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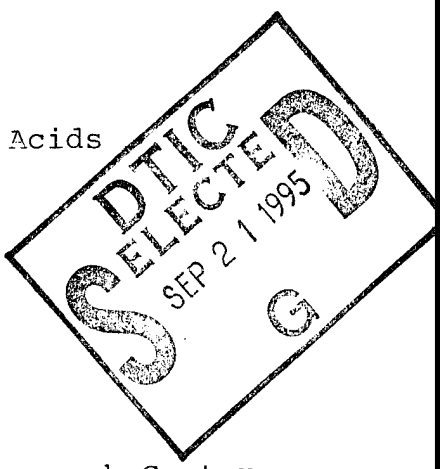

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13. ABSTRACT (Maximum 200 words)

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Janet L. Stanford 8/9/95

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Fatty Acids and Breast Cancer: Is There an Association?

I. ABSTRACT

Experimental studies have shown that omega-6 fatty acids enhance and omega-3 fatty acids suppress oncogenesis. Correlational studies also indicate that breast cancer incidence is positively linked to omega-6 consumption but is negatively related to intake of omega-3 fatty acids, derived mainly from marine sources. To evaluate whether or not these fatty acids are associated with risk of breast cancer, the authors collected pre-treatment blood samples from 138 cases with histologically confirmed incident breast cancer diagnosed between 5/90 and 4/93, and 141 control women frequency matched on age who participated in a population-based case-control study of breast cancer in women under age 45. Erythrocytes (RBCs) were washed and stored at -70°C. Fatty acids were extracted from RBC membranes and gas-liquid chromatography was used to determine the relative proportions of specific fatty acids. Univariate and multivariate analyses were completed. The relative proportions of the major omega-6 fatty acids (18:2n-6, linoleic acid; 20:4n-6, arachidonic acid), and omega-3 fatty acids (20:5n3, eicosapentaenoic acid; 22:6n-3, docosahexaenoic acid) were similar in cases and controls. Compared to the lowest quartiles (OR=1.0, reference group) of total omega-6 and total omega-3 fatty acids, women in the upper quartiles had relative risk estimates of 0.54 (95% confidence interval, CI 0.3-1.1) and 1.9 (95% CI 0.9-4.0), respectively. These results do not support the hypothesis that omega-3 fatty acids reduce the risk of breast cancer in young women.

II. INTRODUCTION

Breast cancer is the most frequently diagnosed cancer among women in the United States, accounting for 32% of all incident cancers diagnosed in 1995 [Wingo et al., 1995]. The descriptive epidemiology of breast cancer and evidence regarding established and suspected risk factors for the disease have been extensively reviewed [Kelsey and Gammon, 1991][Kelsey and Horn-Ross, 1993]. At the present time, known risk factors for breast cancer can explain only about 50% of its occurrence in the population, and many of the stronger risk factors such as age, race, family history of breast cancer, benign proliferative breast disease, and late age at first full-term pregnancy, are not amenable to change. Dietary fat intake, however, has been associated with risk of breast cancer and provides a promising area of research for cancer prevention.

Experimental and observational studies suggest that dietary fat influences the development of breast cancer. Polyunsaturated fats appear to have a more adverse effect than saturated fats on tumor occurrence, and within the family of polyunsaturated fatty acids, omega-6 fatty acids appear to enhance risk while omega-3 fatty acids reduce risk [Karmali, 1987a][Rogers and Longnecker, 1988][Yetiv, 1988]. Whether or not such associations with fatty acids exist in women, however, is unclear since few studies have evaluated the composition of dietary fat. If dietary intake of omega-3 fatty acids is found to reduce the risk of breast cancer, this could have substantial public health implications.

To evaluate the possible relationship of fatty acids and breast cancer, we conducted an ancillary study as part of a larger population-based case-control study of breast cancer in women under age 45 years. The primary aim of the study was to determine whether or not specific or total levels of omega-3 and omega-6 fatty acids measured in erythrocyte (red blood cell) membranes, a biomarker of recent fat intake, are associated with breast cancer in young women.

A. Dietary Fat and Breast Cancer

Experimental evidence linking dietary fat to the genesis of mammary tumors was first reported over 50 years ago [Tannenbaum, 1942]. Data consistent with a role for

dietary fat in the etiology of breast cancer include: 1) animal studies demonstrating a consistent and strong link between the incidence and number of mammary tumors and the amount and composition of dietary fat [Carroll et al., 1986][Rogers and Longnecker, 1988]; 2) international correlational studies showing that breast cancer incidence and mortality rates are directly related to the per capita consumption of dietary fat estimated from food disappearance data [Rose et al., 1986][Prentice et al., 1988]; and 3) migrant studies indicating that women who migrate from low-incidence to high-incidence countries for breast cancer soon acquire rates that approach those of women in the high risk areas [Muir and Staszewski, 1986][Thomas and Karagas, 1987].

Findings from case-control and cohort studies of diet and breast cancer have been reviewed, and provide further clues that dietary fat may influence risk [Rohan and Bain, 1987][Hulka, 1989][Howe et al., 1990][Hunter and Willett, 1993], although results are inconsistent. Methodologic limitations related to measurement error in estimating past dietary exposures from food questionnaires may explain the lack of consistency in findings from previous studies [Willett, 1990]. Another problem with past research has been reliance on food frequency questionnaires to estimate dietary exposures, rather than more objective biomarkers to quantify and qualify fat intake.

B. Fatty Acids and Breast Cancer

Experimental studies with animals indicate that the type of fat may be more important than the amount of fat in the diet in causing mammary tumors [Rogers and Longnecker, 1988]. Few observational studies, however, have considered the fatty acid composition of the diet in relation to breast cancer incidence. Given the varied physiological roles of different fatty acids, a more specific investigation of dietary fat according to the amount of unsaturation and type of the component fatty acids may help clarify the role of fat in breast cancer etiology.

