduction (33).

Studies have investigated the effect of omega 3 PUFA and its metabolites as an adjuvant therapy along with chemotherapy and radiotherapy to achieve the highest anti-proliferative effect on cancer patients (87). These PUFA are shown to have a synergetic effect with chemotherapy drugs in breast cancer; DHA has been shown to increase the sensitivity of cancer cells to anti-cancer agent arsenic trioxide (As_2O_3) (87).

Yee et al investigated the dose-dependent effects of EPA/DHA supplement on breast adipose tissue and serum in women at high risk of breast cancer for 6 months. They showed that EPA/DHA doses >=2.52 g/d up to 7.52 g/d increased the tissue levels of EPA/DHA and led to a maximum tissue response. The desired incremental changes in serum were obtained at doses 5.04 to 7.52 g/d. The supplements were well tolerated by subjects up to 7.52 g/day and none of their subjects developed any serious adverse changes from the supplements which was

investigated by measuring alanine aminotransferase (ALT), LDL cholesterol and platelet activity (88). They also concluded that changes in tissue levels of fatty acids are negatively correlated to the body mass index (BMI) and baseline fatty acid concentrations. So the higher the BMI and baseline fatty acid concentrations, the less incremental change would be observed in tissue levels when receiving the supplements (88).

It should be noted that although n-3 PUFA have not shown any harmful effects by itself especially at the recommended dosage (the recommended nutritionally achievable daily intake of DHA+EPA is between 250 and 500 mg/d (89)), but its marine source can be contaminated by pesticides and mercury and cause toxicity (87).

3. Summary of Rationale for the study

The effects of dietary fat on breast cancer have been investigated in many experimental studies. It is evident that tissue composition of fatty acids particularly the essential fatty acids is influenced by the fat intake. However, the changes in the fatty acid composition of breast cancer tissue from the normal breast tissue have not been thoroughly investigated.

We hereby examined and compared the profile of fatty acids in normal, tumoral and its adjacent normal looking breast tissues. We also studied the plasma and red blood cell composition of fatty acids and its correlation with stage and grade of the disease in breast cancer patients.

3.1. Hypothesis and objectives

3.1.1. Study Hypothesis

The fatty acid composition varies in tumoral tissue, surrounding marginal tissue and normal breast tissue in female breast cancer patients.

3.1.2. Study Objectives

1- **To study tissue composition of fatty acid in tumor, adjacent marginal and normal looking breast tissue.**
2- **To investigate the association of fatty acid composition of plasma and red cells with stage of the disease.**
3- **To investigate the association of fatty acid composition of plasma and red cells with grade of the disease.**

3.1.3. Ethics

This study has received approval from the St. Boniface General Hospital Research Review Committee at Winnipeg, Manitoba, Canada.

3.2. Materials and Methods

3.2.1. Study Patient

Patients were identified through ACF (Ambulatory Care Facility) surgery clinic at St. Boniface General Hospital, Winnipeg. Patients were examined by the study surgeon and sent for core biopsy and further excision of the lesion.

3.2.2. Consent form and questionnaire

Patients signed an informed consent form and completed a questionnaire consisting of general information, medical history and dietary data. Information was obtained about age, menopausal status, body mass index, education, number of children, employment, family history and personal history of cancer. Dietary data was obtained by a self-administered food frequency questionnaire (FFQ), which contained questions about the intake and frequency of consumption of various food items, fast food, bakery, pastry, fish and other sea food, different types of nuts, supplements and types of oil used for either cooking or dressing and also drinking and smoking habits. Please see appendix for FFQ and consent form.

3.2.3. Blood collection

Five ml of blood was drawn from patients in sodium heparin anticoagulant tube either at the time of surgery or a day or two before the surgery as part of the pre-operative assessment. It was then transported on ice to the research laboratory and plasma and red blood cells (RBC) were separated by centrifugation. Red blood cells were washed three times in 0.9% NaCl to remove the trapped plasma by centrifuging at 5000 rpm for 15 minutes at 4°C. Plasma and washed RBC were kept frozen at –80°C until analyzed.

3.2.4. Tissue collection

Tissue specimens were obtained after surgical removal of the breast lesion. First the required sections for pathological diagnosis were excised and stored in 10% formalin for fixation. Then an approximate 2x2x0.3 cm slice of each of the three regions (Figure 7) of the breast cancerous tissue, the adjacent marginal tissue (tissue surrounding the tumor) and normal looking breast tissue (outside the tumor) was obtained, transported on ice to research laboratory and kept frozen at -80°C until analyzed for fatty acid composition.

Currently tissues selected for lipid analysis are kept frozen rather than formalin fixed since formalin fixation has shown to decrease the concentration of some lipid components (sphinganine and sphingosine concentration) (90).

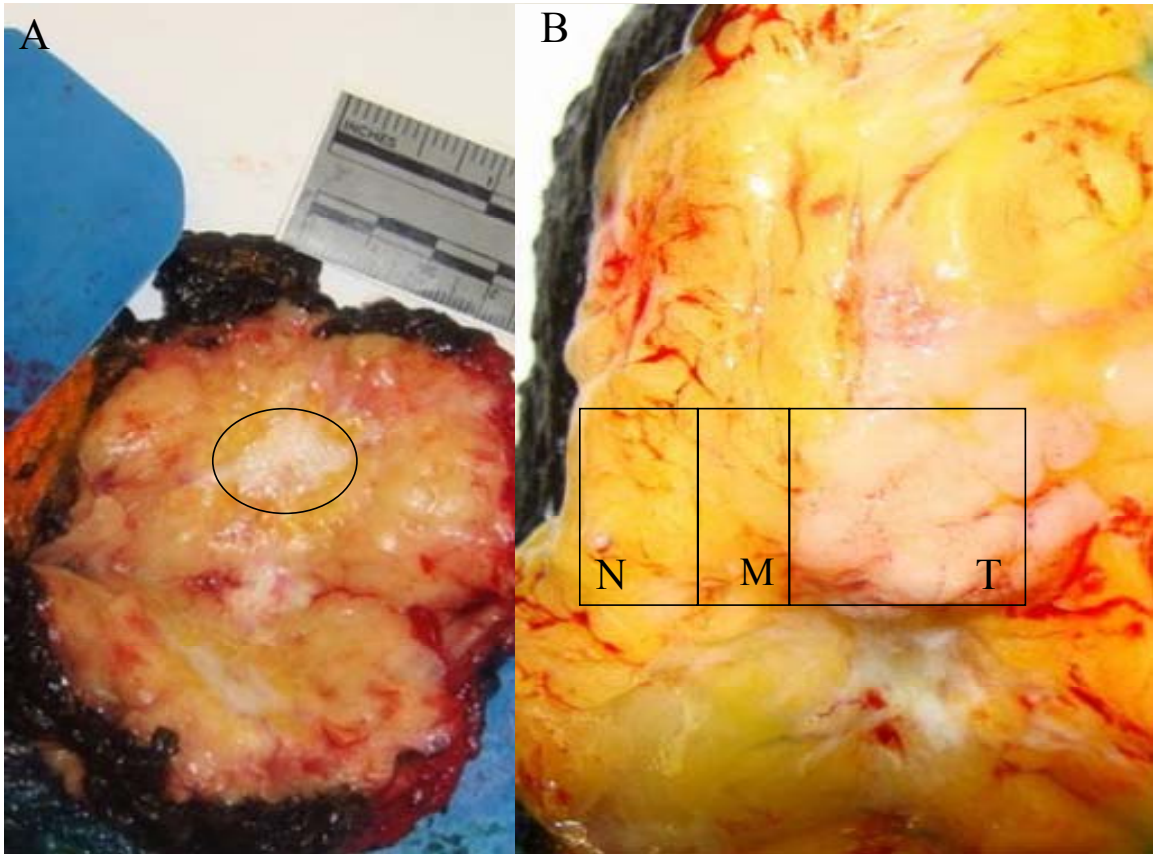


Figure 7. Gross (macroscopic) appearance of breast tumoral tissue. (A) Cut section of breast tissue after lumpectomy shows the whitish tumoral lesion (circle). (B) Closer view of the breast tissue shows the three sections of Tumoral (T), Marginal (M) and Normal (N) breast tissue.

3.2.5. Histology

Part of the specimen fixed in 10% buffered formalin was sent to diagnostic laboratory for histological evaluation.

Samples were processed in series of graded ethanols and embedded in paraffin. Five micron sections were prepared and stained with Hematoxylin and Eosin.

The study pathologist reviewed the slides, made the diagnosis and graded the tumors based on Nottingham modification of the Scarff-Bloom-Richardson system (SBR). The pathology report consisted of the histologic type of the tumor (ductal , lobular, mixed or metaplastic), percentage

of *in situ* carcinoma present, status of the lymph nodes, extension to the margins, lymphovascular invasion and size of the tumor.

Immunostaining was performed for hormonal (estrogen and progesterone) and HER2 receptors. The slides were accordingly scored as negative, borderline or positive.

Staging of the breast cancer patients was performed clinically based on the 2009 7th edition of American Joint Committee of Cancer using the TNM classification (91).

3.2.6. Total lipid, phospholipid and lipid class analysis for tissue, plasma and red blood cells

3.2.6.1. Tissue lipid extraction

Lipid extraction was carried out according to Folch method (92). The frozen tissue was thawed and then crushed into fine powder. 0.1 gram of the powder was transferred to a 12 ml acid washed glass tube that contains 50 μL of 17:0 (1mg/ml) as the internal standard. One ml of 0.1 molar potassium chloride was added to each tube. It was vortexed for one minute after adding four ml of freshly prepared chloroform: methanol (2:1).

After an overnight stay at 4°C, the samples were spun at 1000 rpm for 10 minutes (at 21°C) to separate phases. The lower chloroform layer was pipetted into labeled pre-weighed acid washed 15 ml glass tube using a long Pasteur pipette. The chloroform was dried down under gentle steam of nitrogen at low heat for 10 to 15 minutes.

The tubes were weighed with dried lipid and reconstituted in chloroform for a concentration of 10 mg/ml. The sample was then stored at –80 °C freezer in glass GC vials for total lipid, phospholipids analysis by TLC and separation of lipid classes by TLC (100 μL each).

3.2.6.2. Plasma and RBC lipid extraction

For plasma or RBC, fifty μL of the sample were transferred to a 12 ml glass acid-washed tube with 950 μL of 0.1 molar solution of potassium chloride. It was vortexed for 1 minute after

adding 4 ml of freshly prepared chloroform:methanol (2:1). The process was then continued as the above mentioned tissue lipid extraction.

3.2.6.3. Total lipid

Ten microliter of 17:0 standard (1mg/ml) and 100 μL of lipid sample were added to the bottom of a 15 ml acid washed glass tube. After adding 2 mL 0.5M KOH in methanol, tubes were votexed and flushed with nitrogen.

