# THE OMEGA-6 AND OMEGA-3 POLYUNSATURATED FATTY ACIDS AND MODIFIABLE BREAST CANCER RISK FACTORS

by

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Experimental evidence suggests that omega-6 (n-6) fatty acids have mammary tumor promoting effects whereas omega-3 (n-3) fatty acids inhibit tumor growth. These two families of fatty acids may influence breast cancer development by impacting prostaglandin E2 (PGE2) formation and consequently estradiol synthesis. Whether this effect on estrogen production can be observed in the circulation or in breast tissue, as reflected on a mammogram, is unknown. Therefore, using fatty acids in erythrocytes as a biomarker of recent dietary intake, we sought to establish the relationship between the n-6 and n-3 fatty acids with both serum estradiol and mammographic breast density, two well-established modifiable breast cancer risk factors. We hypothesized that n-6 fatty acids are positively related and n-3 fatty acids negatively related to both risk factors. Nonsteroidal anti-inflammatory drugs (NSAIDs) also inhibit PGE2 formation, therefore we further hypothesized that estradiol levels would be lower among NSAID users. NSAID data was not available at the time of mammogram; hence the relationship between NSAID use and mammographic density could not accurately be assessed. To test our hypotheses we conducted several investigations ancillary to the Mammograms and Masses Study (MAMS), a case control study of the determinants of mammographic breast density. Participants were eligible for this compilation of studies if they were breast cancer-free, postmenopausal and not taking exogenous

hormones. We observed significantly lower levels of serum estradiol among current users of NSAIDs as compared to non-users of NSAIDs. Further, as hypothesized, estradiol concentration decreased with increasing erythrocyte composition of total n-3 fatty acids and rose with increasing erythrocyte composition of total n-6 fatty acids. However, these findings were noted only among non-users of NSAIDs and not among NSAID users. No relationship was observed between any of the n-6 or n-3 fatty acids measures and mammographic breast density. In summary, lowering consumption of n-6 fatty acids, increasing n-3 intake, or taking a NSAID may result in reduced estradiol synthesis and potentially breast cancer risk. Further research is needed to validate our results. If confirmed, these findings could have a substantial impact on public health as it could lead to the development of chemopreventive guidelines, and ultimately prevent the development of estrogen-dependent breast cancer.

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# 1.0 INTRODUCTION

## 1.1 BREAST CANCER EPIDEMIOLOGY

Breast cancer incidence rates, in the United States, are among the highest in the world **(Figure 1)** [1]. The American Cancer Society estimates that approximately 178,480 new cases of invasive and 62,030 cases of in situ breast cancer will be diagnosed in women residing in the United States in 2007 [2]. Although early diagnosis and adjuvant chemohormonal therapy have resulted in significant improvements in breast cancer survival rates, breast cancer still ranks second in female cancer mortality in the United States, and is expected to account for approximately 40,460 deaths in 2007 [2].

With the exception of gender, age is generally the most acknowledged and scientifically proven risk factor to be linked with breast cancer. Breast cancer is seldom diagnosed before age 25; however, soon after this age the incidence rates rise linearly until around the age of menopause, where rates begin to plateau [3]. Even though the rising breast cancer rate tapers after menopause, older women remain at increasing risk over time, with more than 80% of breast cancer cases occurring in women 50 years of age and older [4].

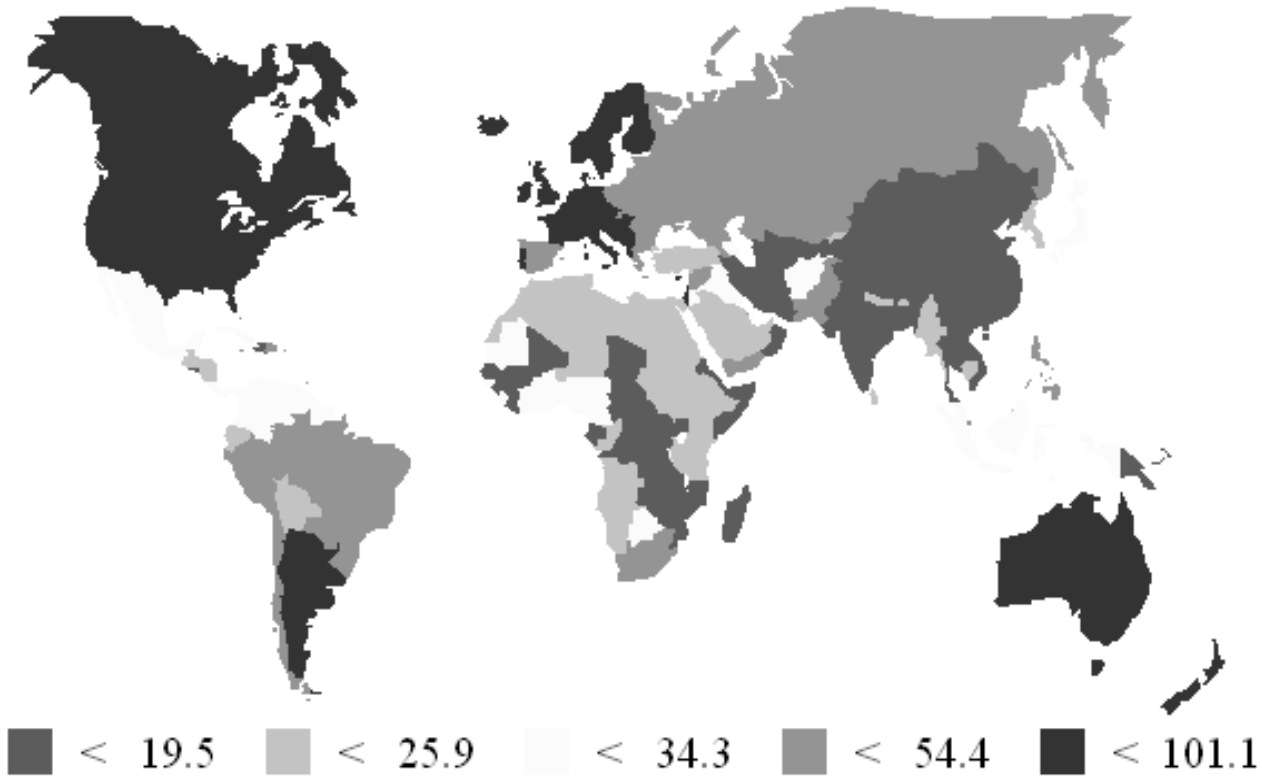


**Figure 1. Global incidence of female breast cancer: ASR, 2002**

Age-adjusted breast cancer incidence rates vary greatly around the globe, with approximately a 3-5 fold difference between high and low risk countries [1]. Greater risk is associated with the industrialized nations of North America and Western Europe, and far lower rates are found among the developing nations in Asia and Africa [5]. Unfortunately, the huge geographical variation between countries is difficult to explain.

For decades, studies have shown breast cancer rates rise among women that migrate from countries with low incidence rates to countries with high breast cancer rates. For instance, Asian women have one of the lowest breast cancer rates of any population in the world. In contrast, Asian women who have migrated to North America acquire incidence rates similar to the women

in the host country [6-8]. Although these women share a common genetic background, they experience different rates of disease when living in dissimilar geographic and cultural settings. Thus, it appears that exposure to the "Western" lifestyle has a significant impact on breast cancer risk, and therefore breast cancer may be a preventable disease.

### 1.1.1 Breast cancer risk factors

While the etiology of breast cancer is poorly understood, it is believed to be the result of environmental, reproductive, hormonal, and genetic factors. Epidemiological research has identified a number of factors that may either predispose or protect a woman from developing this disease (**Table 1**). However, it should be noted that many individuals who develop breast cancer have established risk factor values very similar to the population average. Furthermore, the majority of women exposed to multiple well-established breast cancer risk factors never develop breast cancer, and some women that develop this disease have no apparent breast cancer risk factor [9].

Risk factors fall into one of two categories, modifiable or non-modifiable. Modifiable risk factors include diet and obesity, whereas nonmodifiable risk factors include one's age and family history of breast cancer. Of particular interest in cancer prevention research, are those that are modifiable; however, a major modifiable lifestyle risk factor has yet to be recognized which can be used as a means for primary prevention.

**Table 1. Selected established and potential breast cancer risk factors**

| RISK FACTOR | REFERENCE GROUP | COMPARISON GROUP | OR, RR, HR* (95%CI) † |
|---|---|---|---|
| **Family History** [10] | | | |
| One first degree relative | Women without an affected relative | Women with 1 1st degree relative‡ | OR: 1.80 (1.69, 1.91) |
| Two first degree relatives | Women without an affected relative | Women with 2 1st degree relatives | OR: 2.93 (2.36, 3.64) |
| **Benign Breast Disease** [11] | | | |
| Atypical hyperplasia | Iowa SEER registry population | Women with proliferative fibrocystic changes with atypia | RR: 4.24 (3.26, 5.41) |
| Proliferative no atypia | Iowa SEER registry population | Women with proliferative fibrocystic changes without atypia | RR: 1.88 (1.66, 2.12) |
| Nonproliferative | Iowa SEER registry population | Women with non-proliferative fibrocystic changes | RR: 1.27 (1.15, 1.41) |
| **Reproductive Factors** | | | |
| Age at menarche [12] | <12 years of age | 15+ years of age | RR: 0.84 (0.70, 1.02) |
| Full-term pregnancy [12] | Women with no full-term pregnancy | Women with 1 full-term pregnancy | RR: 0.76 (0.61, 0.95) |
| Age at first pregnancy [12] | Women with first full term pregnancy < 22 years of age | Women with first full term pregnancy > 30 years of age | RR: 1.46 (1.18, 1.81) |
| Breast-feeding [13] | Parous women that never breastfed | Parous women that breastfed for a median of 12 months | OR: 0.94§ |
| Abortion [14] | | | |
| Spontaneous | No record of a spontaneous abortion | Record of a spontaneous abortion | OR: 0.98 (0.92, 1.04) |
| Induced | No self-reported induced abortion | Self-reported induced abortion | OR: 0.93 (0.89, 0.96) |
| Age at menopause [15] | <40 years of age | ≥55 years of age | OR: 1.71 (1.37, 2.12) |