The first experimental evidence that omega-3 fatty acids have anticancer activity was reported about 10 years ago [Karmali et al., 1984]. Subsequently, animal studies comparing omega-3 (fish oil) with omega-6 (corn oil) exposures have confirmed that omega-3 fatty acids suppress oncogenesis [Karmali, 1987a][Szeluga et al., 1987]. In contrast, a requirement for linoleic acid, an omega-6 fatty acid, has been demonstrated in mammary carcinogenesis [Carroll et al., 1986][Ip, 1987][Rogers and Longnecker, 1988]. Omega-6 polyunsaturated fatty acid-containing oils (e.g., corn oil, safflower oil) fed to rats given chemical carcinogens (e.g., DMBA, MNU) increase significantly the incidence and number of tumors and decrease the tumor latency period [Rogers and Longnecker, 1988].

There is also evidence from epidemiologic studies that certain fatty acids may be associated with risk of breast cancer. Analyses of ecologic data by Prentice and coworkers [Prentice et al, 1988] implicate polyunsaturated fats, especially the omega-6 fatty acids, in breast cancer development. More recently, Hursting and associates [Hursting et al., 1990] analyzed breast cancer incidence from 20 countries according to estimates of per capita disappearance of polyunsaturated fats. These investigators found that total polyunsaturated fat was positively correlated with age-standardized breast cancer incidence rates. Fish omega-3 polyunsaturated fat was negatively associated with the incidence of breast cancer [Hursting et al., 1990].

Additional support for a role of fatty acids comes from the observation that Alaskan natives have an age-adjusted breast cancer incidence rate that is about 60% lower than the rate in Connecticut [Lanier et al., 1976]. Investigators postulate that the high omega-3 intake of Eskimos is partly responsible for their low rates of cancer [Karmali, 1987b] [Yetiv, 1988]. The omega-3 fatty acid intake of Eskimos is exceptionally high,

making up 13% of total fatty acids compared to 0.8% in the Danish diet [Bang et al., 1976], which is similar to the typical western diet consumed in countries with high incidence rates of breast cancer. In Greenland, Eskimo women have a low incidence of breast cancer compared to women in Denmark, despite a total fat intake that is similar [Nielsen and Hansen, 1980]. The major difference between the two groups is the type of fat consumed, with Eskimos eating large amounts of fish oil [Rose,1986].

Insull et al. [Insull et al., 1969] compared Japanese with Americans according to consumption of dietary fat, and found that Japanese consume more unsaturated fat than Americans. These investigators did not determine omega-3 fatty acid intake, but higher consumption of fish by Japanese women compared to U.S. women may be one explanation for the lower breast cancer incidence rate in Japan. Other researchers compared fatty acid profiles of breast adipose tissue from Japanese and American women treated for breast cancer [Hill and Wynder, 1987]. Postmenopausal patients from the U.S. had higher levels of linoleic acid (18:2n-6) than their Japanese counterparts, but no difference was observed in premenopausal women. Omega-3 fatty acids were not measured in that study.

Only a few epidemiologic studies of breast cancer have attempted to use biomarkers of polyunsaturated fatty acid intake. London et al. [London et al., 1993] determined fatty acid composition of subcutaneous adipose tissue in a series of postmenopausal women from five Boston area hospitals who sought evaluation for breast abnormalities or symptoms. Using women who were diagnosed with nonproliferative breast disease or who did not undergo biopsy as the control group, these researchers found no overall associations between polyunsaturated fatty acids and breast cancer. Women in the highest quintile of linolenic (18:3n-3) and eicosapentaenoic (20:5n-3) acid, however, had risk estimates that were 10% and 30% lower, respectively, compared to women in the lowest category of exposure. There were no associations with omega-6 fatty acids.

Two other studies measured fatty acid levels in adipose tissue from breast cancer patients and women with non-malignant breast diseases [Caleffi et al., 1987][Eid and Berry, 1988]. The study by Caleffi et al. [Caleffi et al., 1987] included 23 breast cancer cases and 30 control subjects, and the one by Eid and Berry [Eid and Berry, 1988] involved 37 cancer cases and 48 controls. No associations were found in these small studies, which measured only a few fatty acids. Both had insufficient numbers of study subjects to accurately estimate risk of breast cancer in relation to fatty acid profiles. Furthermore, none of the above studies were population-based, and most of them used as controls women presenting for evaluation of some breast abnormality. These methodologic problems may have obscured true associations of fatty acid profiles with breast cancer.

A single epidemiologic study measured erythrocyte membrane fatty acid profiles in relation to risk of breast cancer. Zaridze et al. [Zaridze et al., 1990] calculated mean values for five fatty acids (16:0, 18:0, 18:1n-9, 18:2n-6, 20:4-n6) and found inverse associations between risk of premenopausal breast cancer and levels of linoleic acid and between risk of postmenopausal breast cancer and arachidonic acid. All breast cancer patients (25 premenopausal, 21 postmenopausal) had localized disease at diagnosis. Control women were seen at the same out-patient clinics as the cases, and blood samples were collected on 20 premenopausal and 33 postmenopausal controls. These data are difficult to interpret given the small numbers of subjects and the few fatty acids measured. Omega-3 fatty acid levels were not determined in the study.

In summary, epidemiologic studies of fatty acids in relation to breast cancer are insufficient and findings are difficult to interpret. Researchers have generally not evaluated individual omega-3 and omega-6 fatty acids separately, even though they apparently have opposing physiologic effects. Further, most investigations relied on dietary questionnaires to measure fatty acid exposures, rather than potentially more accurate biomarkers.

C. Biomarkers of Dietary Fat Intake

Biochemical techniques are now available to accurately estimate exposure to dietary fat in qualitative terms. Modern gas chromatographic methods permit reliable separation and measurement of a range of individual fatty acids [Holman, 1986].

All cell membranes and most body fluids contain fatty acids, which can be measured in plasma, erythrocyte (red blood cell) membranes, or adipose tissue. Plasma fatty acids reflect relatively short-term dietary intake. Plasma triglycerides represent hourly or daily changes, cholesterol esters reflect a 1-2 week period, and phospholipids a 5-6 week interval. Erythrocyte membranes are about 95% phospholipids and red cell turnover is about 120 days. Thus, erythrocyte membrane fatty acid profiles reflect dietary exposures over the past two to three months. Adipose tissue is a long-term storage site for fatty acids and reflects dietary intake during approximately the previous two years [Dayton et al., 1967].