Samples were saponified at 100 °C for 1 hour in the oven and then cooled for 10 minutes at room temperature in fume hood. Two ml hexane and 2 ml 14% BF3-MeOH were added and the tubes were flushed with nitrogen. The tubes were methylated at 100 °C for 1 hour in the oven and cooled for 10 minutes at room temperature in the fume hood. Tubes were then centrifuged at 1000 rpm for 10 minutes after adding 2 ml double distilled water (ddH2O).

The top hexane layer was extracted and dried down under nitrogen. It was then reconstituted in 100 μL hexane and transferred to a GC insert to be run on the 6890 GC for trans-fat analysis or 7890B for quick run.

3.2.6.4. Phospholipid TLC separation of extracted lipid

One hundred microliter of sample was transferred and spotted on an activated 20x20 cm H-plate (placing it against wall of oven at 100 °C for an hour). Plate was run in the 20x5 cm tank filled with solvent mixture of 30ml chloroform, 9 ml MeOH, 25 ml 2-propanol, 6ml KCL (0.25% w/w) and 18 ml triethylamine. It takes about 2 hours until solvent reaches approximately 4 cm from top of plate.

The bands were visualized and scored under UV light after applying a fine mist of 0.1% (w/v) ANSA with TLC nebulizer. The scored bands were then scraped for methylation.

The internal standard C17:0 FFA was added to the very bottom of acid washed labeled leak proof 15 ml glass tubes; 10 μL C17:0 for PC fractions and 5 μL for lysoPC, SM and PE was added.

3.2.6.5. Methylation from Silica

Two ml hexane and 2 ml freshly prepared14% BF3-MeOH (trifluoroborane in methanol) were added to each tube and vortexed. Methylation was done at 100 °C for 1.5 hours. It was then cooled down for 10 minutes at room temperature. The process was stopped by adding 2 ml double distilled water. The phases were separated by spinning down at 1000 rpm for 10 minutes at 21 °C. The extracted upper hexane layer was then dried down under nitrogen. PC fractions were reconstituted in 50 μL of hexane and lysoPC/SM/PE fractions in 25 μL of hexane and stored at –20 °C until ready for use.

3.2.6.6. Lipid class thin layer chromatography (TLC) separation of extracted lipid

The procedure is similar to the phospholipid TLC separation mentioned above with some changes. G-plate is used instead of H-plate and the TLC solvent mixture is 80 mL petroleum ether, 20 mL ethyl ether and one ml acetic acid. After methylation from silica, fractions were reconstituted in 50 μL of hexane and stored at –20 °C for later GC analysis.

3.2.6.7. Gas chromatography

An Agilent 6890 gas chromatograph (GC) was used to separate the fatty acid methyl esters (FAME). It has a flame ionization detector and a SP2560 fused silica capillary column of 100m, 0.25-mm film thickness and 0.25-mm internal diameter). Peaks were identified from C14:0 to C 22:6 (n-3) and expressed as percentage of total lipids (93).

3.2.6.8. Plasma lipid assays

Quantitative assessment of plasma triglyceride and cholesterol levels was done using the enzymatic method. The reagents and standards were purchased from Diagnostic Chemicals Limited. Plates were then read quantitatively by a microplate reader (autoreader EL311; Bio-tek instruments).

3.2.6.9. Statistics

Data are shown as means ± standard deviation (SD). The Student's *t* test was performed to analyze differences between the two variables and analysis of variance (ANOVA) with a post hoc-Tukey test was used for 3 or more variables. Pearson's correlation coefficients were used to assess the relations between stage and grade of the tumor and individual fatty acids in plasma and red blood cells. SPSS (Statistical Package for the Social Sciences) software version 11.5 (SPSS Inc, Chicago, IL) was used for conducting the analyses. The level of significance was set at $p<0.05$.

4. Results

4.1. Study subjects

There were 14 female subjects enrolled into the study. Mean age of the subjects was 61.5 years old. Selected demographic and reproductive risk factors of the patients are summarized in Table 2. Table 3 depicts the highlights of the food questionnaire and the smoking and drinking habits of the study subjects.

Table 2. Demographic and reproductive characteristics of the subjects

Variables	Mean±SD
Age (yrs)	61.5±13.5
Weight (pounds)	163.5±46.8
Height (cm)	162.4±8.2
BMI (kg/m2)	28.1±6.7
Location	n (%)
Winnipeg	12(85.8)
Rural Manitoba	2(14.2)
Marital Status	
Married	10 (71.6)
Single	2(14.2)
Divorced or Widowed	2(14.2)
Education	
>=High school diploma	11 (78.5)
<High school diploma	3 (21.5)
Parity	
Para 0 (nulliparous)	4(28.5)
Para >=1	10 (71.6)
Menopausal Status	
Post-menopausal	9 (64.3)
Pre-menopausal	5 (35.7)
Family History of breast cancer	
Yes	5 (35.7)
No	9 (64.3)

Table 3. Selected characteristics of study subjects obtained from the study questionnaire

Characteristic	n(%)
Alcohol Consumption	
Non-Drinker	6(42.9)
2-5 drinks/wk	4(28.5)
1 drink/month	2(14.3)
1-2 drink/day	2(14.3)
Smoking history	
Non-smoker	12(85.8)
Smoker	2(14.2)
Fish Consumption	
Rarely	4 (28.5)
1-2 servings/month	5 (35.7)
1 serving / wk	2 (14.3)
>2 servings/ wk	3 (21.5)
Fast food consumption	
Rarely to none	7 (50)
1 /month	2 (14.3)
1-2 / wk	4 (28.6)
Daily	1 (7.1)

4.2. Pathology Report

The most common type of breast cancer observed in the study patients was ductal (57%) followed by lobular (28%) carcinoma. The side of the tumoral involvement was equally distributed to right and left breast among subjects. The greatest diameter of half of the lesions was equal to or less than 2.5 cm whereas 42% of the lesions size was between 2.5 to 5 cm and the rest were over 5 cm in diameter. There was equal number of patients with intermediate and high grade cancer (five patients in each group) and only two patients showed low grade cancer. Clinical staging showed equal number of patients in stage one and two (5 patients in each group) and four patients in stage three of breast cancer. Two of our patients didn't show any cancer in the breast tissue itself; one had breast cancer in the chest wall muscle and the other in the axillary

lymph nodes. Therefore for these patients grading and assessment of the size of tumor were not performed. Further pathology information about immunostaining, grade and stage of the tumor has been shown in Table 4.

We performed a correlation analysis on the hormonal and HER2 receptors and stage and grade of the cancer. There was a negative correlation between estrogen receptor activity and stage of the disease (R^2=0.3, correlation coefficient=0.547, $P<0.05$). Therefore estrogen receptor positive cancers were associated with early low-stage of the disease. No correlation was found between other biomarkers and disease stage or grade.

Table 4. Characteristics of breast carcinoma addressed in pathology report

Clinical Characteristics	**n(%)**
Histologic type	
Ductal	8 **(57.14)**
Lobular	4 **(28.58)**
Mixed	1 **(7.14)**
Metaplastic	1 **(7.14)**
Tumor size*	
<= 2.5 cm	6**(50)**
2.5-5 cm	5**(41.67)**
>5cm	1**(8.3)**
Tumor grade*	
Low	2 **(16.7)**
Intermediate	5 **(41.67)**
High	5 **(41.67)**
Stage	
1	5 **(35.71)**
2	5 **(35.71)**
3	4 **(28.58)**
ER	
Negative	2 **(14.28)**
Positive	11 **(78.58)**
Borderline	1 **(7.14)**
PR	
Negative	4**(28.58)**
Positive	9**(64.28)**
Borderline	1**(7.14)**
HER2	
Negative	9**(64.28)**
Positive	2**(14.28)**
Borderline	3**(21.44)**

* Only for lesions inside the breast (the ones in the muscle and lymph node are excluded)

4.3. Tissue Lipids

4.3.1.Extracted tissue lipids

The extracted lipid (Figure 8) from the three sections of breast (normal, marginal and tumor) was significantly different in each group ($p<0.05$). Marginal tissue showed the highest amount of extracted lipid (37.5 ± 20.5) whereas tumoral tissue yielded the least lipid (5.53 ± 4.76).

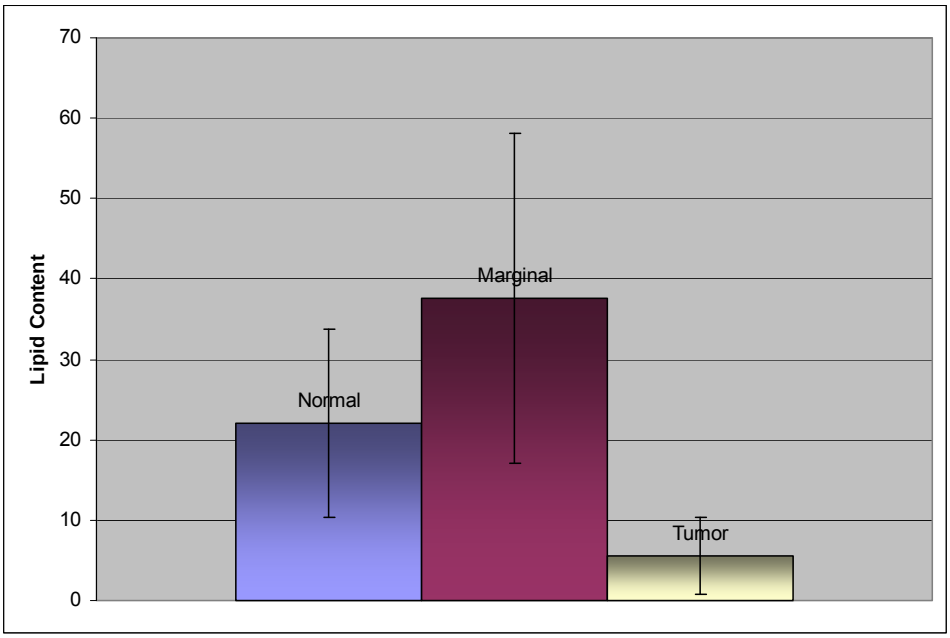


Figure 8. Lipid contents of normal, marginal and tumoral tissues. Marginal tissue showed significantly higher extracted lipid compared to normal and tumoral tissue (* P< 0.05).

4.3.2.Fatty acid composition of breast tissue

More than fifty fatty acids were detected in the tissues; these include the following 14:0, 14:1, 15:0, 15:1 c10, 16:0,16:1n–9t, 16:1n–9c, 17:1 c10, 18:0, 18:1 t4, 18:1 t5, 18:1 t6-8, 18:1 t9, 18:1 t10, 18:1 t11, 18:1 t12, 18:1 t13, 18:2n–6tt, 18:2n–6ct, 18:2n–6tc, 18:2n–6cc, 18:1 c9, 18:1 c11, 18:1 c12, 18:1 c13, 19:00, 18:2 t9,t12, 18:2 c9t13,18:2 c9, t12, 18:2 t9, c12, 19:1 c10, 18:2 n-6, 18:2 c9 c1, 18:2 c9 c15, 20:0, 18:3 n-6, 20:1 c5, 20:0, 18: 3 n-6, 20:1 c5, 20:1 c8, 20:1 c11, 18:3 n-3, 18:2 c9 t11, 18:2 c11 t13, 18:2 t10 c12, 18:4 n-3, 18:2 CLA, 21:0, 20:2 n-6, 22:0, 20:3 n6, 22:1 n-9, 20:3 n-3, 20:4 n-6, 22:0, 20:3 n-6, 22:1 n-9, 20:3 n-3, 20:4 n-6, 22:2 n-6, 24:0, 20:5 n-3, 24:1, 22:3 n-3, 22:4 n-6, 22:5 n-6, 22:5 n-3, 22:6 n-3.