**Table 1 (continued)**

| RISK FACTOR | REFERENCE GROUP | COMPARISON GROUP | OR, RR, HR (95%CI) |
|---|---|---|---|
| **Hormonal Factors** | | | |
| Exogenous hormones | | | |
| Oral contraceptives [16] | Never user of oral contraceptives | Current user of oral contraceptives | OR:1.24 (1.15, 1.33) |
| Hormone Therapy [17] | Randomized to placebo | Randomized treatment with E+P[#] | HR: 1.24 (1.02, 1.50) |
| Endogenous hormones | | | |
| Sex steroid hormones | | | |
| Premenopausal [18] | | | |
| Estradiol | Lowest quartile | Highest quartile | OR: 1.00 (0.66, 1.52) |
| Estrone | Lowest quartile | Highest quartile | OR: 1.16 (0.72, 1.85) |
| SHBG** | Lowest quartile | Highest quartile | OR: 0.95 (0.65, 1.40) |
| Testosterone | Lowest quartile | Highest quartile | OR: 1.73 (1.16, 2.57) |
| Postmenopausal [19] | | | |
| Estradiol | Lowest quintile | Highest quintile | RR: 2.00 (1.47, 2.71) |
| Estrone | Lowest quintile | Highest quintile | RR: 2.19 (1.48, 3.22) |
| SHBG | Lowest quintile | Highest quintile | RR: 0.66 (0.43, 1.00) |
| Testosterone | Lowest quintile | Highest quintile | RR: 2.22 (1.59, 3.10) |
| Estrogen metabolites [20] | | | |
| Premenopausal | | | |
| 2-OHE†† | Lowest quintile | Highest quintile | OR: 1.23 (0.45, 3.35) |
| 16-OHE†† | Lowest quintile | Highest quintile | OR: 1.90 (0.68, 5.31) |
| 2:16OHE | Lowest quintile | Highest quintile | OR: 0.55 (0.23, 1.32) |
| Postmenopausal | | | |
| 2-OHE | Lowest quintile | Highest quintile | OR: 1.61 (0.66, 3.94) |
| 16-OHE | Lowest quintile | Highest quintile | OR: 1.34 (0.55, 3.27) |
| 2:16OHE | Lowest quintile | Highest quintile | OR: 1.31 (0.53, 3.18) |

**Table 1 (continued)**

| RISK FACTOR | REFERENCE CATEGORY | COMPARISON CATEGORY | OR, RR, HR (95%CI) |
|---|---|---|---|
| IGF-1 [21] | | | |
| Premenopausal | 25th percentile | 75th percentile | OR: 1.93 (1.38, 2.69) |
| Postmenopausal | 25th percentile | 75th percentile | OR: 0.95 (0.62, 1.33) |
| IGFBP-3 [21] | | | |
| Premenopausal | 25th percentile | 75th percentile | OR: 1.96 (1.28, 2.99) |
| Postmenopausal | 25th percentile | 75th percentile | OR: 0.97 (0.53, 1.77) |
| Factors effected by hormones | | | |
| Bone mineral density [22] | lowest quartile of bone density at 3 skeletal sites | highest quartile of bone density at 3 skeletal sites | RR: 2.70 (1.4, 5.3) |
| Breast density [23] | lowest quartile of density | highest quartile of density | OR 4.04 (2.12, 7.69) |
| **Anthropometry** [24] | | | |
| Premenopausal | | | |
| Weight (kg) | <60.0 | ≥80.0 | RR: 0.58 (0.40, 0.83) |
| Height (cm) | <1.60 | ≥1.75 | RR: 1.42 (0.95, 2.12) |
| BMI ($kg/m^2$) | <21.0 | ≥33.0 | RR: 0.58 (0.34,1.00) |
| Postmenopausal | | | |
| Weight (kg) | <60.0 | ≥80.0 | RR: 1.25 (1.02, 1.52) |
| Height (cm) | <1.60 | ≥1.75 | RR:1.28 (0.94, 1.76) |
| BMI ($kg/m^2$) | <21.0 | ≥33.0 | RR:1.27 (1.03, 1.55) |
| **Environmental Factors** | | | |
| Ionizing Radiation [25] | never exposed to radiation to treat or monitor a condition | radiation exposure between 10 and 19 years of age | OR: 1.6 (0.5, 2.5) |
| Physical Activity [26] | | | |
| Premenopausal | lowest tertile of average lifetime activity | highest tertile of average lifetime activity | OR: 0.74 (0.52,1.05) |
| Postmenopausal | lowest tertile of average lifetime activity | highest tertile of average lifetime activity | OR: 0.81 (0.64, 1.02) |

**Table 1 (continued)**

| RISK FACTOR | REFERENCE CATEGORY | COMPARISON CATEGORY | OR, RR, HR (95%CI) |
|---|---|---|---|
| Smoking [27] | | | |
| Postmenopausal | Never smoker | Smoked >40 years | RR: 1.5 (1.2, 1.9) |
| Dietary Factors | | | |
| Alcohol [28] | 0g/day | ≥45g/day | OR: 1.46 (1.33, 1.61) |
| Total Fat [29] | lowest quantile | highest quantile | OR: 1.13 (1.03, 1.25) |
| Saturated Fat [29] | lowest quantile | highest quantile | OR: 1.19 (1.06, 1.35) |
| Meat intake [29] | lowest quantile | highest quantile | OR:1.17 (1.06, 1.29) |
| Soy [30] | lowest quantile | highest quantile | OR: 0.86 (0.75, 0.99) |
| Calcium (dietary) [31] | ≤500 mg/d | >1,250 mg/d | RR: 0.80 (0.67, 0.95) |
| Vitamin D (dietary) [31] | ≤100 IU/d | >300 IU/d | RR: 0.89 (0.76, 1.03) |
| Folate (total) [32] | 150-299µg/d | ≥600µg/d | RR: 0.93 (0.83, 1.03) |
| Total Fruit [33] | lowest quintile | highest quintile | RR: 1.09 (0.94, 1.25) |
| Total Vegetables [33] | lowest quintile | highest quintile | RR: 0.98 (0.84, 1.14) |
| Night work [34] | no nightshift work | any nightshift work | OR: 1.48 (1.36, 1.61) |

* OR=Odds Ratio; RR=Relative Risk; HR=Hazards Ratio

†CI=Confidence Interval

‡ First degree relative=mother, sister, or daughter

§CI not provided

# E+P= estrogen + progestin

**Sex hormone binding globulin

††2-OHE=2-hydroxyestrone; 16-OHE=16-hydroxyestrone

### 1.1.2 Circulating estradiol

Experimental data strongly support the hypothesis that estrogen plays a causal role in the development of some breast cancers. The mechanisms through which estrogens contribute to the carcinogenic process are complex; however, evidence exists confirming estrogens cause both normal and malignant breast cell proliferation [35]. Many established breast cancer risk factors can be attributed to some means of elevated estrogen exposure. For example, both an early age of menarche and a late age of menopause are related to prolonged exposure to the high levels of estrogen that occur during the menstrual cycle, and both are associated with increased breast cancer risk [12, 15]. Surgical menopause, which results in an abrupt arrest of estrogen secretion by the ovaries, is protective against breast cancer [36]. Moreover, the rate of age specific breast cancer slows around the time of menopause, a time when estrogen levels decline [3]. Increased bone mineral density, a potential reflection of cumulative estrogen exposure, is associated with increased breast cancer development in menopausal women [22]; and obesity, which is positively correlated with circulating estrogen levels is associated with postmenopausal breast cancer risk [22, 24].

Treatment with estrogens may cause an increase in risk of postmenopausal breast cancer. A meta-analysis found an increased risk of breast cancer risk associated with the use of estrogen-replacement therapy (ET) [37]. In contrast, the Women's Health Initiative Study did not find increased risk associated with participants receiving ET; however, the study may not have been long enough [38]. Within the Nurse's Health Study breast cancer risk increased with the duration of ET use, the multivariate relative risks (RRs) and 95% confidence intervals (CIs) for breast cancer with current ET use for less than 5 years, 5 to 9.9 years, 10 to 14.9 years, 15 to 19.9 years, and 20 years or more were, respectively, 0.96 (0.75-1.22), 0.90 (0.73-1.12), 1.06 (0.87-1.30),

1.18 (0.95-1.48), and 1.42 (1.13-1.77) (P for trend <0.001) [39]. The relationship was more notable among estrogen receptor positive (ER+) and progesterone receptor positive (PR+) tumors, and became statistically significant after 15 years of use (RR, 1.48; 95% CI, 1.05-2.07). Further, there is ample evidence that endogenous levels of estradiol are strongly linked with breast cancer in postmenopausal women [40]. Ten of 11 prospective studies reported higher circulating estradiol concentrations in postmenopausal women who subsequently developed breast cancer than in controls **(Table 2)**. However, 4 of the 11 risk estimates were not significant, as to be expected given the small number of cases. A reanalysis pooling data from 9 of these studies found an OR=2.0 (95%CI: 1.4, 2.1) for breast cancer risk when comparing women in the upper versus the lowest quintile of estradiol levels [41].

In addition to the observational studies linking circulating estradiol concentrations and breast cancer risk, convincing data from large clinical trials exist showing drugs that block the action of estrogen reduces breast cancer incidence. The risk reduction is more pronounced in women with higher estrogen levels than in those with lower levels; thus further strengthening the evidence that estrogen exposure is associated with the development of breast cancer [42, 43]. In the Multiple Outcomes of Raloxifene Evaluation (MORE) trial, it was found that women in the highest tertile of estradiol levels had a 2.1 fold risk of breast cancer in comparison to women with the lowest levels of estradiol [44]. Women in the placebo arm of the trial had 6.8 times the risk of developing breast cancer than women with estradiol levels lower than the assays detection limit (0.6%/year, 95%CI: 0%-1.1%), and women with circulating levels of estradiol >10pmol/L in the Raloxifene group had a breast cancer rate 76% lower (95%CI: 53%, 88%) than women with similar levels of estradiol in the placebo group; thus, inhibiting the action of estrogen plays an obvious role in the risk reduction of breast cancer [43].