Dougherty et al. [Dougherty et al., 1987] have shown that erythrocyte membrane fatty acid composition is a better, more stable indicator of dietary fatty acid intake than plasma fatty acid composition. By comparison, collection of adipose tissue is a more invasive procedure than venipuncture, which makes adipose sample collection not feasible for most epidemiologic field studies. For these reasons, erythrocyte membranes appear to be the best biomarker available for assessment of fatty acid profiles in the context of epidemiologic studies.

Dietary polyunsaturated fatty acids are the only source of essential fatty acids, which cannot be synthesized by humans [Karmali, 1987a]. Two important classes of essential fatty acids, linoleic (18:2n-6) and linolenic (18:3n-3) acid, are parent compounds for omega-6 and omega-3 polyunsaturated families, respectively, which undergo various elongations and desaturations (Figure 1). Although the metabolic pathways of these two families of fatty acids share the same enzymes, there is no interconversion between omega-6 and omega-3 fatty acids [Holman , 1986].

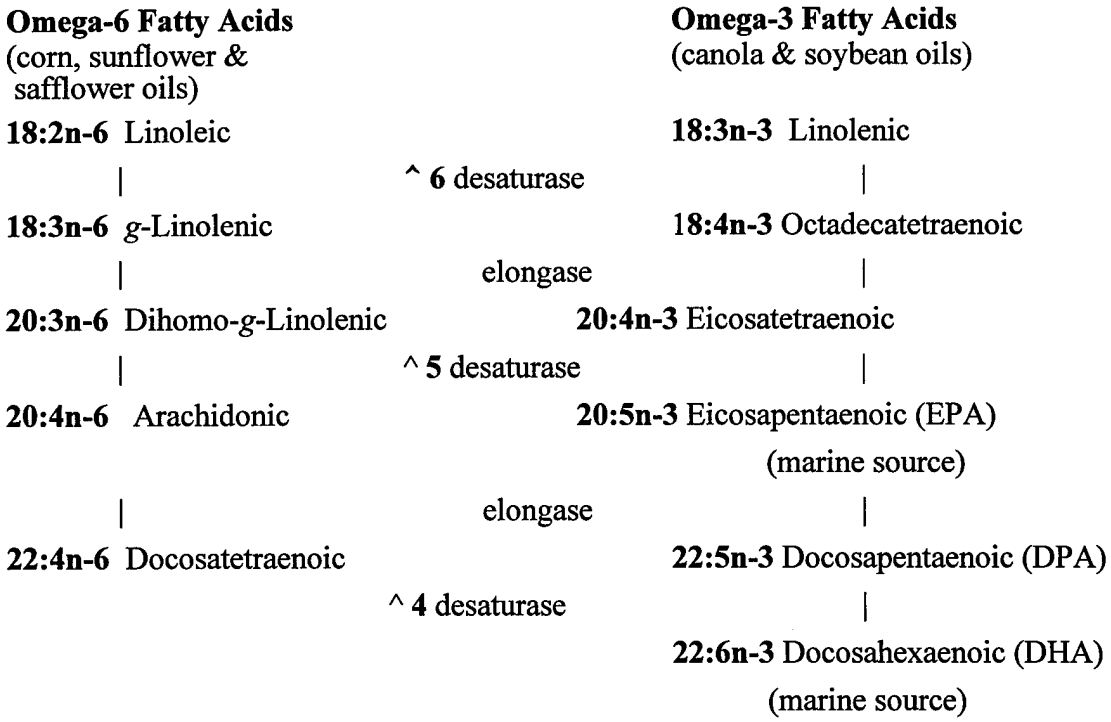

Figure 1. Metabolic pathways of omega-6 and omega-3 fatty acids.

Some fatty acids are more susceptible to dietary influences than others. Studies confirm that increasing the dietary intake of linoleic and linolenic acid increases the levels of omega-6 and omega-3 polyunsaturated fatty acids, respectively, in tissue lipids [Holman, 1986]. Linoleic acid, the most common polyunsaturated fatty acid found in the U.S. diet (6-7% of kcal) is strongly associated with dietary intake. The tissue levels of arachidonic acid (20:4n-6), the major metabolic regulator, are influenced both by diet and metabolic interactions with other polyunsaturated fatty acids [Lands, 1992]. Increasing the intake of dietary linoleic acid may increase tissue levels of arachidonic acid [Kinsella et al., 1990]. Although dietary intake of arachidonic acid is low (<0.2% kcal) and is mainly derived from animal origin, dietary sources have a greater impact on tissue stores of arachidonic acid than endogenous production [Sanders et al., 1978][Phinney et al., 1990]. Tissue levels of arachidonic acid can also be modulated by the omega-3 fatty acids. Linolenic acid ingested regularly as part of a normal diet will significantly reduce tissue levels of arachidonic acid, and will increase tissue levels of linolenic acid as well as the longer chain omega-3 fatty acids eicosapentaenoic (EPA) and docosahexaenoic (DHA) [Sanders and Younger 1981][Renaud and Nordoy, 1983]. Tissue levels of EPA and DHA are mainly influenced by dietary intake of marine sources such as fatty fish (e.g., salmon, mackerel) and fish oils [Salem ,1989].

D. Biological Mechanisms: Fatty Acids and Breast Cancer

Many biochemical mechanisms have been proposed by which dietary fatty acids, particularly omega-6, may exert a stimulatory effect on the development and progression of breast cancer. These mechanisms include changes in essential fatty acid metabolism, eicosanoid production, cell membrane structure and function, cell-cell communications, and immune and endocrine system functions [Karmali et al., 1984][Erickson, 1986][Welsch, 1987][Kinsella, 1990].

Long-chain polyunsaturated fatty acids are precursors of eicosanoids, prostaglandins, thromboxanes, and leukotrienes [Lands, 1992]. Eicosanoids, produced by arachidonic acid, are elevated in malignant tissues and are thought to be involved in tumor initiation and promotion [Karmali, 1987b]. Animal studies show that both eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) suppress arachidonic acid metabolism, which may explain why omega-3 fatty acids inhibit tumorigenesis [Hill and Wynder, 1987].