A number of fatty acids showed significantly less amounts in tumoral tissue compared to marginal and normal tissue including the following: 14:0, 17:1 c10, 18:1 t11, 18:1 c9, 18:3 n-3 and 18:2 n-6.

On the other hand, fatty acids 18:0, 18:1 t9, 18:1 t12, 18:1 t13, 19:0, 22:6 n-3, 22:5 n-3, 22:5 n-6, 24:0, 20:4 n-6, 20:3 n-6, 20:2 n-6, 22:0, 18:2 tt CLA and 18:2 t10 c12 CLA showed significantly increased amounts in tumoral tissue than in normal or marginal tissue.

Selected fatty acids are shown in tissue specimens and compared with their values in plasma and red blood cells (Table 5).

There were no significant differences between fatty acid composition of marginal and normal breast tissue except in one fatty acid. Alpha linolenic acid 18:3 n-3 was significantly less in marginal tissue compared to normal tissue ($P<0.05$).

Table 5-Selected fatty acid composition of plasma, RBC and breast tissue

Fatty acids	Name	Plasma^	RBC^	Tissue^		
				Normal	Marginal	Tumor
14:0	Myristic acid	0.78±0.27	0.38±0.09	2.53±0.51*	2.34±0.44	1.91±0.66*
16:0	Palmitic acid	20.38±1.95	21.31±1.26	19.79±2.19	19.95±2.76	19±2.6
18:0*	Stearic acid	6.98±0.74	13.01±0.87	4.45±1.39*	4.73±1.83*	6.84±2.88*
18:1n9c*	Oleic acid	21.03±2.44	14.95 ±0.9	44.57±3.22*	42.96±5.02	38.17±8.18*
18:1n-7	Cis-Vaccenic acid	1.92±0.81	1.61 ±0.44	2.58±1.13	2.3±0.82	2.71± 0.96
18:2n-6*	Linoleic acid	27.76±4.36	12.64±3.56	13.16±2.09*	12.97±2.74*	10.31±2.78*
18:3n-6	γ Linolenic acid	0.56±0.18	0.15±0.08	0.08±0.04	0.09±0.05	0.13±0.1
20:3n-6*	Dihomo-γ-linolenic acid	1.55±0.49	1.58±0.47	0.27±0.12*	0.33±0.17*	1.32±1.5*
20:4n-6*	Arachidonic acid (AA)	6.73±1.9	12.36±3.3	0.58±0.76*	0.79±1.1*	3.13 ±3.74*
18:3n-3*	α-linolenic acid	0.80±0.19	0.28±0.08	1.75±0.31*	1.22±0.59*	0.77±0.41*
20:5n-3	Eicosapentaenoic acid	1.27±0.9	1.07±0.72	0.07±0.05	0.09±0.07	0.15±0.19
22:5n-3*	Docosapentaenoic acid	0.56±0.09	2.07±0.58	0.23±0.13*	0.31±0.22	0.54±0.38*
22:6n-3*	Docosahexaenoic acid	1.84±0.73	3.6±1.4	0.12±0.1*	0.22±0.2*	0.45±0.28*
Total omega-3 PUFA		4.47	7.02	2.17	1.84	1.91
Total omega-6 PUFA		36.54	26.73	14.09	14.18	26.65
Total saturated		28.14	34.7	26.77	27.02	27.75
Total monounsaturated		22.95	16.56	47.15	45.26	40.88
Total n-3/n-6 ratio		0.12	0.26	0.15	0.12	0.07

** Significant difference between the normal, marginal and tumoral tissue*

^ Percentage to total by weight

4.3.3. Fatty acid composition of phospholipid subclasses in tumoral, marginal and normal breast tissue

The fatty acid composition of tumoral, marginal and normal tissue was investigated in different phospholipid subclasses (Table 6).

Palmitic acid showed a significantly increased level in lysoPC, PC, SM of tumoral tissue and significantly decreased levels in PS of tumoral tissue compared to marginal and normal tissue. (Figure 9). Oleic acid incorporation in to PI and PS of tumoral tissue was significantly greater than in its levels in marginal and normal breast tissue (Figure 10).

Arachidonic acid showed significantly increased levels in PC, PE and PI of tumoral tissue compared to normal and marginal tissue (Figure 11).

Table 6. Tissue incorporation of fatty acids in different phospholipid subclasses in breast normal, marginal and tumoral tissue (* P<0.05)

Fatty acids	Tissue (% to total weight)		
	Normal	Marginal	Tumor
16:0 (palmitic)			
Lysophosphatidylcholine	15.76±7.48	17.14±6.05	25.58±8.13*
Phosphatidylcholine	16.99±6.57	22.36±4.85	30.07±3.32*
Phosphatidylethanolamine	7.44±3.2	6.81±2.48	5.75±2.66
Phosphatidylinositol	11.29±5.57	8.33±3.41	8.44±2.13
Phosphatidylserine	13.16±4.69	9.62±3.91	5.48±3.6*
Sphingomyeline	12.95±4.68	15.88±6.53	37.24±9.14*
18:0 (Stearic)			
Lysophosphatidylcholine	16.9±8.03	16.92±6.53	21.32±6.53
Phosphatidylcholine	16.57±8.56	12.88±2.35	11.37±2.41
Phosphatidylethanolamine	17.17±8.28	17.27±10.15	19.23±6.89
Phosphatidylinositol	19.56±7.85	25.77±19.68	35.46±3.67*
Phosphatidylserine	15.93±5.72	20.08±10.05	36.81±5.82*
Sphingomyeline	14.9±6.7	16.37±4.71	10.42±3.83
20:4 n-6 (Arachidonic)			
Lysophosphatidylcholine	2.56±1.84	2.17±1.28	2.35±1.62
Phosphatidylcholine	3.88±1.83	6.25±2.19	7.46±2.22*
Phosphatidylethanolamine	10.93±7.32	14.7±7.42	18.02±5.83*
Phosphatidylinositol	10.41±10.8	6.32±3.62	19.62±6.8*
Phosphatidylserine	3.38±1.7	5.41±8.95	3.64±0.81
Sphingomyeline	2.34±1.25	1.71±1.49	1.31±0.62

Figure 9. Differential tissue incorporation of palmitic acid in phospholipid subclasses in breast normal, marginal and tumoral tissue (* P<0.05)

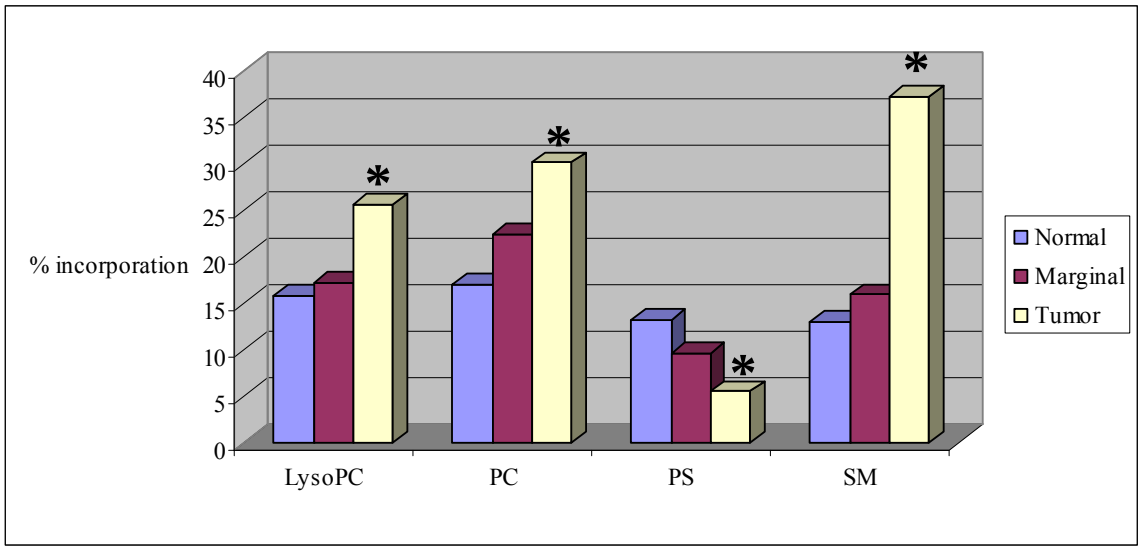


Figure 10. Differential tissue incorporation of oleic acid in phospholipid subclasses in breast normal, marginal and tumoral tissue (* P<0.05)