**Table 2. Nested case control studies of circulating estradiol and breast cancer risk in postmenopausal women**

| Author (year) | Country | Cases/ Controls | Partition (pmol/L) | Specimen Type | Adjustments & Matching | OR (95%CI)* |
|---|---|---|---|---|---|---|
| Garland (1992) [45] | USA | 15/400 | <36.7 vs. ≥62.4 | Plasma | Adjusted for age | OR=0.6† |
| Helzlsouer (1994) [46] | USA | 29/58 | <44.1 vs. >66.1 | Serum | Matched on age, time since last natural menstrual period, time of blood draw, and fasting status at the time of blood draw | OR=4.0 (0.4, 34.8) |
| Berrino (1996) [47] | Italy | 24/88 | <66.8 vs. >89.6 | Serum | Matched on study recruitment center, date of enrollment, daylight savings period at time of blood draw, location of freezer storage (i.e. freezer and level on freezer); adjusted for age | OR= 5.5 (0.8, 37.6) |
| Dorgan (1996) [48] | USA | 71/133 | <28.6 vs. ≥88.1 | Serum | Matched for age, date of blood draw, and time of blood draw; adjusted for years since last natural menstrual period, height, weight, first degree family history of breast cancer, and parity | OR=2.7 (0.8, 9.1) |

**Table 2 (continued)**

| Author (year) | Country | Cases/ Controls | Partition (pmol/L) | Specimen Type | Adjustments & Matching | OR (95%CI)* |
|---|---|---|---|---|---|---|
| Thomas (1997) [49] | Guernsey | 61/179 | <30.8 vs. >41.1 | Serum | Matched for age, year of blood draw and number of years postmenopausal | OR= 5.0 (2.0, 12.5) |
| Hankinson (1998) [50] | USA | 154/306 | ≤18.4 vs. ≥ 44.1 | Plasma | Matched for age, month and time of day and fasting status of blood draw; adjusted for BMI at age 18, family history of breast cancer, age at menarche, parity, age at first birth, age at menopause, and past HT use | OR=1.9 (1.1, 3.5) |
| Cauley (1999) [40] | USA | 97/243 | <18.4 vs. ≥29.4 | Serum | Adjusted for age, BMI, age at menarche, first birth, and menopause, nulliparity, family history of breast cancer, physical activity, surgical menopause, and alcohol consumption | OR=2.9 (1.2, 7.2) |
| Kabuto (2000) [51] | Japan | 26/56 | Lowest quintile vs. highest quintile | Serum | Matched for city, age (±3 years), date (±3 months) of blood collection, and radiation dose | OR=2.5 (0.2, 40.2) |

**Table 2 (continued)**

| Author (year) | Country | Cases/ Controls | Partition (pmol/L) | Specimen Type | Adjustments & Matching | OR (95%CI)* |
|---|---|---|---|---|---|---|
| Missmer (2004) [52] | USA | 319/637 | Batch specific cutpoints:<br><br>1990-1995<br><22.0 vs. ≥40.1<br><br>1996-1998<br><18.4 vs. ≥33.0 | Plasma | Matched for age, month and time and fasting status of blood draw; adjusted for BMI at 18 years, family history of breast cancer, age at first birth, parity, age at menopause, duration of HT use | OR= 2.1 (1.5, 3.2) |
| Zeleniuch-Jacquotte (2004) [53] | USA | 294/558 | <62.9 vs. >116.3 | Serum | Matched on age, date of enrollment, number and dates of subsequent blood draws; adjusted for age at menarche, family history of breast cancer, parity, age at first birth, surgical menopause, previous breast biopsy, BMI, and height | OR=2.1 (1.2, 3.6) |
| Kaaks (2005) [54] | 9 European countries | 672/1297 | Lowest quintile vs. highest quintile | Serum | Matched on study center, age, time of day of blood draw, fasting statu at blood draw | OR=2.3 (1.6, 3.2) |

* OR=Odds ratio; CI=Confidence interval

† Not statistically significant. CI not reported

### 1.1.3 Mammographic breast density

The mammographic image of the breast differs according to the amounts of each type of breast tissue. Fatty breast tissue is radiolucent, allowing x-ray beams to pass through resulting in dark areas on a mammogram whereas fibroglandular tissue, which is comprised of stroma, ductal, and glandular tissue, is denser and absorbs x-rays, thus appearing lighter on the film. The dense tissue in the breast decreases the visibility of tumors thus hindering mammogram interpretation and consequently reducing mammography sensitivity and specificity [55].

Mammographic breast density is an estimate of the proportion of dense tissue in the breast as opposed to fatty tissue. There is not a standardized means to assess the degree of density in the breast, and a range of subjective and semi-objective classification systems have been developed. Common qualitative measurements include Wolfe's patterns and Tabar's categories. The less subjective quantitative breast density measurements include visual estimation, planimetry, and computerized thresholding.

Wolfe's parenchymal patterns classify the breast into four categories (N1, P1, P2, and DY) according to the relative amounts of fat, epithelial and connective tissue observed on the mammogram: N1 signifies breast tissue predominantly comprised of fatty tissue and no duct pattern is visible; P1 category refers to mainly fatty tissue, but ductal prominence is visible in up to 25% of the breast; P2 displays a prominent ductal pattern in more than 25% of the breast; and DY, denotes dense tissues spread throughout the majority of the breast [56]. Similarly, Tabar's classification consists of categories, as follows: breast image composed of scalloped contours with some lucent areas of fatty replacement, and 1mm evenly distributed nodular densities (represents the typical appearance of a premenopausal woman's breast); composed almost

entirely of lucent areas of fatty replacement, and 1 mm evenly distributed nodular densities; prominent ducts in the retroareolar area; extensive nodular and linear densities, with nodular size larger than normal lobules, and; homogeneous, ground glass-like appearance with no perceptible features [57].

In hopes to improve interrater agreement, more objective measurements to assess the radiographic appearance of the breast were established. Among the first of these methods was visual estimation of the proportion of the breast area occupied by dense tissue [23]. More recent studies have used planimetry, both manual and computerized, to assess breast density. This method involves tracing the total breast area and areas of dense tissue. The percentage of density is then calculated by dividing the area of the dense breast tissue by the total area of the breast [58]. Another adopted method for the quantitative assessment of breast density is through interactive thresholding. In this method, using digitized images of the breast, an observer selects a gray-level value as a threshold to define the edge of the breast from the darker background, and subsequently selects the region of dense tissue [59]. The areas defined are then measured by the computer, and total breast area, dense breast area, nondense breast area and percent density are calculated.

Regardless if density is defined by qualitative or quantitative measurement, numerous epidemiological investigations have shown breast density to be a strong risk factor for breast cancer in the general population; however, studies using a quantitative measure of breast density usually report a stronger association with breast cancer risk [60, 61]. The risk of developing breast cancer is estimated to be 2 to 6 times greater among women with the highest partition of density as compared to women with little or no visible density **(Table 3)**. A recent meta-analysis found an increased risk (OR=4.64; 95%CI=3.64-5.91) for breast cancer among women with a

breast density of 75% as compared to women with breast density <5% [61]. This phenomenon is dose dependent and notable in both pre- and postmenopausal women [23]. For each 1% increment in percent breast density an estimated 1.5-2% increase in breast cancer risk occurs [62, 63]; thus, mammographic density is a stronger predictor of breast cancer risk than most traditional risk factors.

Mammographic density is believed to be a result of both genetic and lifestyle factors. Aside from age and BMI [64], two well-established breast cancer risk factors among postmenopausal women, several reproductive and hormonally-related breast cancer risk factors are positively associated with mammographic density. Increased density has been linked to an early age at menarche, late age of menopause, nulliparity and late age at first full-term birth [64-70]. Mammographic density can also be modified with treatments known to alter breast cancer risk such as increased density with HT use, with combined formulations of estrogen and progesterone having the most obvious effects [71-85] and decreased density with use of estrogen receptor modulators (SERMs) [86-90]. The effects of both HT [83] and SERMs [91] cease with discontinuation of use. Additionally, changes in density are observed among oral contraceptive users and by phase of the menstrual cycle [92-94]. Because of the above-mentioned associations between reproductive and hormonal exposures and breast density, it has long been hypothesized that mammographic density may, in part, be a marker of estrogen and other hormonal effects on the breast tissue.

Contrary to what would be expected, the limited cross-sectional information available on the relationship between circulating endogenous estradiol concentrations and breast density suggests a strong relationship between the two factors does not exist. Among postmenopausal women, one study found a positive association between estradiol levels and percent

mammographic breast density, two studies found an inverse relationship between estradiol and mammographic breast density, and two observed null relationships [95-99]. It has been suggested that localized estrogen production in the breast tissue may be more relevant to breast density than circulating estrogen levels. As with endogenous estrogens and breast density, the relationship between breast density and bone mineral density, an established breast cancer risk factor and surrogate marker of estrogen exposure, remains unclear [100-102] .

### 1.1.4 Potential intermediate markers

Circulating estradiol and mammographic breast density have been proposed as potential intermediate markers of postmenopausal breast cancer. Unlike most other established breast cancer risk factors they have also been shown to be modifiable. Factors that alter estradiol levels and mammographic breast density may also alter breast cancer risk; hence the identification of such factors may lead to a greater understanding in the etiology of breast cancer and the development of prevention strategies. In addition, assessment of endogenous estradiol levels and mammographic breast density is not very invasive and both can be objectively measured, which strengthens the use of these endpoints in epidemiological studies.

**Table 3. Summary of quantitative studies of mammographic density and breast cancer risk**

| Author (year) | Country | Cases/ Controls | Age Range | Partition (%) | Method of Measurement | Adjustments & Matching | Adjusted OR or RR (95%CI)* |
|---|---|---|---|---|---|---|---|
| ***Case Control Studies*** | | | | | | | |
| Boyd (1982) [103] | Canada | 183/183 | 40-65 | <10 vs. ≥ 75 | Observer estimation | Age at first birth, parity, family history | OR=2.8† (1.4, 5.6)<br>OR=3.7 (1.7, 4.1)<br>OR=6.0 (2.5, 14.1) |
| Brisson (1982) [104] | USA | 408/1,021 | 20-69 | 0 vs. ≥ 60 | Observer estimation | Parity, age at first birth, family history of breast cancer, age at menopause, HT use | OR=3.8‡ (1.6, 8.7)<br>OR=5.4‡ (2.5,11.4) |
| Brisson (1984) [105] | USA | 362/686 | -------- | 0 vs. ≥ 60 | Observer estimation | Weight and height | OR=4.4 (2.5,7.9) |
| Wolfe (1987) [58] | USA | 160/160 | 30-85 | <20 vs. ≥ 70 | Manual planimetry | Parity | OR=4.3 (1.8, 10.4) |
| Brisson (1989) [106] | Canada | 290/645 | 40-67 | 0 vs. ≥ 60 | Observer estimation | Age, parity, education, weight, height | OR=3.2 (1.6, 6.5)§<br>OR=4.6 (2.4, 8.5)§<br>OR=5.5 (2.3, 13.2)§ |

**Table 3 (continued)**

| Author (year) | Country | Cases/ Controls | Age Range | Partition (%) | Method of Measurement | Adjustments & Matching | Adjusted OR or RR (95%CI) |
|---|---|---|---|---|---|---|---|
| (2000) [107] | USA | 647/647 | -------- | <10 vs. > 50 | Computer assisted thresholding | Matched by age, ethnicity, year of mammogram; adjusted for age at menarche, parity, age at first birth, menopausal status, HT use, family history of breast cancer, prior breast problems | OR=1.8 (1.1, 3.0) |
| Ursin (2003) [63] | USA | 622/443 | 35-64 | <1 vs. > 75 | Computer assisted thresholding | Age, BMI, HT use, age at menarche, family history of breast cancer, parity, age at first birth, menopausal status | OR=5.2 (1.7, 16.1) |
| ***Nested Studies*** | | | | | | | |
| Saftlas (1991) [108] | USA | 266/301 | 35-74 | <5 vs. ≥ 65 | Manual planimetry | Age, weight, parity | OR= 4.3 (2.1, 8.8) |
| Boyd (1995) [23] | Canada | 354/354 | 40-59 | 0 vs. ≥ 75 | Observer estimation & computer assisted | Age, parity, age at first birth, weight, height, age at menarche, family history of breast cancer | OR=4.0 (2.1, 7.7)# OR= 6.0(2.8, 13.0)# |