Arachidonic acid is a precursor for prostaglandins, substances that enhance cell proliferation rates [Welsch, 1987]. Inhibitors of prostaglandin biosynthesis oppose the tumor promoting effect of dietary fat in experimental models, and eicosapentaenoic acid (EPA) in fish oil has been shown to block prostaglandin formation from arachidonic acid [Carroll et al., 1986].

Since cell membrane composition is dependent to a large extent on the amount and type of fatty acids, membrane fluidity also can be altered by dietary fat [Spector and Yorek, 1985][Welsch, 1987]. Cell membrane fluidity represents molecular motion of proteins and lipids in the membrane structure. Compared to saturated fatty acids, polyunsaturated fat increases the fluidity of membranes, and increased cell division has been observed with increased membrane fluidity. Higher levels of unsaturated fatty acids, particularly linoleic, have been found in cell membranes derived from proliferating mammary cells compared to non-proliferating cells [Welsch, 1987]. More importantly, the fatty acid architecture of cell membranes can influence membrane protein functions [Murphy, 1990] and enzyme activities [Kinsella, 1990]. Therefore, dietary fatty acids may modulate membrane receptors and regulate membrane bound enzymes such as adenyl cyclase and 5'-nucleotidase [Kinsella, 1990].

Intercellular communications modulate cell growth and differentiation and may play a role in tumor promotion. One type of cell-cell communication is via the passage of

low-molecular weight compounds such as growth factors through the gap junctions in membrane structures. This communication appears to be blocked by many tumor promoters, and polyunsaturated fatty acids have been shown to inhibit cell-cell communication [Welsch, 1987].

The role of dietary fat in modulation of immune function is another way in which fatty acids may alter risk of breast cancer. Current evidence suggests that diets high in omega-6 fatty acids are more immunosuppressive than diets high in omega-3 fatty acids [Erickson , 1986]. The immune response also may be mediated by changes in cell membrane architecture or by modulation of eicosanoid production [Karmali, 1987b].

Finally, several hormones are known to be influenced by the amount and type of dietary fat [Clandinin et al., 1991]. There is some evidence that omega-3 fatty acids lower the production of estrogen and prolactin, as well as that of other growth factors such as epidermal growth factor (EGF), transforming growth factor (TGF), and insulin-like growth factor (IGF) [Galli and Butrum, 1991]. The omega-3 fatty acids may also decrease the 16-alpha-hydroxylation of estrogen, a process which may reduce the risk of breast cancer [Osbourne et al., 1988].

E. Study Objectives

Gas-liquid chromatography was used to measure erythrocyte fatty acid levels in 138 breast cancer patients and 141 population controls who were recruited and interviewed for a larger population-based case-control study of breast cancer in women under age 45. The specific aims of this investigation of fatty acids in relation to breast cancer were to test the following hypotheses:

1) Levels of omega-3 fatty acids (eicosapentaenoic, docosahexaenoic, total omega-3) are higher in controls as compared to women with breast cancer.

2) Levels of omega-6 fatty acids (linoleic, arachidonic, total omega-6) are higher in women with breast cancer as compared to controls.

The hypotheses were tested by determining whether levels of omega-3 and omega-6 fatty acids, as measured by the fatty acid composition of erythrocyte membranes, biomarkers of dietary fat intake, were negatively and positively associated with breast cancer risk, respectively. In addition to the above aims, we evaluated whether or not the relative proportions of total saturated fat, total polyunsaturated fat, total monounsaturated fat or trans-fatty acids determined from erythrocyte membranes were associated with breast cancer status.

III. BODY

A. Overview

Blood samples available for this project were a unique resource obtained from participants in a population-based case-control study of breast cancer in women under age 45, which included an extensive in-person interview on risk factors for breast cancer, a detailed food frequency questionnaire, anthropometric measurements, and blood collection. Blood samples were collected prior to treatment and within 90 days of diagnosis date for all breast cancer patients eligible for this analysis, and at the time of interview for control subjects. Washed erythrocyte (RBC) samples were frozen at -70^{o} C, and were available from 138 incident breast cancer cases and 141 control subjects.

Gas-liquid chromatography was used to determine the relative proportions of 38 individual fatty acid components extracted from red cell membranes. Multivariate regression will be used to test for differences between cases and controls in levels of the main exposures of interest (18:2n-6, 20:4n-6, 20:5n-3, 22:6n-3, total omega-3, total omega-6), controlling for potential confounding factors. Fatty acid levels were examined as continuous and categorical variables. Questionnaire data on risk factors, dietary intake, and anthropometric measurements was linked to the fatty acid data so that possible confounding or interacting effects of breast cancer risk factors and dietary exposures on study results could be evaluated.

B. Experimental Methods

The blood samples for this case-control study of fatty acids in relation to risk of breast cancer were collected as part of a multicenter population-based case-control study of breast cancer in women under age 45, which was sponsored by the National Cancer Institute (Women's Interview Study of Health, N01-CP95671;P.I. Janet R. Daling, PhD). The Seattle component of the study involved identification of women immediately following a positive biopsy for breast cancer, so that a blood sample and anthropometric measures could be obtained prior to any surgery or other treatment for the breast cancer. On average, case bloods were drawn within 2-4 weeks of diagnosis date, and control bloods were obtained at the time of interview. Population controls for the study were identified through random digit telephone dialing, and were frequency matched to cases on age. Control women eligible for the study were randomly sampled (40%) and asked to provide a blood sample. Bloods were collected and processed between February 1991 and April 1993. Washed erythrocyte membranes were frozen at -70^{o}C and were available on a total of 138 cases and 141 controls.

Blood samples were processed and stored in the core laboratory, Public Health Sciences, Fred Hutchinson Cancer Research Center. Gas-liquid chromatography was used to determine RBC membrane fatty acid profiles, which included measurement of 38 individual fatty acid components. Batches with both case and control samples were processed in a blinded fashion (i.e., laboratory personnel did not know the case-control status of samples), and case and control samples were selected in such a way that batched samples from each group were similar with respect to time in storage. Blood samples are identified by unique specimen numbers that correspond to a subject's unique study number. Both numbers reside in the main database, which made it easy to blind laboratory personnel as to the case-control identity of the samples.