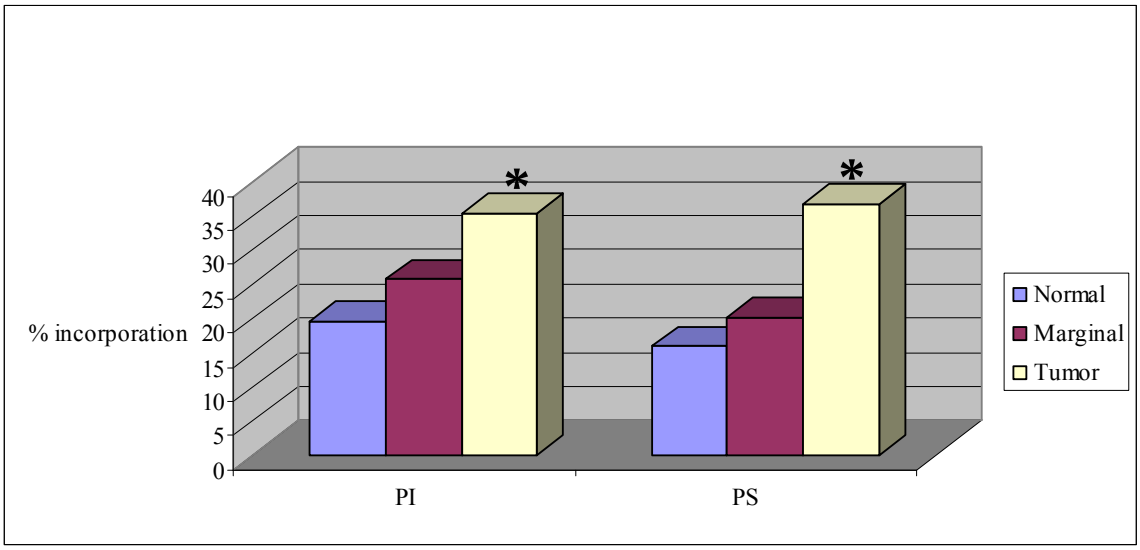
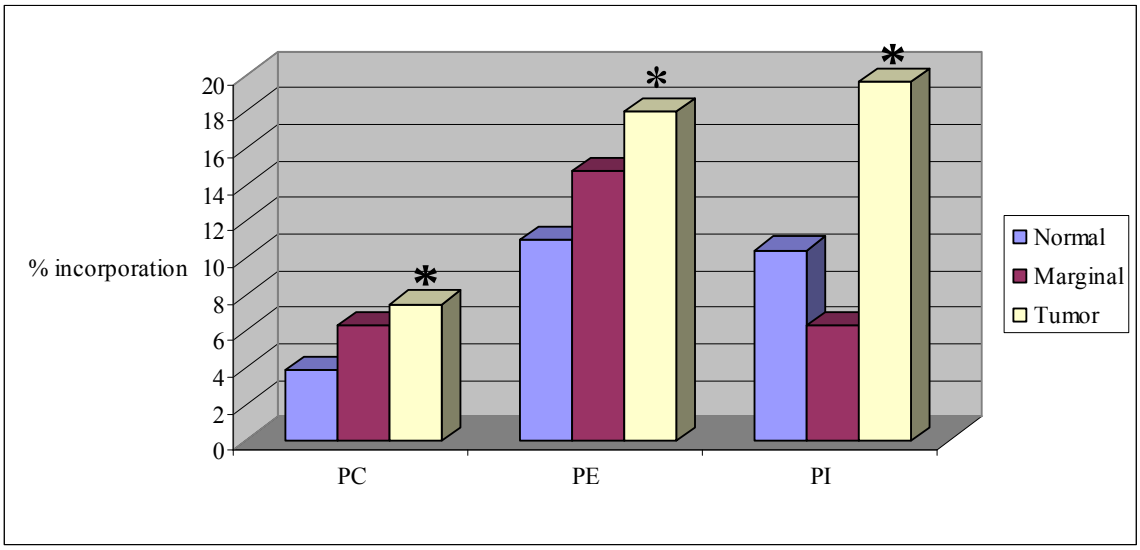


Figure 11. Differential tissue incorporation of arachidonic acid in phospholipid subclasses in breast normal, marginal and tumoral tissue (* P<0.05)



4.4.Plasma lipids

4.4.1.Plasma Lipid classes

Plasma samples were analyzed for lipid contents. Plasma cholesterol levels were 167.1 ± 35.6 mg/dl (mean±SD) and triglyceride levels were 95.8 ± 57.6 mg/dl. Cholesteryl ester, free fatty acids, phospholipids and triglyceride were measured and are shown in Figure 12. Between different lipid classes, phospholipid has the greatest amount whereas the free fatty acid has the least amount in plasma.

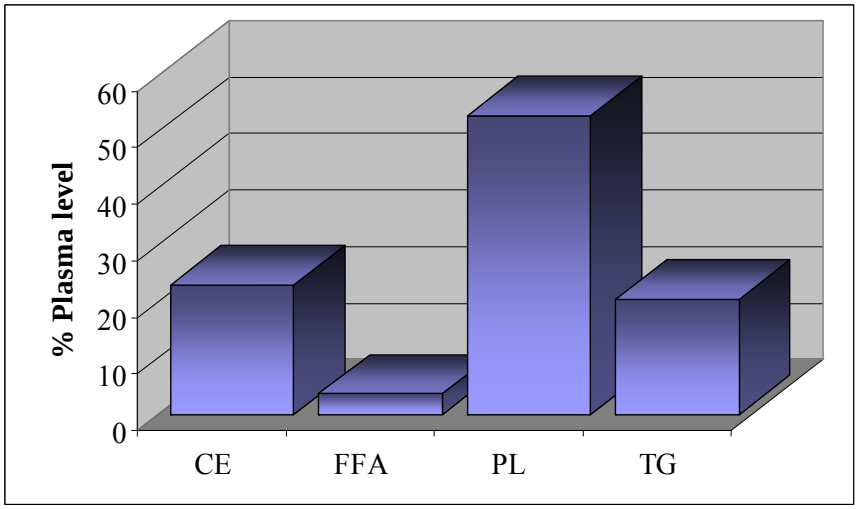
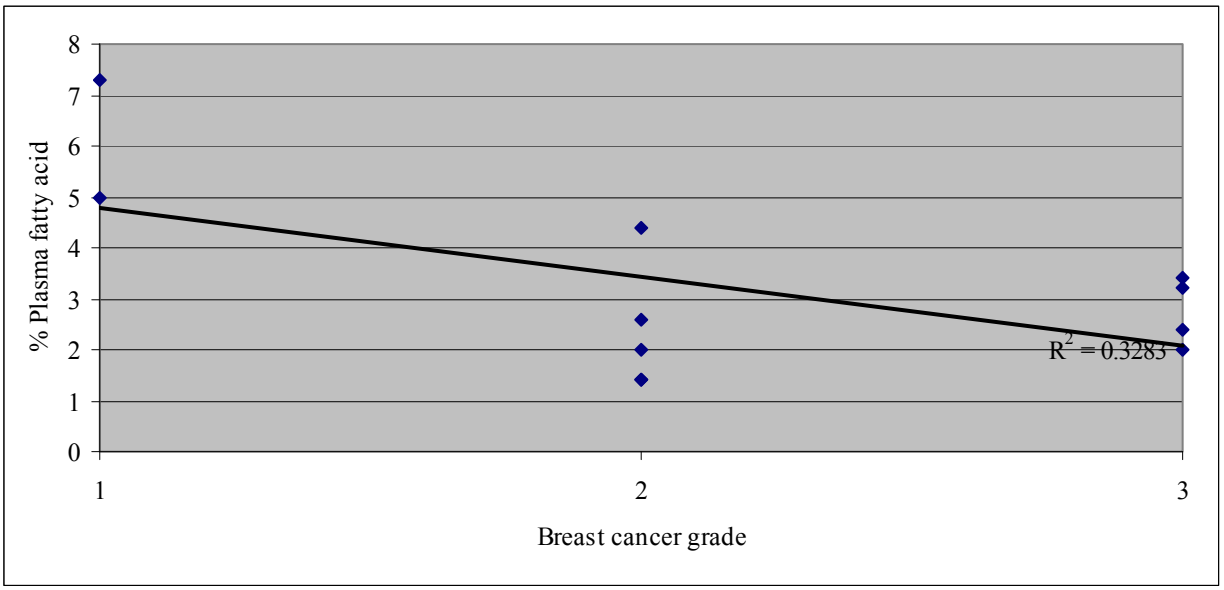


Figure 12. Plasma lipid contents

Correlation analysis was performed to show the strength of association between plasma lipid classes (PL, CE, FFA and TG) and severity of the cancer in terms of stage and grade of the disease. A negative association was observed between grade of the tumor and level of free fatty acids in plasma ($p<0.05$) (Figure 13).

There was no correlation between plasma lipid classes and stage of the disease.

Figure 13. Correlation of plasma free fatty acids and grade of breast cancer



4.4.2. Fatty acid composition of plasma lipid classes

Fatty acid contents of plasma lipid classes (PL, CE, FFA, TG) were studied and summarized in Tables 7 to 10 and the major fatty acid constituents of each class were shown in the representative graph. (Figure 14 to 17). The major fatty acid constituents of plasma phospholipid were 16:0, 18:0, 18:1 c9, 18:2 n-6, 20:4 n-6, 22:6 n-3 and 20:3 n-6.

16:0, 16:1 c9, 18:0, 18:1 t10, 18:1 c9, 18:1 c11 and 18:2 n-6 constitute the main fatty acids of plasma cholesteryl ester.

16:0, 18:0, 18:1 c9, 18:2 c9 t11CLA, 22:4 n-6 and 22:6 n-3 were shown to be the major free fatty acids in plasma. The following fatty acids constitute major fatty acid content of plasma triglyceride: 16:0, 16:1 c9, 18:0, 18:1 c9, 18:2 n-6.

Table 7. Plasma fatty acid composition of total phospholipid

Fatty acid	% of total by weight	Fatty acid	% of total by weight
14:0	0.40 ± 0.09	**18:2c9t13**	0.07 ± 0.05
14:01	0.11 ± 0.08	**18:2c9t12**	0.13 ± 0.04
15:0	0.27 ± 0.11	**18:2n6**	18.48 ± 2.38
16:0	25.35 ± 2.00	**20:0**	0.13 ± 0.04
16:1t9	0.28 ± 0.08	**18:3n6**	0.13 ± 0.07
16:1c9	0.93 ± 0.32	**20:1c11**	0.28 ± 0.06
17:1c10	0.12 ± 0.11	**18:3n3**	0.28 ± 0.06
18:0	13.75 ± 0.94	**18:2c9t11 CLA**	0.28 ± 0.20
18:1 t4	0.04 ± 0.05	**18:2tt CLA**	0.13 ± 0.06
18:1t6-8	0.14 ± 0.06	**20:2n6**	0.44 ± 0.09
18:1t9	0.54 ± 0.27	**20:3n6**	3.13 ± 0.93
18:1t10	0.55 ± 0.32	**22:1n9**	0.10 ± 0.06
18:1t11	0.18 ± 0.09	**20:3n3**	0.07 ± 0.03
18:1 t12	0.25 ± 0.08	**20:4n6**	10.43 ± 1.55
18:1 t13	0.17 ± 0.07	**22:2n6**	0.13 ± 0.11
18:1c9	12.05 ± 2.13	**24:0**	0.18 ± 0.12
18:1c11	1.79 ± 0.64	**20:5n3**	1.86 ± 1.36
18:1c12	0.23± 0.05	**24:01**	0.34 ± 0.16
18:1 c13	0.18 ± 0.09	**22:4n6**	0.32 ± 0.10
18:1 c14	0.13 ± 0.05	**22:5n6**	0.25 ± 0.10
19:0	0.19 ± 0.11	**22:5n3**	0.98 ± 0.16
18:2t9,t12	0.07 ± 0.10	**22:6n3**	3.49 ± 1.20