**Table 3 (continued)**

| Author (year) | Country | Cases/ Controls | Age Range | Partition (%) | Method of Measurement | Adjustments & Matching | Adjusted OR or RR (95%CI) |
|---|---|---|---|---|---|---|---|
| Kato (1995) [109] | USA | 197/521 | 35-65 | ≤28.5 vs. ≥ 44.0 | Manual planimetry | BMI, parity, menopausal status | OR=2.1 (1.1, 3.8) |
| Byrne (1995) [110] | USA | 1,880/2,152 | -------- | 0 vs. ≥ 75 | Computerized planimetry | Matched for age and race, adjusted for age first birth, weight, family history of breast cancer, education, alcohol use, number breast biopsies, reproductive years | OR= 4.3 (3.1, 6.1) |
| van Gils (1999) [111] | Netherlands | 108/400 | 50+ | <5 vs. >25 | Computerized | BMI, menopausal status | OR= 3.3 (1.5, 7.2) |
| Kerlikowske (2005) [102] | USA | 200/431 | 28+ | <23.9 vs. ≥ 66.8 | Computer assisted thresholding | Age, family history of breast cancer, age first birth, BMD, race, BMI | OR= 2.7 (1.4, 5.4) |
| Maskarinec (2005) [112] | USA | 607/667 | -------- | <10 vs. ≥ 50 | Computer assisted thresholding | Ethnicity, age at mammogram, BMI, age at first birth, parity, age at menarche, age at menopause, HT use, family history of breast cancer | OR= 3.1 (2.0, 4.9) |

**Table 3 (continued)**

| Author (year) | Country | Cases/ Controls | Age Range | Partition (%) | Method of Measurement | Adjustments & Matching | Adjusted OR or RR (95%CI) |
|---|---|---|---|---|---|---|---|
| ***Prospective Studies*** | | | | | | | |
| Thomas (2002) [113] | USA | 472/547 | 50+ | <26.7 vs. ≥70.3 | Manual Planimetry | Age and study | RR= 4.4 (3.0, 6.7) |
| Torres-Mejia (2005) [114] | UK | 111/3,100 | 35+ | <18.7 vs. ≥45.9 | Computer assisted thresholding | Age, age at leaving full time education, social class, job status, parity, height, BMI, and change in BMI | RR=3.5 (1.7, 7.2) |

* OR=Odds ratio; RR=Relative risk; CI=Confidence interval

† OR estimation for each of 3 observers who estimated percent density. OR for total density unless noted otherwise

‡ OR for percentage of nodular density and percentage of homogenous density, respectively

§OR for percentage of nodular density, percentage of homogenous density, and percentage for total density, respectively

#OR for observer estimation and computer estimation, respectively

## 1.2 OMEGA-6 AND OMEGA-3 FATTY ACIDS

### 1.2.1 Biological properties and nomenclature of fatty acids

As a class, fatty acids are simple hydrocarbon structures and can be grouped into well defined families. All fatty acids have a common basic structure, consisting of a chain of carbon atoms with hydrogen atoms attached, a methyl group ($CH_3$) at one end of the chain, and a carboxylic acid group at the other end (COOH) (**Figure 2)**. Although, there are four common chemical naming systems for fatty acids, only one will be reviewed in this section, the "n-minus" system, which denotes the chain length and the number and positions of any double bonds. The number of carbon atoms in the fatty acid chain is the first number, followed by a colon with the second number denoting the number of double bonds (unsaturation level) in the chain. After the second number, "n -" and a third number may appear, this specifies the position of the first carbon double bond from the methyl end (omega end) of the molecule. Thus, 20:5n-3 denotes a 20-carbon fatty acid with five double bonds, the first of which is located three carbons from the terminal methyl group of the fatty acid **(Table 4)** [115].

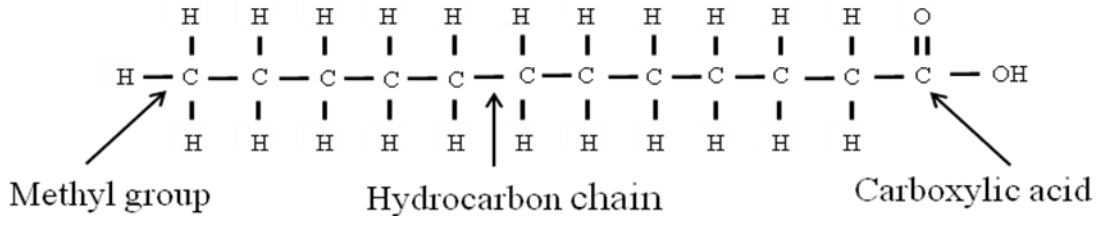


**Figure 2. Structure of a fatty acid**

**Table 4. Naming of omega-6 and omega-3 polyunsaturated fatty acids**

| Common name | Shorthand | Systematic name |
|---|---|---|
| Linoleic acid | 18:2 n-6 | 9,12-octadecadienoic acid |
| Gamma-linolenic acid | 18:3 n-6 | 6,9,12-octadecatrienoic acid |
| Eicosadienoic acid | 20:2 n-6 | 11,14-eicosadienoic acid |
| Dihomo-gamma-linolenic acid | 20:3 n-6 | 8,11,14-eicosatrienoic acid |
| Arachidonic acid | 20:4 n-6 | 5,8,11,14-eicosatetraenoic acid |
| Docosapentaenoic acid | 22:5 n-6 | 4,7,10,13,16-docosapentaenoic acid |
| Alpha-linolenic acid | 18:3 n-3 | 9,12,15-octadecatrienoic |
| Eicosatetraenoic acid | 20:4 n-3 | 8,11,14,17-eicosatetraenoic |
| Eicosapentaenoic acid | 20:5 n-3 | 5,8,11,14,17-eicosapentaenoic |
| Docosapentaenoic acid | 22:5 n-3 | 7,10,13,16,19-docosapentaenoic |
| Docosahexaenoic acid | 22:6 n-3 | 4,7,10,13,16,19-docosahexaenoic |

Fatty acids fall into one of three major categories saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids (PUFAs). The difference between these families of fatty acids is in the type of chemical bonds they contain. Saturated fatty acids have no double bonds in the carbon chain and carry the maximum number of hydrogen atoms, monounsaturated fatty acids have one double bond, and PUFAs have more than one double bond that is methylene-interrupted.

There are only two essential fatty acids encountered in the diet critical to human health, both of which are PUFAs. The omega-6 (n-6) fatty acid linoleic acid (18:2n-6; LA) and the omega-3 (n-3) fatty acid, alpha-linolenic acid (18:3n-3; ALA), are necessary fats that must be obtained through the diet, because humans cannot synthesize these fatty acids de novo. Moreover, the human body cannot interconvert these fatty acids because it lacks enzymes for forming double bonds (desaturase enzymes) past the delta 9 position

(9th carbon atom from carboxyl group). The remaining PUFAs in the n-6 and n-3 families are not necessarily essential because the human body can produce a modest amount on its own, via the desaturase and elongase enzymes (enzymes for lengthening the carbon chain) given that an adequate supply of the parent fatty acids, LA and ALA, are readily available (**Figure 3)**. Both the n-6 and n-3 families of PUFAs play vital roles in diverse biological processes, including serving as substrates for a number of biological actions that oversee a wide range of functions, including blood pressure regulation, diuresis, muscle contractions, blood platelet aggregation, inflammatory responses to injury and infection, and the manufacturing and repair of cellular membranes [116].

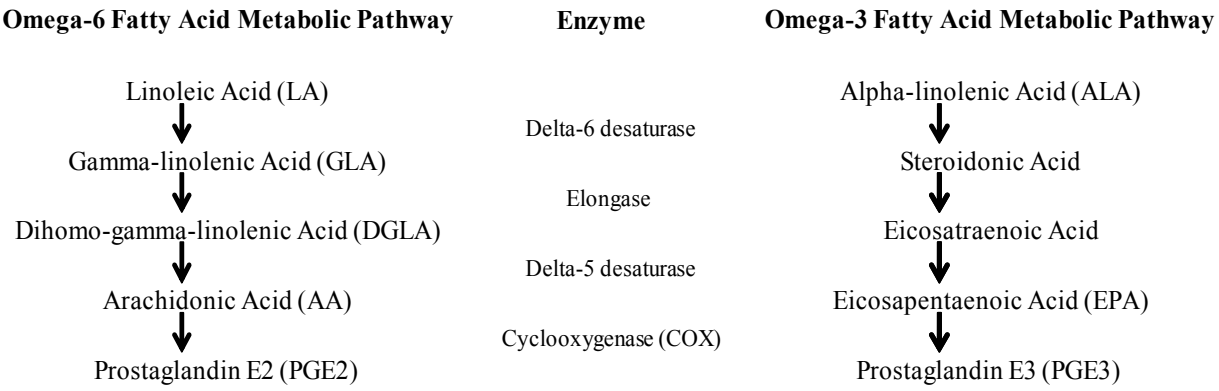


**Figure 3. Metabolic pathways of the omega-6 and omega-3 fatty acids**

### 1.2.2 Sources of the omega-6 and omega-3 fatty acids

The intake of various fatty acids differs widely among countries. In the typical "Western" diet, approximately 5-20 times more n-6 fatty acids are consumed compared to n-3 fatty acids [117], whereas coastal countries, such as Japan, consume considerably lower quantities of n-6 fatty acids, and much higher intakes of the n-3 PUFAs [118].

This substantial excess of n-6 fatty acids found in the United States population diet, is largely due to the high intake of LA. Approximately 85-95% (approximately 12 to 17g/day for men and 9 to 11g/ day for women) of total n-6 PUFA consumption, is in the form of LA [119]. The majority of LA intake is hidden in prepackaged foods such as cereals, snack foods, and baked goods.