Demographic factors and other information on established and suspected breast cancer risk factors (family history of breast cancer, menstrual and reproductive characteristics, socioeconomic variables, oral contraceptive and other exogenous hormone use, body weight, physical activity, alcohol and smoking) were available from standardized, in-person interviews completed on all study subjects. Information on dietary intake during the year before diagnosis date (and a similar time period for controls) also was available on all subjects. The food frequency instrument had four separate questions on fish intake as well as a question on how a woman's diet within the past year compared to her diet 10-15 years age with respect to intake of meat, fish, butter, cream and milk. Anthropometry was done (height, weight, waist and hip measurements, skin-folds) on all consenting subjects at the time of interview. Laboratory, questionnaire, and anthropometry data were linked prior to analysis.

C. Ascertainment of Study Subjects: Women's Interview Study of Health (WISH)

Cases

Eligible cases included all female residents of King, Pierce, or Snohomish County who were diagnosed with primary *in situ* or invasive breast cancer during the period May 1, 1990 and April 30, 1993, and who were less than 45 years of age at the time of diagnosis. Two systems were utilized for case ascertainment: 1) a network of breast surgeons in Seattle who identified potentially eligible patients immediately following a positive biopsy for breast cancer, but prior to definitive surgery or other treatment; and, 2) the Cancer Surveillance System (CSS), a population-based cancer registry that has covered the 13-county area of northwestern Washington State since 1974. The CSS is part of the National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) program.

A total of 767 eligible cases were identified for the WISH Study, and 644 (84%) agreed to participate in the interview phase of the study. Since control ascertainment was through random digit telephone dialing, cases (n=12) without a residential telephone at diagnosis were excluded. Of the remaining 632 cases, 430 were eligible for the blood draw component of the study and 401 (93.3%) agreed to provide a sample. Of these, 192 patients had pre-treatment blood samples drawn and washed erythrocytes stored. For the present analysis, only cases (n=138) who had pre-treatment blood drawn within 90 days of diagnosis date are included. This latter decision regarding eligibility was made to maximize the likelihood that the fatty acid profiles truly reflected pre-diagnostic levels, since the lifespan of erythrocytes averages 60-120 days.

Controls

A general population group of control women without a prior history of breast cancer was identified by random digit telephone dialing [Waksberg, 1978]. Random digit dialing is an effective method to select population samples for epidemiologic studies, particularly in areas such as Seattle where residential telephone coverage is estimated to be over 98% [Bureau of the Census, 1992]. During the study period, a total of 789 eligible control subjects, frequency matched to the age distribution of breast cancer patients, was identified. Of these, 610 (77.3%) participated in the interview phase of the study. One control with a prior history of breast cancer was subsequently excluded. A random sample of 402 control women from the Seattle area were asked to provided a blood sample, and 257 (83.2%) agreed. Washed erythrocyte samples were available on 144 of these controls for the proposed fatty acid research.

Data Collection

Several types of data were available for the project. In addition to the fatty acid profiles determined from red cell membranes, data were available from detailed in-person interviews which asked about all known and suspected risk factors for breast cancer, anthropometry measurements, and for cases clinical information from the cancer registry.

Risk Factor Data

Trained interviewers completed in-person questionnaires with breast cancer cases and controls. Information collected focused on menstrual, reproductive, and medical histories, use of contraceptive and non-contraceptive hormones, family history of breast cancer, history of benign breast disease, body weight at different ages, physical activity,

alcohol and tobacco use, and sociodemographic variables such as education and occupation. Following the interview, anthropometry measurements were taken (height, weight, waist and hip circumference, skin-folds) and a food frequency questionnaire that focused on dietary intake over the prior 12 months was completed. Four specific questions on fish consumption were included, and women were asked about how their recent intake of specific food items including meat, fish, cream, butter, and milk compared with their consumption of these foods 10-15 years ago.

Clinical Information

All of the breast cancer patients included in the WISH Study were also ascertained by the Cancer Surveillance System (CSS), our population-based cancer registry. The registry routinely abstracts detailed information on all cancer cases diagnosed in the 13-county area of northwestern Washington State. Information on histologic type, tumor grade, stage of disease at diagnosis, and hormone receptor (estrogen and progesterone) status was available for breast cancer patients in the registry.

Blood Collection and Processing

Patients newly diagnosed with breast cancer were identified through a network of surgeons in the Seattle area who collaborated on the WISH Study. These surgeons' offices were contacted each week to identify all women who had a positive biopsy for breast cancer. Following physician permission, these women were contacted immediately and asked about their willingness to participate in the early blood draw component of the WISH Study, prior to undergoing definitive surgery or other treatment for the cancer. A total of 192 patients agreed to the early (pre-treatment) blood draw. Control bloods were obtained from a random sample of eligible women following the study interview. For the proposed study, 138 cases and 144 controls had frozen, washed erythrocyte samples available for analysis.

Blood was drawn by the WISH Study interviewers, and was collected in 10-ml vacutainer tubes containing liquid potassium EDTA as an anticoagulant. Unique blood sample numbers, linked to different unique subject identification numbers, were used to label blood tubes (i.e., each woman has a unique blood number and study number that are linked in the computer). After removal of plasma and buffy coat, the tube was shipped on ice to the Public Health Sciences core laboratory for further processing. In the core laboratory, erythrocytes (RBCs) were washed 3 times with 5 volumes of isotonic saline. Washed RBCs were then divided into two l-ml aliquots, which were stored in 1-ml cryovials filled to the top to exclude as much air as possible. Both cryovials were then stored at -70^{o} C. For this study, samples were analyzed in 13 batches, which consisted of both case and control samples that had been in storage for approximately the same period of time. Laboratory personnel were blinded as to the case-control status of samples.