Figure 14. Major fatty acid contents of plasma phospholipid

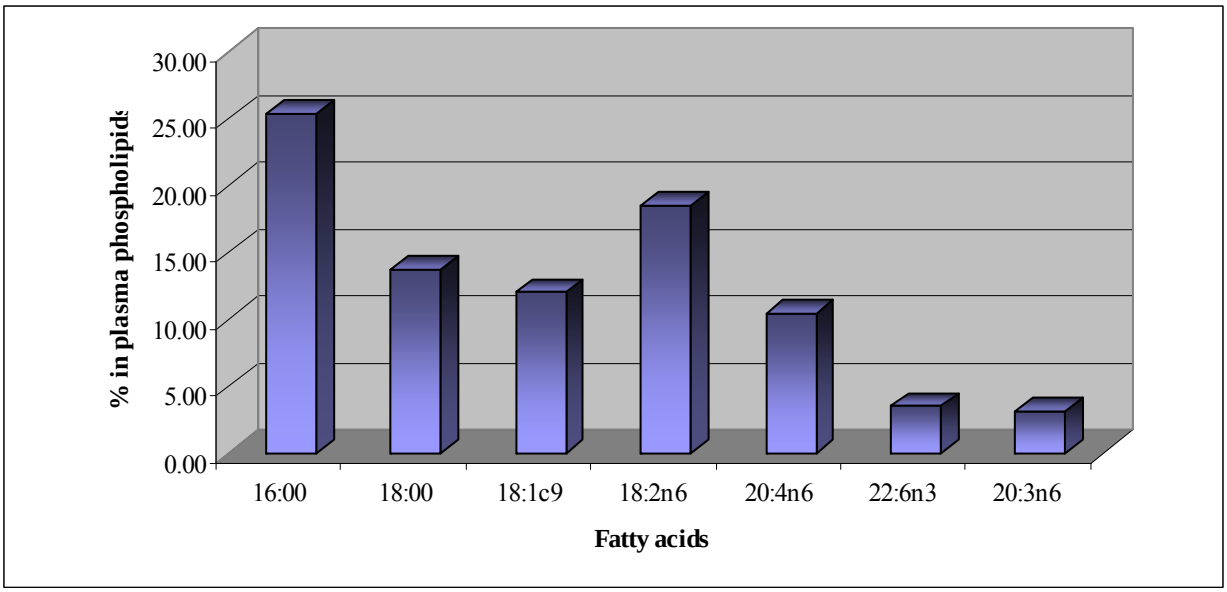


Table 8. Plasma Fatty acid composition of cholesteryl ester

Fatty Acid	% of total by weight	Fatty Acid	% of total by weight
16:0	18.31 ± 4.68	**20:0**	0.10 ± 0.09
16:1t9	0.55 ± 0.13	**18:3n6**	0.27 ± 0.18
16:1c9	3.55 ± 1.06	**20:1c11**	0.58 ± 0.20
18:0	5.42 ± 1.26	**18:3n3**	1.22 ± 1.52
18:1t6-8	0.41 ± 0.26	**18:2tt CLA**	0.37 ± 0.22
18:1t9	2.04 ± 1.16	**20:2n6**	0.32 ± 0.23
18:1t10	2.27 ± 1.28	**22:00**	0.16 ± 0.08
18:1t11	0.65 ± 0.40	**20:3n6**	0.24 ± 0.08
18:2c9t12	0.44 ± 0.13	**22:1n9**	0.31± 0.15
18:1 t13	0.27 ± 0.13	**20:3n3**	0.07 ± 0.07
18:1c9	39.47 ± 2.30	**20:4n6**	0.94 ± 0.44
18:1c11	2.65 ± 0.89	**22:2n6**	0.58 ± 0.67
18:1c12	0.39 ± 0.15	**20:5n3**	0.84 ± 0.67
18:1 c13	0.64 ± 0.43	**22:4n6**	0.41± 0.17
18:2tt	0.02± 0.08	**22:5n3**	0.30 ± 0.14
18:2t9,t12	0.19 ± 0.26	**22:6n3**	0.59 ± 0.37
18:2c9t13	0.32±0.11	**18:2n6**	10.79 ± 4.30

Figure 15. Major fatty acid contents of plasma cholesteryl ester

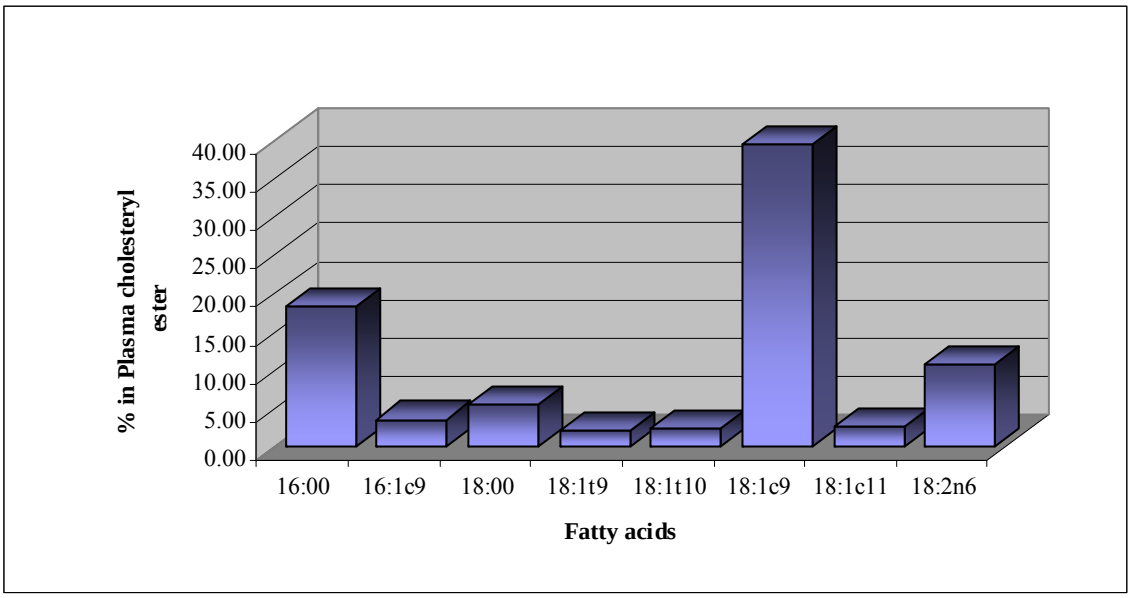


Table 9. Free fatty acid composition of plasma

Fatty acid	% of total by weight	Fatty acid	% of total by weight
14:0	1.92 ± 0.44	**18:1t6-8**	0.35± 0.18
15:0	1.04± 0.51	**18:1t9**	2.35 ± 1.33
16:0	8.56 ± 2.22	**18:1t10**	3.99± 1.10
16:1c9	2.76 ± 0.61	**18:1t11**	1.93± 0.40
18:0	6.56 ± 2.59	**18:1 t12**	0.25± 0.12
22:1n9	0.26 ± 0.32	**18:1 t13**	0.35± 0.12
20:4n6	2.85± 4.33	**18:1c9**	34.82± 5.91
20:5n3	1.10± 0.75	**18:1c11**	2.03± 0.30
22:4n6	4.80± 2.98	**18:1c12**	0.22± 0.30
22:5n3	2.60± 2.15	**18:1 c13**	0.61± 0.86
22:6n3	5.80± 3.50	**19:0**	0.92± 1.21
18:2c9t11 CLA	6.14± 6.36	**18:2n6**	3.01± 0.48
18:2tt CLA	0.95± 0.40	**18:3n3**	4.04± 3.37

Figure 16. Major plasma free fatty acids

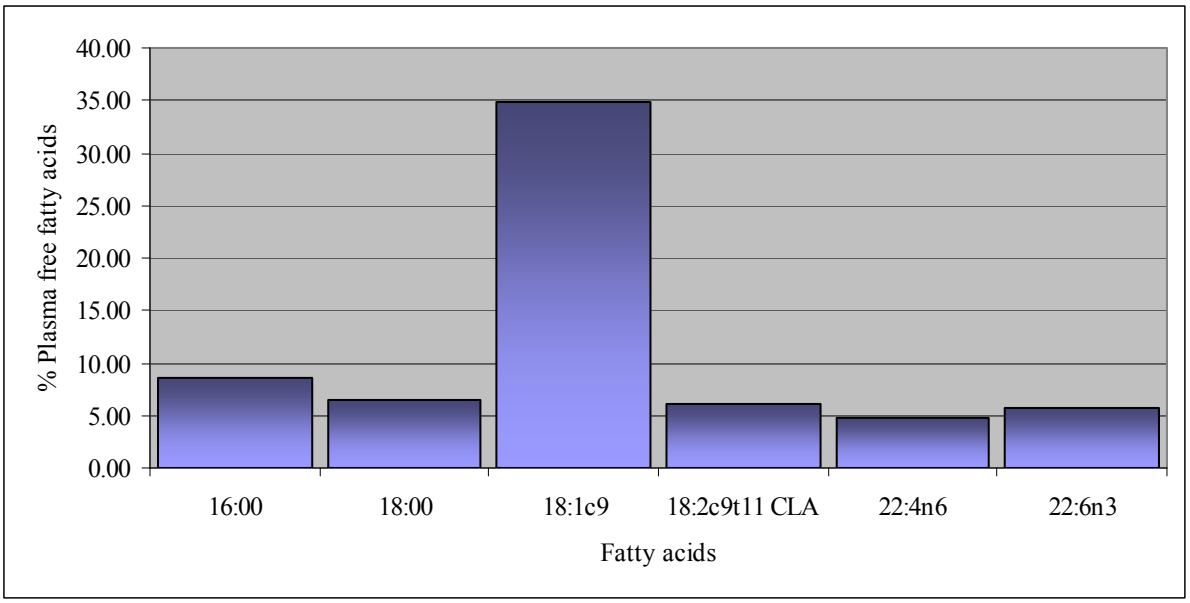
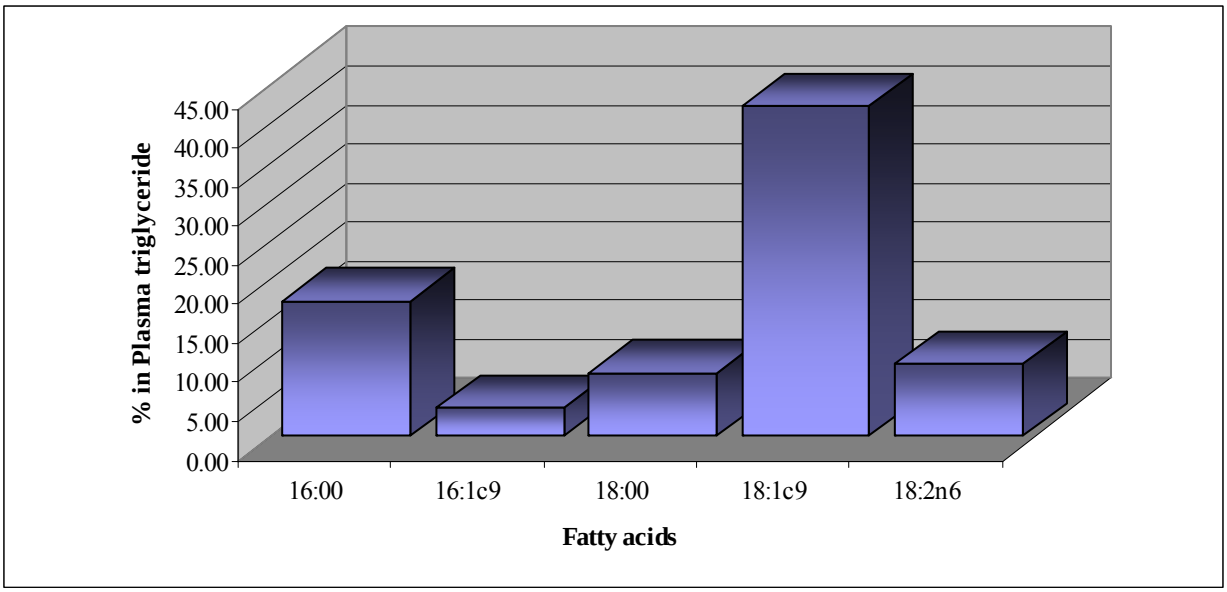