LA is the principal member of the n-6 PUFA family, and from this fatty acid, the human body can manufacture all other members of the n-6 PUFA family. LA is predominantly found in commonplace seed and vegetable oils such as safflower oil, sunflower oil, sesame oil, cottonseed and corn oil; certain nuts (e.g. peanuts, pistachios, almonds); seeds (e.g. pumpkin, sesame); meat from corn-fed animals (e.g. chicken), and dairy products. Blackcurrant seed oil, borage oil, and evening primrose oil are particularly rich sources of the n-6 PUFA, gamma-linolenic acid (18:3n-6; GLA). The long chain n-6 fatty acid, dihomo-gamma-linolenic acid (20:3n-6; DGLA) is found in high doses in liver and certain fish. However, GLA is not typically consumed in large quantities in the United States, and DGLA is near non-existent in the diet. LA, GLA, and DGLA can be converted in the human body, through a serious of enzymatic reactions, to form the n-6 PUFA arachidonic acid (20:4n-6; AA). AA can also be obtained through dietary intake and is derived from the consumption of animal products including poultry, meat, dairy, eggs and some tropical fish, but not from plant-derived fats and oils. AA is not found in plant products because unlike animal cells, plant cells lack enzymes that are capable of the conversion of LA to AA.

The essential fatty acid, ALA, is the predominant plant-derived dietary n-3 PUFA in the United States diet, and given this fatty acid, the human body can make all other

members of the n-3 PUFA family. In contrast to LA, ALA is not consumed in great quantity in the United States (approximately 1.3 to 1.8g/day for men and 1.0 to 1.2g/day for women) [119]. A selection of fats (e.g. margarine, shortening); vegetable and seed oils such as soybean, canola, and flaxseed oils; legumes, primarily soybeans and navy beans; and leafy green vegetables (e.g. kale, broccoli, salad greens) are rich sources of ALA. ALA is the metabolic precursor for eicosapentaenoic acid (20:5n-3; EPA) and thence to docosahexaenoic acid (22:6n-3; DHA); however, the elongation rate of ALA to these longer chained (20 or more carbon atoms) highly unsaturated n-3 PUFAs is believed to be inefficient (0.2-15% conversion); thus, ALA is not believed to be a viable source of either EPA or DHA [120, 121]. The major food sources for the human supply of EPA and DHA, are marine plants (kelp and seaweed), shellfish, and cold water fatty fish (salmon, mackerel, anchovies and sardines) [122, 123]. Another important source of EPA and DHA in the Western diet is egg-yolks; however, concentrations of these fatty acids are dependent upon the feed given to the animals [124]. Adult intake of EPA (approximately 0.004 to 0.007 g/day for men and 0.052 to 0.093 g/day for women) and DHA (approximately 0.066-0.093 g/day for men and 0.052-0.069 g/day for women) is minimal in the United States [119].

N-3 fatty acid levels vary substantially among different types of fish and have a propensity to be found in much higher concentrations in fatty fish as compared to lean fish [125]. Moreover, the n-3 fatty acid composition within a single species of fish is likely to differ, as it is effected to a great extent by the eating habits, geographic location and maturity of the fish as well as the canning oils and preparation and cooking methods used [126]. The differences in fatty acid profiles within species is particularly

pronounced when comparing cultivated and wild fish [127]. For example, wild catfish contain twice the amount of EPA as catfish bred in captivity **(Table 5).** Additionally some fish types, such as farmed Atlantic salmon, contain substantially more n-6 AA than either the long chain n-3 PUFAs, EPA and DHA.

**Table 5. Fatty acid composition of various foods**

| Food* | n-3 | n-6 | Food* | n-3 | n-6 |
|---|---|---|---|---|---|
| ***Finfish*** † | | | ***Oils***‡ | | |
| **Catfish** | | | Canola | 1.302 | 2.842 |
| Farmed, raw | 0.067 | 0.085 | Corn | 0.714 | 5.530 |
| Wild, raw | 0.130 | 0.149 | Flaxseed | 7.249 | 1.727 |
| **Cod** | | | Rapeseed | 0.014 | 9.466 |
| Atlantic, raw | 0.064 | 0.022 | Olive | 0.103 | 1.318 |
| Pacific, raw | 0.080 | 0.017 | Peanut | 0.000 | 4.320 |
| **Flounder** | | | Safflower | 0.000 | 10.149 |
| Raw | 0.093 | 0.038 | Sunflower | 0.000 | 8.935 |
| **Halibut** | | | Walnut | 1.414 | 7.194 |
| Atlantic and Pacific, raw | 0.071 | 0.139 | ***Vegetable/Fruits***† | | |
| Greenland, raw | 0.526 | 0.061 | Apple | 0.009 | 0.043 |
| **Herring** | | | Avocado | 0.125 | 1.674 |
| Atlantic, raw | 0.709 | 0.060 | Banana | 0.027 | 0.046 |
| Pacific, raw | 0.969 | 0.096 | Broccoli | 0.021 | 0.017 |
| **Mackerel** | | | Cauliflower | 0.104 | 0.029 |
| Atlantic, raw | 0.898 | 0.183 | Lettuce, Romaine | 0.113 | 0.047 |
| King, raw | 0.136 | 0.136 | Kale | 0.180 | 0.138 |
| **Salmon** | | | Peas | 0.035 | 0.152 |
| Atlantic, farmed, raw | 0.618 | 1.152 | Raspberries | 0.126 | 0.249 |
| Atlantic, wild, raw | 0.287 | 0.267 | Spinach | 0.138 | 0.026 |
| Pink, raw | 0.096 | 0.078 | | | |
| **Trout** | | | ***Animal Products***† | | |
| Rainbow, farmed, raw | 0.260 | 0.025 | Beef | 0.015 | 0.227 |
| Rainbow, wild, raw | 0.167 | 0.109 | Cheese, Swiss | 0.352 | 0.620 |
| **Tuna** | | | Chicken | 0.090 | 1.980 |
| Bluefin, raw | 0.283 | 0.043 | Milk, 2% | 0.028 | 0.043 |
| Yellowfin, raw | 0.037 | 0.028 | Turkey | 0.190 | 3.180 |

* Data from the USDA Nutrient Database for Standard Reference
†Per 100g
‡Per 1Tbsp

### 1.2.3 Erythrocytes as markers of the omega fatty acids

Dietary semiquantitative food frequency questionnaires, dietary records, and dietary recalls are standard tools used in nutritional epidemiological investigations. Subject to widespread misreporting of fat intake, these instruments may play a role in the conflicting results of dietary fat and breast cancer. In fact, a recent publication from a prospective study found a statistically significant association between dietary fat intake and breast cancer risk when analyzing participants' food records, but did not find a smiliar association when analyzing the same populations' food frequency questionnaires [128].

Food preparation is often not entirely conducted by the participant under study making it difficult, if not impossible, to determine the quantity of each fatty acid in all foods consumed, through the use of a questionnaire [129]. Additionally, dietary instruments for the long chain n-3 PUFAs, such as EPA, typically do not make a distinction between types of fish consumed, but rather measure total fish consumption; thus, they do not capture the large differences in n-3 fatty acid composition within and between species of fish. Use of biochemical indicators of fat reduces error resulting from human perceptions, opinions, and memories [130, 131]. Therefore, studying biological specimens rather than self-reported estimates could possibly reduce the chance of over or underestimation of dietary fat intake, which has been shown to be particularly vulnerable to bias [132].

Fatty acid levels in erythrocytes, represent dietary intake of fatty acids that cannot be produced endogenously (LA and ALA). Furthermore, because erythrocytes have a long half-life (~120days) and lack the ability for de novo fatty acid elongation and desaturation, they are a good reflection of medium term intake of the "nonessential" n-6

and n-3 PUFAS which are derived from elongation of both LA and ALA as well as from the diet [133, 134]. Hence measures of all erythrocyte n-3 and n-6 fatty acids reflect dietary intake, and perhaps more importantly, internal dose [135-137].

Intervention with flaxseed, one of the richest dietary sources of n-3 ALA, results in raised ALA levels in erythrocyte membranes [138, 139]. Comparison among two populations, one with high cold water fatty fish intake and the other without, demonstrated higher n-3 PUFA levels in the erythrocyte phospholipid membranes of the population with greater marine fat intake [133]. Supplementation with fish oil, high in EPA and DHA, results in a rise of these fatty acids in erythrocytes [140-145], and for EPA incremental increases were proportional to the amount supplied [144]. Randomization to consumption of either fresh fish, fish oil, DHA oil, as compared to the control group, resulted in an elevation in total n-3 PUFA, EPA and DHA in erythrocytes [146]. In an experimental study, a strong correlation ($r = 0.91$ at 6 weeks) was found between EPA measured in erythrocytes and the amount of EPA ingested over a 6-week period [147]. Not only does supplementation of EPA result in elevated erythrocyte levels of this fatty acid, but erythrocyte EPA levels return to baseline values after 18 weeks of discontinuation [148]. EPA and DHA in erythrocytes are also correlated with fatty fish consumption, estimated via a food frequency questionnaire [149].

Less interest has been taken in the association between self-reported dietary intake of the n-6 PUFAs and fatty acids measured in erythrocytes. The correlation of LA in erythrocytes of postmenopausal women with their reported LA intake in food frequency questionnaires is good ($r=0.44$ and $r=0.40$) [148, 150]. Supplementation with corn oil, which is high in LA, results in a rise in this fatty acid in erythrocytes, and to a

much lesser extent, LA's elongated product, AA [151]. In pregnant women erythrocyte AA levels were related to reported dietary intake (β=0.49; p=0.04) [152]. Furthermore, AA which is found only in animal products, is minimal in the erythrocytes of individuals who eat no products of animal origin [153, 154]. Dietary supplementation with AA, results in an erythrocyte fatty acid profile significantly enriched in AA [151, 155].

Erythrocyte fatty acid levels, unlike plasma and serum fatty acid compositions, do not change rapidly after dietary treatment, and obtaining blood samples from participants is not as an invasive measurement as obtaining fatty acids from adipose tissue [156-159]. Given that fatty acids in erythrocytes are easily obtainable and are able to express habitual dietary patterns of individuals over several weeks, they are a useful molecular tool in epidemiologic studies when assessing n-6 and n-3 PUFA intake [130, 160-164].

## 1.3 OMEGA-6 AND OMEGA-3 FATTY ACIDS AND BREAST CANCER

### 1.3.1 AA, EPA, and prostaglandins

The release of n-6 AA and n-3 EPA from phospholipids in the cell membrane is the first step in their conversion into prostaglandins (PG), short-lived hormone-like lipids, and occurs in all cells except for erythrocytes [165]. The conversion occurs by the addition of molecular oxygen via cyclooxygenase-1 (COX-1) or cyclooxygenase-2 (COX-2) [166], which are the rate limiting enzymes in PG biosynthesis. In general, COX-1 is constitutive, overseeing regular bodily functions such as blood flow in the kidneys; COX-2 is inducible and activated only under certain conditions. Conversion of AA by COX

results in PGE2, primarily known for its role as a pro-inflammatory mediator; whereas conversion of EPA, the n-3 homologue of AA, results in PGE3 which is deemed anti-inflammatory [167].