Fatty Acid Extraction

The samples were allowed to thaw at room temperature for about 20 minutes. The cells (0.5-ml) were mixed with an equal volume of distilled water, and lipids were extracted with isopropanol and chloroform as follows: 5.5-ml isopropanol (containing 5 mg BHT/100 ml isopropanol) was added, vortexed, and allowed to stand one hour with occasional mixing. Next, 3.5 ml chloroform was added, mixed, and allowed to stand another hour with occasional mixing. The sample was then centrifuged at 2000 rpm for 20 minutes, and the extract (top layer) was poured into a Teflon-lined screw-capped Pyrex tube and evaporated under nitrogen on a 45^{o}C water bath. The fatty acid methyl

esters (FAME) were prepared by direct transesterification using the method of Lepage and Roy [Lepage and Roy, 1986].

Gas-liquid chromatography

Gas-liquid chromatography was performed on samples dissolved in hexane. The FAME of individual fatty acids of RBC membranes was separated on a gas chromatograph (model 5890B, series II, Hewlett-Packard, Avondale, PA) equipped with a flame ionization detector (FID), automatic sampler (Hewlett-Packard 7673) and Chemstation software (Hewlett-Packard). FAME was separated on a 30 m by 0.25 mm ID wall-coated open-tubular fused silica column (DB23) with 0.25 micron coating (J & W, Folsom, CA). The carrier gas was helium at 60 psi; make-up gas is nitrogen at 60 psi at the tank. At the detector end, hydrogen was at 30 psi and breathing air was at 20 psi. Column linear velocity was set at 33cm/sec (oven temperature of 200°C). The injector and detector port temperatures (T) were at 250°C and 275°C, respectively. The oven temperature was programmed using 3 ramp settings; T1 = 170, hold 1 (H1) = 9 minutes, rate 1 (R1) = 6, T2 = 188, H2 = 15, R2 = 4, T3 = 248, H3 = 30 minutes.

Quantitative precision and identification was evaluated with the use of model mixtures of known FAME. This identification has been confirmed by a mass spectrophotometric analysis performed by the USDA lipid laboratory in Peoria, IL. Quantitative results are standardized with the National Heart Institute's Fatty Acid Standards A, B, C, D, E, F, and GLC 87 (Nu-Check-Prep, Elysian, MN). Fatty acid levels were expressed as relative weight percents.

D. Data Processing

Interview data and anthropometric data from the WISH Study were computerized and were available for this analysis. Data from the food-frequency questionnaire were not available for the present analysis. Clinical information on all of the breast cancer patients was also available from the CSS cancer registry computerized database. Double entry was used for interview and cancer registry data to minimize key entry errors. Results from the gas-liquid chromatography went directly into a computer file, which was then linked with the other data sources.

E. Analysis

Descriptive, stratified, and multivariate analyses were performed. Two group t-tests were used to examine differences between cases and controls in the main fatty acids of interest [Snedecor and Cochran, 1980]. Univariate analyses, including graphical display, were completed to examine the distributions of the fatty acids in cases and controls. Subgroup analyses examined fatty acid profiles according to time period between diagnosis date and blood draw date for cancer cases, according to whether or not the woman had received general anesthesia for the biopsy that resulted in the breast cancer diagnosis, and according to stage of disease at diagnosis.

To adjust for possible confounding factors and evaluate possible modifying factors, multivariate regression techniques were used [Anderson et al., 1980]. Factors considered as possible confounders of the associations with fatty acids included: age, age at first term birth, number of term births, previous breast biopsy, family history of breast cancer, education, oral contraceptive use, alcohol use, smoking history, and body mass index. Only age (continuous) and body mass index (quartiles) confounded study results and have been included in multivariate models. Unconditional logistic regression was used to estimate the odds ratios and 95% confidence intervals for the associations between fatty acid levels and breast cancer [Breslow and Day, 1980]. Exposures were examined as continuous and categorical variables.

F. Study Results

Selected characteristics of breast cancer cases and control women are shown in Table 1. Cases were somewhat older than controls, more likely to be nulliparous, to have had fewer term births, to have had a later age at first term birth, to have had a prior breast biopsy, to have a family history of breast cancer in a mother or sister(s), to be relatively thin, and to have completed more that 12 years of school compared to controls. Further, in comparison with control women, breast cancer cases were less likely to have used oral contraceptives for 5 or more years, less likely to report never or infrequent alcohol use, and less likely to have smoked for 6 months or longer. These distributions are similar to those observed in the overall data from the Woman's Interview Study of Health [Brinton , Daling et al, 1995].

We also examined the stage of disease at diagnosis for breast cancer cases included in the Seattle component of the WISH Study, according to whether RBCs were available for analysis. For women with known stage of disease, those with (n=138) and without (n=485) RBCs for analysis did not differ substantially by stage: in situ 19.2% versus 15.5%; localized 51.5% versus 48.0%; regional 26.5% versus 34.2%; and distant stage 2.9% versus 2.3%, respectively, for cases with versus cases without fatty acid data.

Graphical display methods and univariate analyses were used to evaluate fatty acid profiles of cases and controls. Because there was no visual departure from normality for the major fatty acids of interest, no data transformations were performed. The relative proportions and standard deviations of specific and grouped fatty acids are shown in Table 2. Cases had slightly lower levels of linoleic acid (18:2n-6), arachidonic acid (20:4n-6), and total omega-6 fatty acids compared to controls. However, cases had slightly higher levels of eicosapentaenoic acid (EPA, 20:5n-3), docosahexaenoic acid (DHA, 22:6n-3), and total omega-3 fatty acids than controls. These overall results were similar when analyses were repeated, but limited to breast cancer cases: 1) who had blood drawn within 31 days after diagnosis date; 2) who had no general anesthesia prior to blood draw; 3) who were diagnosed with in situ or localized stage disease; and 4) who were diagnosed with regional/distant stage breast cancer.

Additional multivariate analyses were completed controlling for age (continuous) and body mass index (quartiles), which were the only variables that appeared to confound the associations between fatty acid levels and risk of breast cancer. Quartiles of fatty acid levels were constructed on the basis of the distribution among control subjects, and were used to categorize exposure levels. Relative risks for breast cancer were estimated according to quartiles of the main fatty acids of interest. As shown in Table 3, no significant associations were observed across quartiles of linoleic acid, arachidonic acid, eicosapentaenoic acid, docosahexaenoic acid, or total omega-3 fatty acids. There was evidence that higher levels of total omega-6 fatty acids were associated with lower risk of breast cancer (RR=0.54 for upper versus lower quartilie; trend test p=0.03). A similar pattern was noted for total polyunsaturated fatty acids (trend test p=0.02), which represent the sum of total omega-3 and total omega-6 fatty acids.