Table 10. Plasma fatty acid composition of triglyceride

Fatty acid	% of total by weight	Fatty acid	% of total by weight
14:0	1.56 ± 0.43	**20:2n6**	0.35± 0.15
14:1	0.18 ± 0.21	**20:3n6**	0.09± 0.07
15:0	0.38 ± 0.09	**22:1n9**	0.19± 0.11
16:0	17.24±3.33	**20:3n3**	0.04± 0.05
16:1t9	0.34±0.16	**20:4n6**	0.45± 0.08
16:1c9	3.52± 1.10	**22:2n6**	0.49±0.46
17:1c10	0.53±0.21	**20:5n3**	0.40±0.21
18:0	8.06± 1.67	**22:5n3**	0.83±0.61
18:1t6-8	0.35±0.21	**22:6n3**	0.16± 0.20
18:1t9	1.72± 1.10	**18:1 c13**	0.55±0.24
18:1t10	2.21± 0.94	**18:2c9t13**	0.32±0.15
18:1t11	0.66± 0.25	**18:2c9t12**	0.36±0.18
18:1 t12	0.30± 0.15	**18:2n6**	9.22±2.62
18:1 t13	0.30± 0.09	**20:1c11**	0.64± 0.17
18:1c9	42.18± 1.88	**18:3n3**	0.88± 0.56
18:1c11	2.84± 0.99	**18:2c9t11 CLA**	0.89± 0.85
18:1c12	0.44± 0.15	**18:2tt CLA**	0.34± 0.28

Figure 17. Major fatty acid constituents of plasma triglyceride



4.4.3. Correlation between grade and stage of breast cancer and plasma and red blood cell fatty acids

We investigated the correlation between plasma and red blood cell fatty acid composition and grade and stage of tumor. There were only two fatty acids in plasma and red blood cells that showed a significant correlation with grade of the tumor.

20:3 n6 and 18:1 t4 showed a positive correlation between their plasma levels and grade of the tumor ($P<0.05$) (Figure 18, 19). On the other hand, 20:3 n-6 and 22:5 n-6 both showed a positive correlation between their red blood cell levels and grade of the breast cancer (Figure 20). A positive correlation between red blood cell fatty acids of 18:1 t9, 18:1 t11, 18:1 t12, 18:1 c12, 22:1 n9 and 19:00 and stage of breast cancer was observed.

Plasma levels of 18:1 t6-8, 18:1 t9, 18:1 t10, 18:1 t12 and 18:1 c12 had a positive correlation with stage of the breast cancer whereas plasma levels of DHA 22:6 n-3 showed a negative correlation with breast cancer stage (Figure 21).

Figure 18. Correlation of plasma levels of 18:1 t4 with breast cancer grade

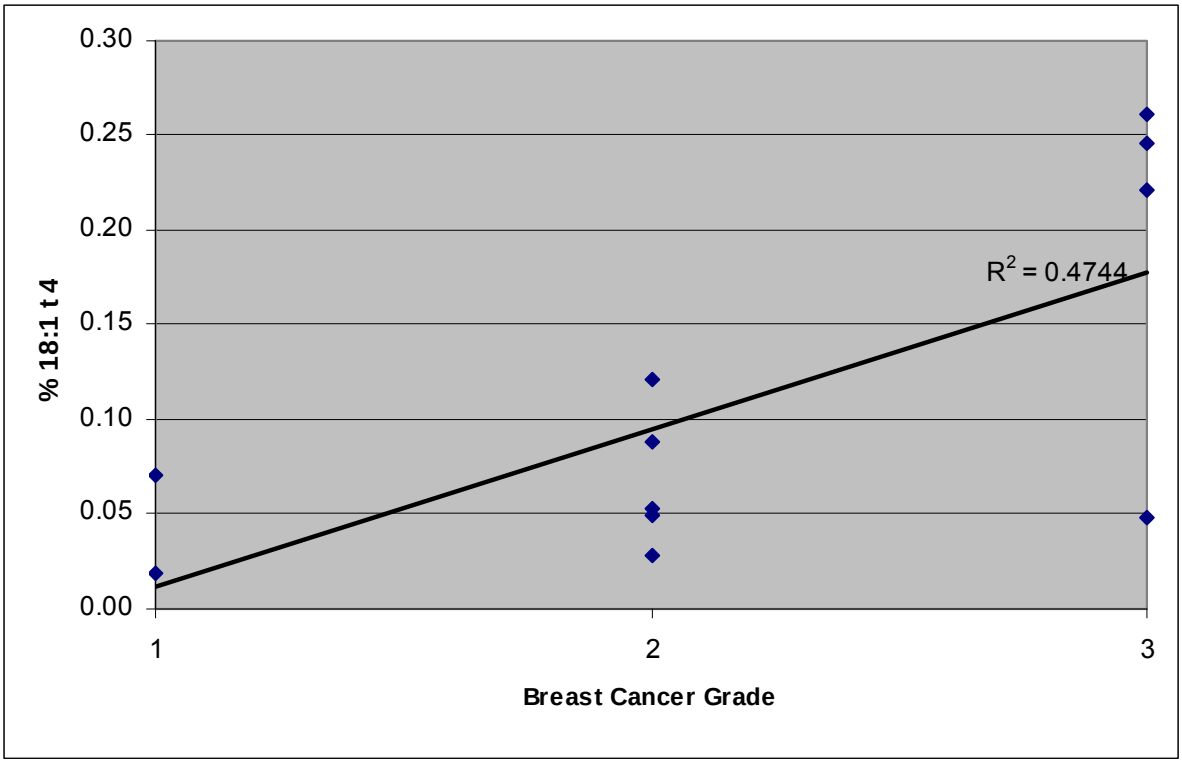


Figure 19. Correlation of plasma levels of 20:3 n-6 and breast cancer grade

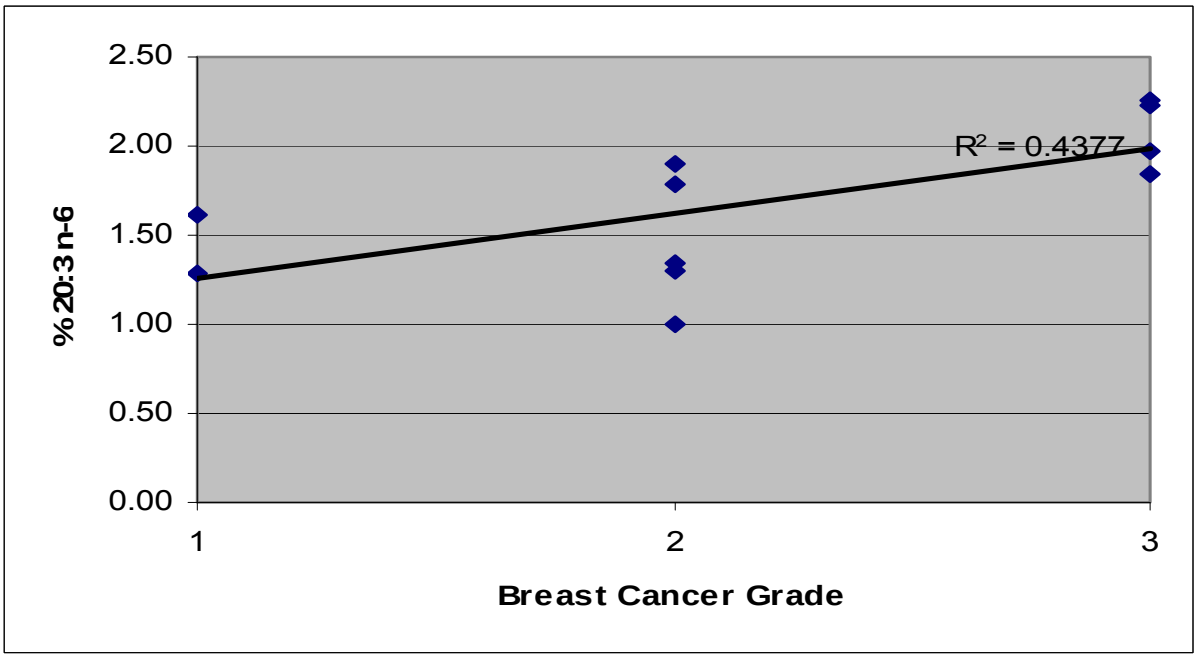


Figure 20. Correlation of red blood cell contents of 20:3 n-6 (blue dot) and 22:5 n-6 (pink dot) with grade of the tumor