AA and EPA are competitive inhibitors of each other. EPA competes with AA at the level of incorporation into cell membrane phospholipids, for desaturase and elongase enzymes, as well as for substrates for COX enzymes once they are incorporated into the membrane [168-171], thus resulting in PGs of different series [171, 172]. An experimental study on rat mammary tumors demonstrated that a diet supplemented with long chain n-3 (EPA+DHA) fatty acids compared to a diet high in n-6 fatty acids suppressed COX-2 protein levels by 36% [170]. Moreover, n-3 supplementation has been shown to significantly lower PGE2 production in cancer cells [173, 174]. In fact, supplementation with fish oil, high in EPA and DHA, not only decreases PGE2, but increases PGE3 levels [175] which have not been linked to processes in carcinogenesis and may be protective [176]. Suppression of PGE2 synthesis in animal models has also been shown to be dependent on a decreased shift of the n-6:n-3 ratio [177, 178], thus, the relative proportion of the n-6 PUFAs to n-3 PUFAs may also be of relevance to breast carcinogenesis. Blood PGE2 levels were 41% lower among individuals taking EPA supplements, in comparison to individuals not supplemented with EPA [179]. Humans prescribed a diet with a low AA:EPA ratio had lower urinary levels of PGE2 in comparison to individuals prescribed a diet with a high AA:EPA ratio [151].

### 1.3.2 Inhibition of prostaglandin E2

COX-2 and PGE2 are overexpressed in a spectrum of human malignant lesions [180-184], including breast cancer [185-188], and are highly correlated with one another [187] as well as with poorer prognosis [188]. Several mechanisms have been proposed by which COX-2 and its by-product, PGE2, might contribute to tumor progression, and include promotion of cell proliferation, inhibition of apoptosis, and increased angiogenesis [189, 190]. The anti-carcinogenic activities of the n-3 PUFAs have been attributed to their ability to inhibit PGE2 synthesis.

Intervention with pharmacological agents that block PGE2 biosynthesis, such as nonsteroidal anti-inflammatory agents (NSAIDs) and COX-2 selective inhibitors, are protective against breast cancer in animal models [191] and possibly in human populations [192-196]. These agents appear to exert their chemopreventive effects by inhibiting COX-2 activity and ultimately PGE2 production [197]. Harris et al. found a reduced risk of breast cancer associated with the use of NSAIDs at least three times a week for one year (RR=0.66; 95%CI 0.52-0.83), and the effect was dose-dependent [198]. Long-term aspirin use is also found to be chemopreventive, specifically towards hormone receptor positive tumors (OR = 0.74; 95%CI, 0.60-0.93) when compared to hormone receptor negative tumors (OR = 0.97; 95%CI, 0.67-1.40), and in postmenopausal women (OR = 0.77, 95% CI, 0.62-0.97) in comparison to premenopausal women (OR = 0.83; 95% CI, 0.56-1.22). Notably when stratified by menopausal status and receptor status a protective effect occurred in postmenopausal, hormone receptor positive breast cancer (OR 0.70; 95%CI, 0.54-0.91), but not among hormone receptor negative postmenopausal breast cancer (OR= 0.91; 95% CI, 0.58-1.42). Therefore, the

chemopreventive effect of PGE2 inhibition may be the result of an effect on estrogen production; particularly since PGE2 has recently been found to increase aromatase activity, which is the primary source of estrogen production in postmenopausal women [199, 200]. Indeed, decreases in aromatase activity were observed in breast cancer cells following treatment with NSAIDs, a COX-1 selective inhibitor, and COX-2 selective inhibitors [201]. Further, experimental evidence has shown that estradiol production is decreased in breast cells that are exposed to a selective COX-2 inhibitor [202]. Considering the profound impact on COX-2 production of PGE2 via the AA cascade, it would be reasonable to assume that dietary reduction of n-6 fatty acids and/or increased intake of n-3 PUFAs could modify one's risk of developing estrogen-dependent breast cancer.

### 1.3.3 CYP19, estrogen and breast cancer

At menopause, when estrogen synthesis of the ovary ceases, estrogen continues to be produced among various tissues of the body from androgens [203]. The aromatase gene, CYP19, catalyzes the conversion of testosterone and androstenedione, to estradiol and estrone respectively by catalyzing three consecutive hydroxylation reactions. The majority of aromatization takes place in peripheral tissues and particularly in adipose tissue and, as previously stated, is the primary source of estrogen exposure in postmenopausal women [204]. Localized estrogen production via aromatase activity has been positively associated with malignant lesions in the breast [205-207] and appears to promote cancerous growth in both an autocrine and a paracrine fashion [208]. In vitro studies demonstrated that 72% of human breast cancer specimens had aromatase activity

greater than that of other tissue [209], therefore, hormonal therapies have been developed targeting this activity. Aromatase inhibitors prevent the development of breast tumors in Sprague-Dawley rats, in a dose-dependent manner [210] and are used in the treatment of postmenopausal estrogen receptor positive breast cancer [211-213]. A 5 year comparison of aromatase inhibitor, Letrozole, found reduced recurrence of breast cancer compared with the placebo group (RR=0.57, CI 0.43-0.75) [214], and Anastrozole reduces serum estradiol levels in postmenopausal Caucasians by 87% [215]. Thus, inhibition of aromatase activity in postmenopausal women has a desirable impact on breast cancer risk.

### 1.3.4 The relationship between COX-2, PGE2, and CYP19

PGE2 within human breast cells significantly increases aromatase activity in adipose stromal cells ($p<0.05$) [216]. Additionally, aromatase is strongly correlated with COX-2 expression in breast tumors ($r=0.80$, $p=0.001$) [217], but neither are strongly expressed in normal breast tissue [185-188, 218, 219]. COX-2, via production of PGE2, rapidly activates aromatase expression by the enhancement of CYP19 transcription [199, 200]. Since CYP19 is responsible for both peripheral and intratumoral estrogen synthesis this upregulation will ultimately lead to increased estrogen production, and potentially breast carcinogenesis [220]. Therefore, the significant linear correlation between COX-2 and aromatase in breast cancer tissues suggests that they may be involved in the advancement of hormonally-dependent breast cancers [221]. NSAIDS and COX-2 inhibitors inhibit aromatase activity in human breast cancer cells in a dose-dependent fashion, likely by diminishing PGE2 production and consequently CYP19 upregulation [222]. Therefore,

blocking n-6 AA conversion to PGE2 by increasing n-3 intake, a competitive inhibitor of n-6 fatty acids, would theoretically inhibit aromatase induction and result in lowered estrogen production (**Figure 4)**. Whether the effects would be systemic or localized to breast tissue is unknown; however, it is critical that this relationship be investigated in human populations.

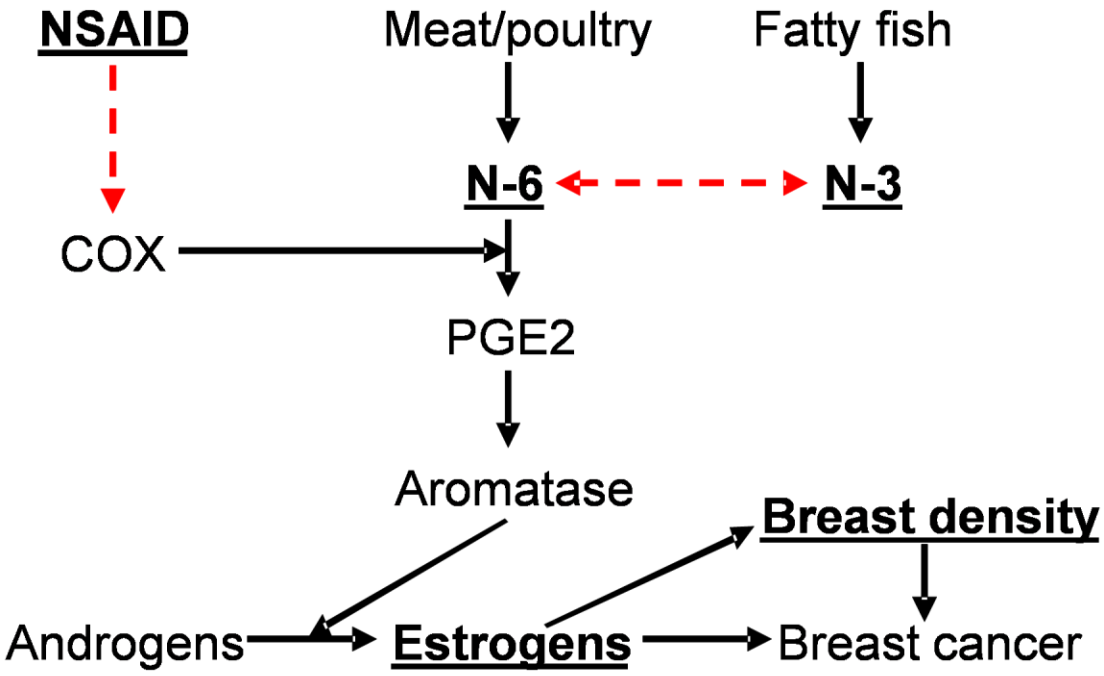


**Figure 4. Potential n-6 and n-3 PUFA pathway leading to breast cancer**

### 1.3.5 Animal and experimental evidence

Extensive laboratory and animal model data suggest that high intake of n-6 PUFAs promotes breast tumor development [178, 223-229], while increasing n-3 PUFAs inhibits mammary tumor growth and metastasis [178, 223, 225-228, 230-236]. N-6 AA, has been

shown to stimulate human breast cancer cell growth [237], and numerous studies have shown EPA to inhibit breast cancer cell growth [226, 227, 230, 232-236, 238] including the growth of estrogen-dependent breast cancer cell lines [231].

Levels of estradiol were approximately 30% higher in rats consuming the largest quantities of n-6 PUFAs in their diet (46% fat) than in the females consuming the lowest n-6 fat (12% fat) ($p < 0.01$) [239], and by week 18 the rats fed the high n-6 PUFA diet developed significantly more mammary tumors than the rats fed a low n-6 PUFA diet (40% vs. 10%; $p<0.05$) [229]. A meta-analysis on mammary tumor incidence in over 12,800 mice and rats, extracted from 97 reports, indicated that n-6 PUFAs had a strong tumor enhancing effect and n-3 PUFAs a protective effect [228]. The results further indicated that the tumor promoting activity of n-6 PUFAs may be abrogated by the competitive inhibition of the n-3 PUFAs. This inhibitory effect has been observed in numerous experimental studies [178, 233, 236, 240]; moreover, the association appears to be dose dependent [178]. Rodents fed a diet with the lowest 6:3 PUFA ratio in comparison to rodents with the uppermost 6:3 ratio had the greatest reduction in tumor growth rate ($p< 0.01$) [178].