Table 1. Distribution of Cases and Controls According to Selected Characteristics

Characteristic	Cases (N = 138)		Controls (N = 141)	
Age, yr.				
<35	18	13.0	31	22.0
35 - 39	39	28.3	53	37.6
40 - 44	81	58.7	57	40.4
Number of Term Births				
0	48	34.8	39	27.7
1	28	20.3	22	15.6
2	45	32.6	47	33.3
≥3	17	12.3	33	23.4
Age at First Term Birth, yr.				
<25	36	40.0	48	47.1
≥25	54	60.0	54	52.9
Previous Breast Biopsy				
No	125	90.6	133	94.3
Yes	13	9.4	8	5.7
First Degree Family History of Breast Cancer				
No	122	88.4	136	96.5
Yes	16	11.6	5	3.5
Quetelet Index*				
≤23.4	81	58.7	63	44.7
23.5 - 27.1	37	26.8	36	25.5
≥27.2	17	12.3	39	27.7
Unknown	3	2.2	3	2.1
Education, yr.				
≤12	22	15.9	30	21.3
>12	116	84.1	111	78.7

Table 1 (continued). Distribution of Cases and Controls According to Selected Characteristics

Characteristic	Cases (N = 138)		Controls (N = 141)	
Oral Contraceptive Use				
None	21	15.2	16	11.3
<5 yrs.	64	46.4	63	44.7
≥5 yrs.	53	38.4	62	44.0
Alcohol				
None/Infrequent	53	38.4	60	42.6
<7 drinks/week	70	50.7	67	47.5
≥7 drinks/week	15	10.9	14	9.9
Smoking				
None/<6 mos.	83	60.1	74	52.5
≥6 mos.	55	39.9	67	47.5

*Weight in kilograms divided by the square of height in meters.

Table 2. Fatty Acid Composition of Erythrocyte Membranes* in Breast Cancer Cases and Controls

Fatty Acids	Cases (N = 138)		Controls (N = 141)	
Saturated				
14:0	0.25	(0.05)	0.26	(0.07)
15:0	0.13	(0.02)	0.13	(0.03)
16:0	18.7	(0.88)	18.5	(0.91)
17:0	0.35	(0.04)	0.34	(0.04)
18:0	14.4	(0.47)	14.3	(1.34)
20:0	0.38	(0.06)	0.38	(0.05)
22:0	1.66	(0.27)	1.67	(0.25)
24:0	3.97	(0.55)	4.01	(0.50)
Monounsaturated				
16:1n-7 trans	0.15	(0.03)	0.14	(0.03)
16:1n-7	0.27	(0.11)	0.26	(0.12)
16:1n-9 trans	0.05	(0.02)	0.06	(0.02)
16:1n-9	0.13	(0.04)	0.13	(0.04)
17:1n-9	1.06	(0.12)	1.07	(0.13)
18:1n-5	0.47	(0.16)	0.50	(0.15)
18:1n-6 trans	0.57	(0.13)	0.59	(0.13)
18:1n-7 trans	0.41	(0.11)	0.43	(0.12)
18:1n-7	0.92	(0.10)	0.94	(0.22)
18:1n-8 trans	0.46	(0.15)	0.48	(0.14)
18:1n-8	0.16	(0.06)	0.15	(0.04)
18:1n-9 trans	0.26	(0.08)	0.27	(0.08)
18:1n-9	10.7	(0.81)	10.6	(0.68)
18:1n-10	0.14	(0.05)	0.15	(0.04)
20:1n-9	0.22	(0.03)	0.22	(0.03)
22:1n-9	0.11	(0.04)	0.10	(0.04)
24:1n-9	3.96	(0.52)	3.88	(0.52)

Table 2 (continued). Fatty Acid Composition of Erythrocyte Membranes* in Breast Cancer Cases and Controls

Fatty Acids	Cases (N = 138)		Controls (N = 141)	
Omega-6				
18:2n-6	9.41	(0.99)	9.56	(1.00)
18:2n-6 trans-cis	0.08	(0.02)	0.09	(0.03)
18:2n-6 cis-trans	0.11	(0.04)	0.11	(0.04)
18:3n-6	0.04	(0.02)	0.04	(0.02)
20:2n-6	0.25	(0.04)	0.25	(0.04)
20:3n-6	1.47	(0.32)	1.47	(0.28)
20:4n-6	14.4	(1.04)	14.5	(1.01)
22:2n-6	0.07	(0.02)	0.07	(0.02)
22:4n-6	3.58	(0.50)	3.73	(0.61)
Omega-3				
18:3n-3	0.13	(0.03)	0.13	(0.03)
20:5n-3	0.48	(0.18)	0.46	(0.20)
22:5n-3	2.17	(0.27)	2.14	(0.26)
22:6n-3	3.94	(0.95)	3.82	(1.00)
Total Saturated	39.8	(0.90)	39.7	(1.20)
Total Monounsaturated	20.0	(1.12)	19.9	(1.02)
Total Polyunsaturated	36.2	(0.98)	36.4	(1.00)
Total Omega-3	6.71	(1.16)	6.55	(1.19)
Total Omega-6	29.5	(1.42)	29.9	(1.52)

*Values are percent weight (standard deviation).