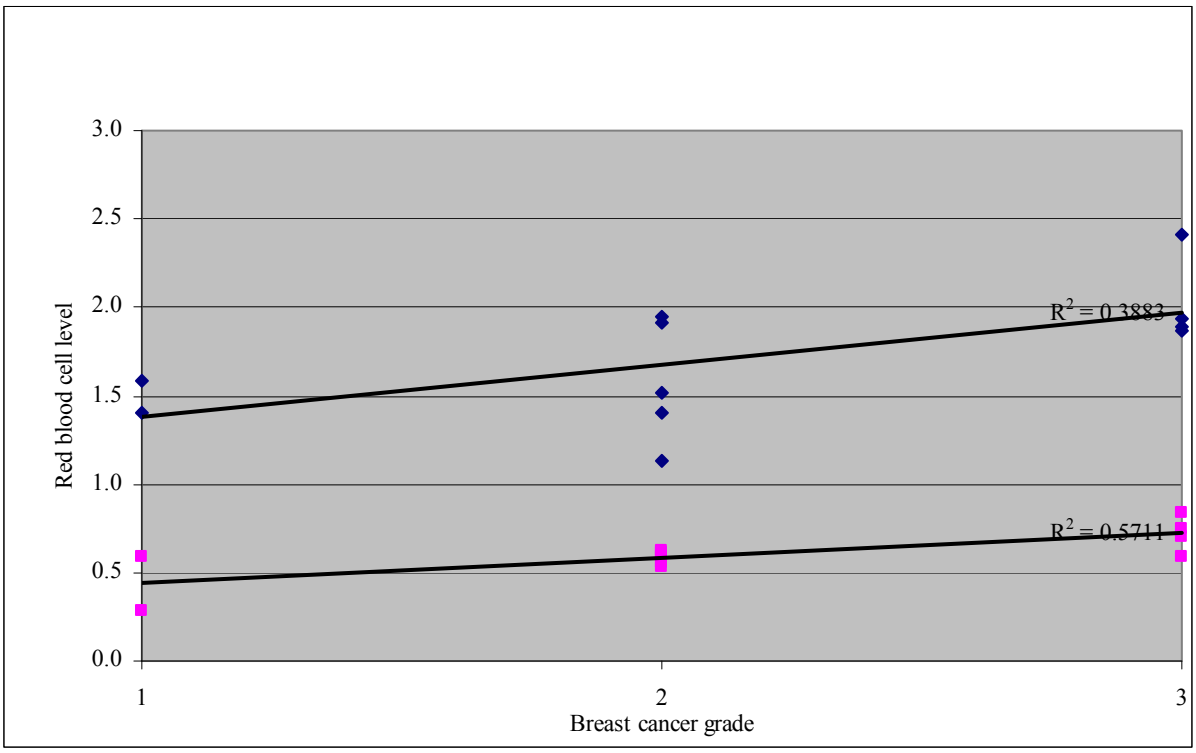
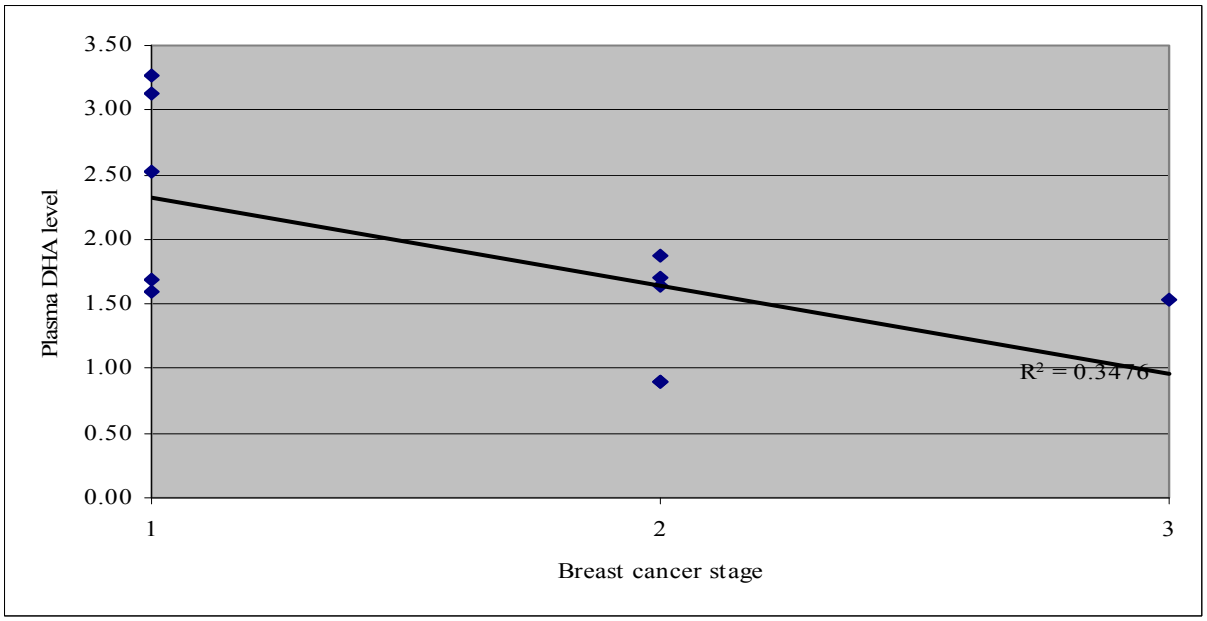


Figure 21. Correlation of plasma DHA level and stage of breast cancer



5. **Discussion**

5.1. **Summary of current knowledge and hypothesis**

Ample experimental evidence supports that consumption of DHA and EPA can inhibit or attenuate the growth of breast cancer(43). The growth inhibiting effects of the aforementioned fatty acids can be explained by their role on altering the tissue lipid composition.

In this study we investigated the tissue lipid profile in tumor, marginal and normal breast tissue in an individual with breast cancer. This is the first study exploring the correlation between stage and grade of the breast cancer and lipid profile of plasma and red blood cell. We hypothesized that lipid composition is different in tumor, marginal and normal breast tissue.

5.2. **Demographic characteristics and cancer characteristics**

Mean age of our study patients was 61.5 years which is very close to median age of breast cancer patients in developed countries like the USA (61 years) and Western European countries (63 years) (94).

The most common type of breast cancer in our study was invasive ductal carcinoma followed by invasive lobular carcinoma which is in agreement with other epidemiologic data(95).

Previous studies on hormonal and growth factor receptors show tumors with positive estrogen and progesterone receptors and negative HER2 receptors have longer survival rates(58). We found out patients with estrogen positive breast cancers presented in early stage of cancer.

5.3. **Tissue lipids**

In our study marginal tissue showed the greatest amount and tumoral tissue the least amount of the lipid. Reviewing the literature, we couldn't find any research study investigating the lipid in marginal, normal and tumoral breast tissue in the same individual. The reductions in lipid

contents in tumoral tissue can be explained by replacement of normal breast adipose tissue by dense tumoral tissue. However, we can not elucidate the alterations in lipid contents of marginal tissue which has an almost similar histology to normal breast. Nevertheless, there is a possibility that some subtle molecular changes lead to its altered lipid content which warrants further investigations.

5.4. **Fatty acid composition of breast tissue**

Major fatty acids in normal breast tissue in our study were 16:0 (19.8%), 18:0 (4.45%), 18:1 c9 (44.57%) and 18:2 n-6 (13.16%) which is in accordance to other studies.

A cohort study carried out in Danish women showed the major fatty acids in the adipose tissue are 18:1n9 oleic acid , 16:0 palmitic acid, 18:2n-6 linoleic acid, 18:0 stearic acid which constitute about 77% of total fatty acids. EPA, DPA and DHA constitute less than 1% of total fatty acid. Eighty six percent of breast cancer cases in their study were ductal, 65% less than 2 cm, 51% node negative and 84% estrogen positive(6). Their multivariable analysis didn't show any significant association between adipose tissue content of marine n-3 PUFA and breast cancer risk (6).

A study in Malaysia reported major fatty acids in breast adipose tissue of subjects with either benign or malignant lesion to be oleic acid (45-46%), palmitic acid (28-29%) and linoleic acid (11-12%) (96).

The EURAMIC Study investigated the subcutaneous gluteal adipose tissue fatty acids in breast cancer cases and controls (62). The level of DHA (% of total fatty acids) in cancer patients and controls was statistically similar between cases and controls (0.10-0.21 vs 0.12-0.20). In our study the DHA level of normal breast tissue was 0.02-0.22 which is also similar to their finding. They showed statistically similar results for alpha linolenic acid (ALA) in cancer patients (0.43-

0.71) and controls (0.42-0.73), while we found the level of ALA in normal breast tissue to be 1.44-2.06 which tends to be higher than their finding.

The level of omega-6 linoleic acid in EURAMIC cancer patients 10.2-15.6, in controls 10-14 and normal breast tissue in our study (11.07-15.25) were similar.

The difference in levels of fatty acids in EURAMIC study and our study can be explained through the results of a study done on 25 women with high risk breast cancer. They showed that gluteal adipose tissue cannot be a good surrogate of studying dietary effect on breast adipose tissue since breast adipose tissue changes more rapidly to dietary modification compared to gluteal adipose tissue(74).

There are non-consistent data on the association of n-3 PUFA in adipose tissue and the development of breast cancer. While a number of studies demonstrated a protective role of n-3 PUFA on breast cancer(97), others indicated that there is no association between n-3 PUFA and cancer development(6).

We found the fatty acid composition of tumoral tissue is totally different from the normal breast tissue with higher amounts of arachidonic acid, 20:3 n-6, 20:2 n-6, 22:5 n-6, EPA, DHA, 18:1 trans and 18:2 CLA isomers and lower amount of 14:0, 17:1 c10, 18:1 t11, 18:1 c9, 18:3 n-3 and 18:2 n-6.

5.5. **Phospholipids and breast cancer tissue**

There are very few studies investigating the lipid content of breast tumoral tissue. Punnonen et al concluded that phospholipid composition of fatty acids is different in breast cancer tissue compared to normal tissue (76). They indicated that cancerous tissue contained high level of 14:0, 16:0, 18:0, 18:1, 18:2 in PE fraction and high levels of 18:0 and 18:1 in PC fraction of phospholipids which could be because of different lipid metabolism in cancerous tissue.

Petrek et al showed that greater subcutaneous (breast and abdominal) adipose tissue content of oleic acid was associated with higher risk of positive lymph nodes(32). On the other hand, we found that the level of palmitic acid in PC, lysoPC and SM are significantly higher while in PS is significantly lower in tumoral tissue compared to normal breast tissue. Also tumoral tissue showed increased levels of oleic acid in PS and PI and increased levels of arachidonic acid in PC, PI and PE fraction. Examining other fatty acids didn't show any significant changes between the groups. The increased amount of arachidonic acid in tumoral cells is possibly due to its role in calcium-mediated angiogenesis(98).

Another study only found a significant difference in 24:1 content of phospholipid in breast adipose tissue samples of cancer cases and controls(78).

This discrepancy in results may have in part resulted from different methods used for fatty acid analysis.

The unique lipid profile of tumoral tissue suggests a special microenvironment for development of breast cancer. Also the subtle change in fatty acid composition of marginal tissue addresses a possible trend of alteration from normal breast tissue which eventually leads its way to cancer formation.

5.6. **Plasma and red blood cell fatty acid composition**

We found that the plasma levels of 20:3 n-6 and 18:1 t4 and red blood cell level of 20:3 n-6 and 22:5 n-6 are positively correlated with grade of breast cancer. These findings are in agreement with other studies which indicated n-6 fatty acids promote the growth of tumoral cells(98). We couldn't find any study addressing the correlation of any fatty acid with breast cancer grade.

In our study, the stage of breast cancer was shown to be positively correlated with plasma and red blood cell levels of 18:1 isomers of t9, t10, t11, t12 and c12 and negatively correlated with plasma levels of DHA.

We didn't find any study evaluating the association of fatty acid composition of red blood cells or plasma and stage of the breast cancer. The majority of previous studies investigated the risk of breast cancer with various plasma fatty acid profiles or compared the lipid content of cancer cases and healthy individuals.

The results of a study showed higher red blood cell membrane content of oleic acid and lower content of stearic acid in breast cancer patients compared to controls(99).

In a case control study, serum levels of fatty acids were compared in 127 incident breast cancer cases and 242 matched controls. It was shown that low serum levels of linoleic acid and high serum levels of MUFA trans-11 18:1 predict high incidence of breast cancer (100).

A study at University of Crete compared the fatty acid composition of gluteal adipose tissue in 94 women with breast cancer and 131 healthy subjects. The level of myristic acid was positively associated whereas the levels of monounsaturated fatty acids and oleic acids were negatively associated with breast cancer risk. They suggested that these associations were mediated mainly through HER-2/new and FAS oncogenes (101).