### 1.3.6 Ecological and epidemiological evidence

In contrast to the abundance of experimental evidence relating n-6 and n-3 PUFAs to mammary carcinogenesis, epidemiological evidence is sparse. The majority of information supporting this hypothesis evolves from international findings of low breast cancer rates in populations with high n-3 fat intake, and elevated rates in populations with high consumption of products containing n-6 PUFAs [241-245]. Significant inverse

associations between n-3 intake and breast cancer risk have previously been reported [246-248]; yet, a recent systematic review of the literature concluded that no association existed between n-3 consumption, primarily from fish, and breast cancer risk [249]. However, this study did not take into account the varying levels of n-3 fatty acids found within and between species of fish. Although explored less intensively than the n-3 fatty acids, the relationship between n-6 PUFAs and breast cancer has also been subject to investigation, and found to be positively associated with risk of breast cancer [250, 251]. Few population based studies have attempted to look at the 6:3 PUFA ratio and breast cancer risk. Nonetheless, epidemiological data using subjective measures of dietary intake suggest a protective effect of a decreased 6:3 PUFA ratio on breast cancer risk [248, 251-253].

N-6 fatty acids and LA in adipose tissue have been positively associated with breast cancer [254, 255], and adipose n-3 fatty acid levels negatively associated with risk of breast cancer [254, 255]. However, null relationships have also been observed between adipose tissue levels of the n-6 PUFAs and n-3 PUFAs and breast cancer risk [254, 256, 257]. In contrast to the relationship observed between adipose n-6 fatty acids and breast cancer, levels of LA and total n-6 in serum have been inversely related to breast cancer risk [258-261]. Further, null relationships have also been observed for n-3 PUFAs in serum and risk of breast cancer [258-260]. The erythrocyte composition of AA has been positively linked to breast cancer risk [262]. In contrast, total erythrocyte n-6 fatty acids have been negatively related to breast cancer [262-265]. The n-3 fatty acid composition of erythrocytes has consistently been inversely linked to breast cancer occurrence [263, 264]. In fact, a review of biomarkers of fatty acids and breast cancer risk assessed the

relationship between fatty acids in biospecimens (adipose, serum and erythrocytes) and risk of breast cancer in studies published from 1966-2002. The authors concluded that a significant protective effect was found for total n-3 PUFAs [266].

The ratio of n-6 to n-3 fatty acids in biospecimens and breast cancer risk has been explored to some extent. The fatty acid composition in adipose tissue was compared between 241 women with breast cancer and 88 women with benign breast disease. Women in the highest tertile of total n-6 to total long chain n-3 PUFAs had an increased risk of breast cancer compared to women in the lowest tertile (OR=3.03; p trend=0.0002) [255]. Bagga et al., observed a positive association between the ratio of n-6 PUFAs (AA+LA) to long chain n-3 PUFAs (EPA+DHA) (OR=1.68; p trend =0.09) in adipose tissue and breast cancer [256]. In the EURAMIC study there was little consistency across the five study sites between n-6 or n-3 fatty acid content in breast adipose tissue and breast cancer risk; however, among four of the sites a nonsignificant positive association was observed between the 6:3 ratio and breast cancer risk (pooled OR =1.4; p=0.19) [254]. This effect was more marked when focusing on the long chain n-3 PUFAs from fish oil. A low total n-6 to long chain n-3 (EPA+DHA) ratio was protective for breast cancer in women in the highest tertile compared to the lowest tertile (OR= 0.33; 95% CI, 0.17-0.66; trend p = 0.0002) [255]. Four studies have investigated the 6:3 PUFA ratio in blood specimens, either serum or erythrocytes, and breast cancer risk. Two studies presented no evidence of a relationship between the 6:3 ratio and breast cancer [258, 265], whereas the other two studies found significant positive relationships between the 6:3 ratio [263, 264]. The ratio of AA to EPA in erythrocytes has also been positively related to breast cancer risk [263].

### 1.3.7 Dietary fat and serum estradiol

Varying levels of fat consumption may influence incidence of hormonally dependent breast cancer by modifying levels of circulating estrogens [267-277]. In fact, free fatty acids added to plasma can significantly increase levels of estradiol in vitro [272, 273]. Among women with a prior history of breast cancer, a significant decrease in estradiol concentration from baseline was observed in those randomized to a one year, low-fat, high-fiber diet (-13pmol/L in intervention group vs. +3pmol/L in control group; $p<0.05$) [274].) A meta-analysis of 13 intervention trials found serum estradiol levels to be 23% (95%CI: -27.7%, -18.1%) lower in healthy postmenopausal women consuming the least amount of dietary fat when compared to women with the highest fat intake [270]. Among these studies, the greatest estradiol reductions occurred in the two trials with the largest reduction in dietary fat, 10-12% of calories [268, 275]. Nonetheless, when these two studies were excluded from the meta-analysis, the results remained significant. The Diet and Androgens (DIANA) Randomized Trial found a non-significant reduction in serum estradiol (-18.0% in intervention group vs. -5.5% in control group; $p=0.13$) among postmenopausal women consuming a low animal fat and high n-3 diet [276]. Additionally, an inverse association between n-3 fatty acids from fish consumption and serum estradiol levels has been observed [277]. However, not all studies evaluating dietary fat and estrogen levels have observed reductions in circulating estradiol levels; it has been hypothesized that inadequate dietary assessment may be one cause of contradiction. No epidemiological study has examined the association between erythrocyte fatty acids in relation to circulating estradiol levels.

### 1.3.8 Dietary fat and mammographic breast density

The few studies that have assessed the role of dietary fat on mammographic density have revealed that diet may influence breast density. A two year intervention with a low-fat, high-carbohydrate diet, reduced breast density by 6.1% in the experimental group as compared to 2.1% in the control group ($p=0.02$) [278]. Positive associations between total fat intake and high mammographic density have been observed [106, 279-281]. However, decreased density and null findings have also been associated with total fat intake [282-285]. As with total fat, positive and null relationships have been found between total PUFA intake and breast density [106, 279-283, 285]. Women with the highest mammographic pattern reported significantly higher consumption of n-6 PUFAs when compared to women with the lowest mammographic pattern (4.7 % energy vs. 3.8% energy; $p<0.001$) [281]. Two studies assessed the effects of total meat intake, which is correlated with n-6 AA intake, and breast density. A nonsignificant, positive association (OR=1.59; 95%CI: 0.83, 3.04.) was observed in one study; however, no relationship was found between total meat intake and density in the other [283, 284]. Fish consumption has not been linked to mammographic density in postmenopausal women; however, these studies measured total fish consumption rather than fatty fish intake which better mirrors intake of n-3 PUFAs [282-284]. There was no difference in means of percent breast density when comparing quartiles of n-3 intake or long chain n-3 intake [279, 283], nor was an association observed between n-3 intake and Wolfe's parenchymal patterns in women with breast cancer [281]. Only one study has investigated the effects of the individual fatty acids of the n-6 and n-3 families with breast density. No association was found between mammographic density and n-6 LA,

but a significant inverse association was observed between n-3 ALA and percent breast density (OR=0.69: 95%CI: 0.47, 0.99) [282]. As is the case with circulating estradiol concentrations, no study has examined the association between erythrocyte fatty acids in relation to mammographic breast density.

## 1.4 SUMMARY OF INTRODUCTION

N-6 and n-3 PUFAs are competitive inhibitors of one another, resulting in different prostaglandin products. PGE2, the result of COX mediated n-6 AA metabolism, stimulates the biosynthesis of estrogen, a causal breast cancer risk factor, by upregulation of the enzyme aromatase. The suppression of aromatase activity via aromatase inhibitors results in lower circulating serum estradiol levels and reduced breast cancer risk in postmenopausal women. Since both low n-6 PUFA consumption and elevated n-3 PUFA consumption reduces the production of PGE2, intake of these fatty acids may influence estradiol synthesis and estrogen dependent breast cancer incidence.

# 2.0 SPECIFIC AIMS AND HYPOTHESES

The relationship between the n-6 and n-3 fatty acids in breast cancer development is well established in animal models. However, the mechanisms by which these factors affect the development of breast cancer are currently unknown. Few epidemiological studies have examined the relationship between the n-6 and n-3 fatty acids and well-established, modifiable breast cancer risk factors, specifically serum estradiol and mammographic breast density. Further, the studies that did assess these relationships did so by measuring dietary intake via self-report dietary assessment instruments, and limitations of these assessment tools and nutrient composition tables are well-known. Measuring n-6 and n-3 fatty acids in biological specimens provides a useful alternative to self-reported dietary intake, and allows for the objective measurement of individual fatty acid levels. The relationship between circulating levels of estradiol, mammographic breast density, and breast cancer risk is greatly documented; hence, identifying agents capable of altering these risk factors could have a substantial impact on public health.

Therefore, the research goal of this body of work was to investigate the relationship between n-6 and n-3 fatty acids in erythrocytes and postmenopausal serum estradiol levels and mammographic breast density, both modifiable, well-established breast cancer risk factors. An additional research goal was to investigate the relationship between current NSAID use and serum estradiol. Similarly to elevated n-3 fatty acid

intake, NSAID use reduces the production of PGE2, thus decreasing upreguation of aromatase and estrogen synthesis. Therefore, if it is through alteration of the PGE2 pathyway that n-6 and n-3 fatty acids influence estradiol synthesis, then we would also expect to observe differences in estradiol levels by NSAID use. NSAID data was not available at the time of mammogram; therefore, the relationship between NSAID use and breast density could not accurately be assessed. The specific research questions addressed and corresponding hypotheses are outlined below.

**1. Are n-6 and n-3 fatty acids in erythrocytes associated with serum estradiol levels in cancer-free, postmenopausal women not using exogenous hormones?** The hypothesis is that erythrocyte n-6 fatty acids are positively related to postmenopausal serum estradiol levels and erythrocyte n-3 fatty acids negatively related to postmenopausal serum estradiol levels.

**2. Is current NSAID use associated with serum estradiol levels in cancer-free, postmenopausal women not using exogenous hormones?** The hypothesis is that current NSAID users have lower circulating serum estradiol levels than NSAID non-users.

**3. Are n-6 and n-3 fatty acids in erythrocytes associated with mammographic breast density in cancer-free, postmenopausal women not using exogenous hormones?** The hypothesis is that erythrocyte n-6 fatty acids are positively

related to mammographic breast density and erythrocyte n-3 fatty acids negatively related to mammographic breast density.