Table 3. Relative Risk Estimates* (95% Confidence Interval) for Breast Cancer According to Levels of Selected Fatty Acids in Erythrocyte Membranes

Fatty Acids

	Quartile								x^2 trend	(P value)
	1**		2		3		4			
Linoleic (18:2n-6)	1.00	0.77	(0.4-1.6)	0.77	(0.4-1.5)	0.64	(0.3-1.3)		1.46	(0.23)
Arachidonic (18:4n-6)	1.00	0.97	(0.5-2.0)	1.12	(0.6-2.2)	1.05	(0.5-2.2)		0.07	(0.79)
Total Omega-6	1.00	0.58	(0.3-1.1)	0.34	(0.2-0.7)	0.54	(0.3-1.1)		4.90	(0.03)
Eicosapentaenoic (20:5n-3)	1.00	0.87	(0.4-1.8)	1.17	(0.6-2.4)	1.63	(0.8-3.2)		2.55	(0.11)
Docosahexaenoic (22:6n-3)	1.00	0.96	(0.5-2.0)	1.21	(0.6-2.5)	1.37	(0.7-2.8)		1.09	(0.30)
Total Omega-3	1.00	2.09	(0.9-4.4)	0.96	(0.4-2.1)	1.91	(0.9-4.0)		0.84	(0.36)
Total Polyunsaturated	1.00	0.75	(0.4-1.5)	0.40	(0.2-0.8)	0.49	(0.2-1.0)		5.77	(0.02)
Total Monounsaturated	1.00	0.95	(0.5-1.9)	0.59	(0.3-1.3)	1.20	(0.6-2.3)		0.05	(0.82)
Total Saturated	1.00	2.52	(1.2-5.3)	1.78	(0.8-3.8)	2.10	(0.9-4.5)		1.74	(0.19)

*Adjusted for age (continuous) and body mass index (quartiles).
**Reference category.

IV. CONCLUSIONS

In this ancillary study to a population-based case-control investigation of breast cancer in women under age 45, we found no evidence that specific omega-6 (linoleic or arachidonic) fatty acids or omega-3 (eicosapentaenoic or docosahexaenoic) fatty acids are associated with risk of breast cancer in young women. Based on experimental, correlational, and limited epidemiological data, we hypothesized that higher levels of omega-3 fatty acids would be associated with lower risk of breast cancer and that higher levels of omega-6 fatty acids would be associated with an elevated risk of breast cancer. If anything, there was a suggestion of an inverse relationship between total omega-6 levels, with women in the highest compared to the lowest quartile of exposure having about a 46% reduction in risk of breast cancer (trend test p=0.03). This study has several strengths and weaknesses, which should be considered when interpreting these findings.

This is the first case-control investigation of fatty acids and breast cancer to use population-based controls. Prior epidemiologic studies that measured fatty acids have used selected series of controls such as blood donors [Vatten et al., 1993], clinic patients [Zaridze et al. 1990], or women presenting with breast problems that required evaluation [London et al. 1993][Petrek et al., 1994]. Those designs may have obscured case-control differences with respect to fatty acid profiles.

Selection bias is also a concern. It is possible that the subset of women included in the fatty acid analysis were not representative of all eligible women, cases and controls, ascertained for the larger study. However, we did observe the standard breast cancer risk factors in this subset of women, and the distributions of risk factors were similar to those of the overall WISH Study [Brinton et al., 1995]. Blood samples on breast cancer patients were collected prior to any treatment for the disease and within 90 days of the biopsy, which resulted in the breast cancer diagnosis. These restrictions were necessary to avoid possible treatment effects on fatty acid levels and to maximize the likelihood that the exposures measured reflected pre-diagnostic fatty acid profiles, since the lifespan of erythrocytes is approximately 60-120 days. However, because of such restrictions, breast cancer patients included in the RBC analysis are only a subset (22%) of all women who were eligible for the Seattle component of the larger case-control study. Eligible, interviewed control women in the Seattle area were randomly selected for the blood draw part of the WISH Study and 83% of those asked consented to provide a sample. RBCs were only available for analysis on 56% of these control women.

A basic limitation of the case-control approach is retrospective ascertainment of exposures, and defining the temporal aspects of exposure in relation to stages of carcinogenesis, i.e., induction, promotion, progression. Erythrocyte samples used in this study reflect dietary exposures during the two to three month period before sample collection. It may be that dietary intake of fatty acids years ago is the most relevant exposure in the development of breast cancer. However, studies show that recent diet is associated with past diet [Willett, 1990], and unless eating patterns have changed substantially over time, erythrocyte fatty acid profiles from our study should be a valid measure of past dietary intake. Further, if dietary fat intake plays a role in tumor promotion and progression, measurement of recent fatty acid levels may be more relevant than levels measured in the distant past.

Although our findings failed to support experimental and correlational data, they are consistent with other epidemiological data, which showed no significant associations between specific omega-6 or omega-3 fatty acids and breast cancer risk [Zaridze et al., 1990][Vatten et al., 1993][London et al., 1993][Petrek et al., 1994]. Only two of these previous investigations reported separate relative risk estimates for premenopausal women [Zaridze et al., 1990] or younger women, aged $\leq$ 55 years [Vatten et al., 1993]. Risk estimates associated with linoleic acid (18:2n-6) and arachidonic acid (20:4n-6) levels were less than one. We also observed a lower risk of breast cancer (OR=0.64; 95%

CI 0.3-1.3) for the upper compared to lower quartile of linoleic acid, but no association with higher levels of arachidonic acid (upper versus lower quartile OR= 1.05). Our results are consistent with those of Vatten et al. [Vatten et al., 1993], who found no associations between levels of eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3) and risk of breast cancer.

In conclusion, our results provide no support for the theory that omega-3 fatty acids, derive mainly from marine sources, reduce the incidence of breast cancer in young women. In addition, these data failed to confirm the notion that omega-6 fatty acids enhance risk of breast cancer.

V. PERSONNEL

The following is a list of individuals who participated in and were paid from this contract:

Name	Role/Title
Burns, Carolyn	Administrative Assistant
Cary, Jeffrey A.	Administrative Assistant
Garry, Michael R.	Research Technician
Jue, Jean W.	Data Entry Technician
King, Irena B.	Co-Investigator
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Malone, Kathleen E.	Co-Investigator
Scholes, Kenneth R. J.	Programmer
Stanford Janet Lee	Principal Investigator
Ure, Carol L.	Administrator

V. PUBLICATIONS

Published Abstracts:

Stanford JL, King IB, Malone KE, Voigt LF, Daling JR, Blumenstein BA, Garry MR. Fatty acids and breast cancer: Is there an association? Am J Epidemiol 1995;141(11):S15.

V. GRADUATE DEGREES RECEIVED

None

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