5.7. Summary of main findings and conclusion

We have shown that the major fatty acids in breast tissue were 16:0 (19.8%), 18:0 (4.45%), 18:1 c9 (44.57%) and 18:2 n-6 (13.16%). We found that the stage of breast cancer was positively correlated with plasma and red blood cell levels of 18:1 isomers of t9, t10, t11, t12 and c12 and negatively correlated with plasma levels of DHA. We discovered an inverse relationship between plasma levels of fatty acids and disease progression.

In our study the plasma level of 20:3 n-6 and 18:1 t4 and red blood cell level of 20:3 n-6 and 22:5 n-6 were positively correlated with grade of breast cancer.

Levels of arachidonic acid, palmitic acid and oleic acid in specific phospholipid fractions were significantly greater in tumor compared to normal or marginal breast tissue. We conclude that the lipid profile of tumoral tissue is different from marginal and normal breast tissue. In addition the level and composition of plasma and red blood cell membrane fatty acids can estimate the severity of breast cancer.

5.8. Strength and limitation of the study

A major strength of this study is that all major fatty acids and lipid classes in plasma, red blood cells and tissue were examined. This study is the first to investigate the lipid profile of marginal breast tissue and compare it to normal and tumoral breast tissue. Also it is the first study to correlate the severity of cancer to fatty acid levels in plasma and red blood cells.

The main limitation in our study was lack of a reference or control population for obtaining breast tissue sample of normal subjects. This is a common limitation in human studies which makes the interventional studies complicated due to ethical issues. Nevertheless we compared the fatty acid composition of three different areas of breast tissue in one individual with identical dietary background. However when tissue composition is compared between controls and cases, the different dietary intake makes the statistical analysis unreliable.

The other limitation in our study was our sample size. Increasing the sample size would in turn increase the power of the study which makes our statistical test more likely to detect a significant difference.

5.9. Future Research and Implications in the field of nutrition

Breast carcinogenesis and its relationship to dietary fat or any specific fatty acid has been a subject of dispute(32). The altered level of certain fatty acids in cancer tissue versus normal breast tissue could be due to a variation in dietary intake or as a result of metabolic interactions between tumor and normal breast cells.

Future studies should examine the effect of changes in fatty acid component of diet on breast cancer cell behavior *in vivo*. Supplementing the proper fatty acids in the diet of high risk breast cancer patients might lower the chance of later development of cancer. Likewise adding certain fatty acids to chemotherapy protocols in cancer patients might enhance the growth inhibiting effect of the drug.

Consequently better understanding of biological behavior of breast cancer would help improve the outcome of breast cancer patients by making new treatment modalities (102).

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Appendix

RESEARCH PARTICIPANT INFORMATION AND CONSENT FORM

Title: Characterization of fatty acid composition of breast cancer tissues in Manitoban subjects

Sponsoring Agency: Manitoba Medical Services Association

Principal Investigator:
Dr. Gabor Fischer

Co-investigator:
Dr. Mohammed Moghadasian

Clinical Investigator:
Dr. Virginia Fraser

Study Coordinator:
Dr. Nazila Azordegan

You are being asked to participate in a human research study. Please take your time to review this consent form and discuss any questions you may have with the study staff. You may take your time to make your decision about participating in this study and you may discuss it with your regular doctor, friends and family before you make your decision. This consent form may contain words that you do not understand. Please ask the study doctor or study staff to explain any words or information that you do not clearly understand.

If you take part in this study, you agree to donate 10 ml (two teaspoons) of your blood (obtained for preoperative workup) and specimens of your tissues (obtained for histologic examination) for research objectives of this study. You will also be asked to complete the attached questionnaire. It should be noted that prior to your surgery your doctor will ask you for blood sample for the treatment/surgery purposes. Samples of that blood collection will be used for this research protocol to determine the levels of certain lipid components.

Similarly, your surgeon will remove your tissue and send it for pathological examinations. Samples of those tissues will be used for this research protocol determine the levels of certain lipid components. Therefore, participation in this research protocol will cause no additional discomfort and inconvenience. All potential risks and discomfort that you may experience are related to your treatment. The collected samples will be coded by the study coordinator using a numeric code system to keep confidentiality, and after the analysis of the results they will be destroyed.

Benefits

By participating in this study, you will be providing information to the study doctors that will show the effects of different types of oil in cancer prevention.

Participation in this study has no cost to you. Similarly, because you will be coming to the hospital for your treatment, no payment will be made to you for your participation in this research study.

Confidentiality

Information gathered in this research study may be published or presented in public forums, however your name and other identifying information will not be used or revealed. Medical records that contain your identity will be treated as confidential in accordance with the Personal Health Information Act of Manitoba. Despite efforts to keep your personal information confidential, absolute confidentiality cannot be guaranteed. Your personal information may be disclosed if required by law.

All study documents related to you will bear only your assigned patient number (or code).

Study doctors, the University of Manitoba Health Research Ethics Board, St. Boniface General Hospital staff, and if required by law, other organizations may obtain access to your records containing personal health information including medical records and research data.

All records will be kept in a locked secure area and only the investigators and the study coordinator will have access to these records. If any of your medical/research records need to be copied and given to the third party, your name and all identifying information will be removed. No information revealing any

personal information such as your name, address or telephone number will leave the St. Boniface General Hospital Research Centre.

Voluntary Participation/Withdrawal from the Study

Your decision to take part in this study is voluntary. You may refuse to participate or you may withdraw from the study at any time. Your decision not to participate or to withdraw from the study will not affect your medical care at this site.

If you are a University of Manitoba/hospital student or staff, your performance evaluation will not be affected by your decision not to participate.

Questions

You are free to ask any questions that you may have about your treatment and your rights as a research participant. If any questions come up, contact the study doctor and the study staff: Dr. Gabor Fischer or Dr. Mohammed Moghadasian or Dr. Azordegan. In case of an urgent problem and in case Drs. Fischer and Moghadasian both are unavailable call Department of Pathology (xxxx) or Pathology Research Laboratory (xxxx).

For questions about your rights as a research participant, you may contact The University of Manitoba Biomedical Health Ethics Board at xxxxxxxxx.

Do not sign this consent form unless you have had a chance to ask questions and have received satisfactory answers to all of your questions.

Statement of Consent

I have read this consent form. I have had the opportunity to discuss this research study with *Dr. Gabor Fischer and/or Dr. Mohammed Moghadasian* and or their study staff. I have had my questions answered by them in language I understand. The risks and benefits have been explained to me. I believe that I have not been unduly influenced by any study team member to participate in the research study by any statement or implied statements. Any relationship (such as employee, student or family member) I may have with the study team has not affected my decision to participate. I understand that I will be given a

copy of this consent form after signing it. I understand that my participation in this clinical trial is voluntary and that I may choose to withdraw at any time. I freely agree to participate in this research study.

I understand that information regarding my personal identity will be kept confidential, but that confidentiality is not guaranteed. I authorize the inspection of my medical records by the University of Manitoba Biomedical Research Ethics Board, St. Boniface General Hospital and/or government authorities, if required by law.

By signing this consent form, I have not waived any of the legal rights that I have as a participant in a research study.

I agree to being contacted in relation to this study. Yes ☐ No ☐

Yes ☐ No ☐

Participant signature_____Date _____
(day/month/year)

Participant printed name: _____

I, the undersigned, attest that the information in the Participant Information and Consent Form was accurately explained to and apparently understood by the participant or the participant's legally acceptable representative and that consent to participate in this study was freely given by the participant or the participant's legally acceptable representative.

Witness signature_____Date_____
(day/month/year)

Witness printed name: _____

I, the undersigned, have fully explained the relevant details of this research study to the participant named above and believe that the participant has understood and has knowingly given their consent

Printed Name: _____Date_____
(day/month/year)

Signature: _____

Food Frequency Questionnaire

This questionnaire is part of a research study program to look at your eating habits. Please take the opportunity to fill out the answers as best as you can. We appreciate your participation in this study and thank you in advance for completing the questionnaire.

1. Age:
2. Menopause? Yes _ No _
3. Weight (Please indicate units: kg or pounds):
4. Height (Please indicate units: cm or feet/inches):

5. What city/town/municipality do you currently live in?

6. What is your current marital status?
Single
Married or common-law
Separated or divorced
Other (please specify)

7. Do you have any children? Yes _ No _
7a. If you have answered yes, please indicate how many children you have: ____

8. What is your current employment status and education level?

9. Do you think your current occupation exposes you to any hazardous pollutants or chemicals? Yes _ No _

9a. If you have answered yes, please indicate what pollutants/chemicals: (if known)

10. What are your current cigarette smoking practices?
daily smoker (___ cigarettes per day)
occasional smoker (___ cigarettes per week)
non-smoker

11. What are your current alcohol consumption practices?

daily (____ alcoholic drinks per day)
weekly (____ alcoholic drinks per week)
monthly (____ alcoholic drinks per month)
Other (please specify _____)
Non-drinker

11a. If you drink alcohol, what is the typical number of drinks you consume on each occasion?

12. Please list any drugs (medications, hormones) that you currently use: (if known)

13. Please name any supplements that you currently take: (for example: fish oil, vitamins, flaxseed, herbs, etc.)

14. Have you had any previous surgeries? Yes _ No _

14a. If yes, please specify what procedure(s) and date of surgery:

15. Do you have any previous history of cancer? Yes _ No _

If yes, what type?

16. Do you have a family history of any cancer? Yes _ No _
If yes, what type?

17. How often do you consume fish?

2 or more times per week
Once per week
Once or twice per month
Rarely

18. If you consume fish, which types of fish do you consume more often?

19. Please name any other seafood you consume at least once a month:
20. How frequently do you consume fast food?
a) Daily (____ times per day)
b) Weekly (_____ times per week)
c) Monthly (_____ times per month)
d) Rarely (please specify: _____)

21. Do you consume commercial bakery items? (For example: crackers, donuts, cakes, cookies, etc.) Yes _ No _

21a. If yes, please indicate which types of commercial bakery items and frequency:

22. Please check if you consume any of the following oils and indicate the frequency: (Please indicate brand consumed if known)
Butter _ ___times per week
Margarine _ ___ times per week
Shortening _ ___ times per week
Canola oil _ ___times per week
Salad dressing _ ___times per week
Mayonnaise _ ___times per week
Walnut oil _ ___times per week
Flaxseed (seed or oil) _ ___times per week
Corn oil _ ___times per week

22a. If you have checked any of the above oils, please indicate how you use the oils in food preparation:

23. What nuts (for example: walnuts, pecans, peanuts, almonds, etc.) do you consume most often? Please indicate which nuts and in what amounts:

23a. How often do you consume the above nuts?
Daily (_____ times per day)
Weekly (_____ times per week)
Monthly (_____ times per month)
Rarely (please indicate frequency: _____)

Thank you for your participation in this valuable research program.