In order to answer these questions, three cross-sectional analyses were undertaken ancillary to the Mammograms and Masses Study (MAMS). The findings from these three investigations are presented in separate manuscripts. These studies are important as the identification of modifiable lifestyle factors that favorably alter breast cancer risk factors may enable us to reduce the onset of breast cancer, via the development of safe, effective and easily adoptable primary prevention strategies.

# 3.0 ARTICLE ONE: ERYTHROCYTE OMEGA-6 AND OMEGA-3 FATTY ACIDS AND POSTMENOPAUSAL SERUM ESTRADIOL

To be submitted for publication

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## 3.1 ABSTRACT

Elevated intake of omega-6 (n-6) polyunsaturated fatty acids (PUFAs) may promote breast cancer, whereas omega-3 (n-3) consumption may inhibit the growth of this disease. The mechanism by which these fatty acids impact breast cancer development is unknown; however, experimental evidence indicates that these two families of fatty acids may influence risk by impacting eicosanoid synthesis. Specifically, when n-3 PUFAs displace n-6 PUFAs, prostaglandin E2 production (PGE2) is reduced, resulting in decreased aromatase activity and ultimately suppression of estrogen synthesis. Thus, in this cross-sectional analysis, we sought to determine whether n-6 and n-3 fatty acids in erythrocytes, expressed as a percentage of total fatty acids, were associated with postmenopausal serum total estradiol concentrations. Because NSAIDs also inhibit PGE2 formation, separate analyses were performed for participants using and not using NSAIDs. Among women not using NSAIDs (n=135), multivariate adjusted estimates revealed that mean estradiol concentrations decreased with increasing tertile of total erythrocyte n-3 fatty acids (24.3 pmol/L vs 18.4 pmol/L; $p<0.05$) and increased with increasing tertile of total n-6 fatty acids (16.0 pmol/L vs. 21.8 pmol/L; $p=0.02$), the total n-6:n-3 ratio (17.6 pmol/L vs. 22.9 pmol/L; $p=0.06$) and the ratio of n-6 arachidonic acid to n-3 eicosapentaenoic acid+docosahexaenoic acid (17.6 pmol/L vs. 24.9 pmol/L; $p<0.01$). Among NSAID users (n=118), mean estradiol was greatest among women in the highest tertile of the n-6 linoleic acid to n-3 alpha-linolenic acid (ALA) ratio as compared to the lowest tertile (21.1 pmol/L vs. 14.2 pmol/L; $p=0.01$). This finding was

primarily due to the inverse association noted between ALA and estradiol. NSAID users in the highest tertile of ALA had a lower mean estradiol concentration than participants in the lowest tertile of ALA (15.2 pmol/L vs. 20.8 pmol/L; p=0.05). No other significant differences were noted among current NSAID users. Because circulating postmenopausal estradiol concentrations are causally related to breast carcinogenesis, these findings provide a mechanism through which the n-6 and n-3 fatty acids may alter breast cancer risk.

## 3.2 INTRODUCTION

Breast cancer is a common form of cancer among women worldwide and despite substantial advances in the treatment of this disease breast cancer remains a leading cause of death among women. Several lines of evidence implicate that dietary intake may influence breast cancer, and for many years it has been postulated that excessive dietary fat consumption may play a role in the etiology of this disease [286]. However, no clear consensus on this topic has been established. One theory that addresses this debate is that a relationship may not exist between breast cancer risk and total fat intake, but an association may exist between breast cancer and the type of fat consumed. Considerable interest has focused on the association between the omega-6 (n-6) and omega-3 (n-3) polyunsaturated fatty acids (PUFAs) and breast cancer risk, largely stemming from analysis of international data [242, 287].

The majority of PUFAs in the United States diet, where breast cancer rates are among the highest in the world, consist of n-6 PUFAs found in abundance in corn and vegetable oils [117, 288]. Populations such as the Greenland Eskimos [289], the Alaskan Natives [290] and the Japanese [118] have high fish consumption, hence high n-3 intake. These populations also have substantially lower rates of breast cancer despite their overall high fat consumption [289, 291, 292]. Therefore, the higher rates of breast cancer observed in the United States may be explained, in part, by the elevated intake of n-6 and/or insufficient intake of n-3 PUFAs. This hypothesis is supported by some

experimental [178, 225, 227, 228, 232-235, 238, 293] and epidemiological data [251, 253, 264, 266].

Perhaps the most acknowledged mechanistic pathway through which the n-6 and n-3 fatty acids may influence breast cancer risk is via eicosanoid biosynthesis. Arachidonic acid (AA; 20:4n-6), which can be ingested or formed endogenously by desaturation and elongation of linoleic acid (LA; 18:2n-6), serves as the substrate for cyclooxygenase (COX) mediated prostaglandin E2 (PGE2) production. PGE2 is an inflammatory eicosanoid that is upregulated in breast tumors [294] and is a potent inducer of aromatase activity [200], the key enzyme in postmenopausal estrogen synthesis. In contrast, prostaglandin E3 (PGE3), an anti-inflammatory eicosanoid, is derived from the metabolism of eicosapentaenoic acid (EPA; 20:5n3). EPA can be consumed, formed from the essential n-3 fatty acid alpha-linolenic acid (ALA; 18:3n-3), or formed from retroconversion of docosahexaenoic acid (DHA; 22:6n-3). Unlike PGE2, PGE3 has not been documented to upregulate aromatase and is signficiantly less mitogenic [176].

The n-6 and n-3 PUFAs compete with each other for enzymes at multiple levels; therefore, increasing n-3 consumption ultimately suppresses the production of PGE2 [176, 179]. This suggests that reducing n-6 or increasing n-3 intake, which results in lowered PGE2 formation [176, 179, 295], may result in lower circulating estradiol levels. Indeed, nonsteroidal anti-inflammatory drugs (NSAIDs), which also inhibit formation of PGE2, are associated with reduced estradiol production in breast cells [202] and lower serum estradiol concentrations in postmenopausal women (Research article 2).

Little is known about the relationship between n-6 and n-3 PUFAs and circulating postmenopausal estrogen levels, likely a result of the methodological issues with the estimation of individual fatty acids from self-reported dietary instruments. Utilizing fatty acids in erythrocytes allows for individual fatty acid assessment, provides an objective measurement, and reflects recent dietary intake of the essential n-6 and n-3 fatty acids. Therefore, we evaluated the association between dietary habits of the n-6 and n-3 PUFAs, as inferred from erythrocyte fatty acid composition, and serum total estradiol concentration in postmenopausal women.

## 3.3 MATERIALS AND METHODS

### 3.3.1 Study Population

Details of the Mammograms and Masses Study (MAMS) have been described previously [296]. The MAMS is a case-control study of estrogen metabolites, mammographic breast density and breast cancer risk. A total of 869 cancer-free women and 264 recently diagnosed breast cancer cases were recruited into the MAMS through the Magee Womens Hospital Mammographic Screening and Diagnostic Imaging Program in the greater Pittsburgh area (Pennsylvania, USA) in 2001-2005. The participants were all women aged 18 years or older and who reported no previous personal history of cancer, with the exception of nonmelanoma skin cancer. Participants in the MAMS include: 1) newly diagnosed breast cancer cases who were recruited from the Magee-Womens Surgical Clinic (n=264); 2) women who were undergoing outpatient needle breast biopsy

through the Breast Biopsy Service at Magee-Womens Hospital, but were not subsequently diagnosed with breast cancer (n=313); 3) cancer-free women who received screening mammography through Magee-Womens Hospital or through Magee Womancare Centers (n=538) and; 4) an additional 18 participants whose blood was dedicated solely to an ancillary study of intra-individual cytokine and hormone concentration reproducibility. To increase recruitment of the "healthy" control group, study flyers were attached to screened negative mammogram reports mailed to patients from 2003-2005. The study was approved by the Institutional Review Board of the University of Pittsburgh and all participants provided written informed consent.

### 3.3.2 Subsample Selection

Inclusion criteria for entry into this ancillary study were as follows: 1) controls recruited only via study flyers through Magee-Womens Hospital or through Pittsburgh Magee Womancare Centers (n=453), as information on these participants was gathered on the day blood was drawn; 2) postmenopausal (having had no menstrual bleeding during prior year or having undergone a bilateral oophorectomy); 3) not using hormone therapy (HT) within three months of enrollment; and 4) not using vaginal estrogen creams, oral contraceptives, selective estrogen receptor modulators (SERMs) or corticosteroids at study enrollment. A total of 270 women met the inclusion criteria for the present analyses. Of those who were excluded, 98 were premenopausal, 84 were using exogenous hormones, SERMs, or corticosteroids, and 1 participant was later diagnosed with breast cancer.

### 3.3.3 Covariate Information

We used a standardized, self-administered questionnaire to gather participants' exposure information at study enrollment. Information on demographic characteristics, current use of medication and supplements, reproductive history, family medical history, past exogenous hormone use, smoking status, and alcohol intake was obtained. Participants were asked to report all prescribed and over-the-counter medications that were currently being used on the questionnaire. Women who listed using aspirin, COX-2 inhibitors, or other non-aspirin NSAIDs were considered "current NSAID users." Participants who did not list using a NSAID were considered "current NSAID non-users." Because acetaminophen is generally reported to be a weak inhibitor of the COX-1/COX-2 enzymes [297], we classified acetaminophen users as non-users of NSAIDs unless they also reported taking a NSAID. Regular alcohol use (g/day) in the past year was calculated as previously described [298]. Age of menopause was defined according to the methods reported by the Women's Health Initiative [299], where age at menopause corresponded to the age of a woman's last natural menstrual bleeding, bilateral oophorectomy, or age a woman began using HT. For a hysterectomized woman without a bilateral oophorectomy, age at menopause was the earliest age at which she began using HT or first had menopausal symptoms. If neither occurred and her age at hysterectomy was 50 years or older, then age at menopause was her age at hysterectomy. Age at menopause could not be determined in 7 participants. Years since menopause were calculated by subtracting a woman's age at menopause from her age at study enrollment. The questionnaire was reviewed for completeness by a trained research nurse.

### 3.3.4 Clinical Measures

After participants removed shoes and heavy clothing, height and weight were measured by the study nurse. Weight was measured at a standing position to the nearest 0.1 kg using a standard balance beam; standing height was measured at full inspiration to the nearest 0.1 cm. Measurements were taken twice and were repeated if the first two measurements differed by more than 0.5 cm or 0.5 kg. The mean of the measurements was used to derive final heights and weights. Body mass index (BMI) was calculated as weight (kg) divided by height squared ($m^2$).

After anthropometric assessment, a 40 ml non-fasting blood sample was collected by the study nurse. Samples were processed on site according to a standardized protocol. After processing, the samples were separated into red blood cell, serum, plasma and buffy coat and stored at or below -70°C until assayed.

### 3.3.5 Measurement of Fatty Acids

Erythrocyte fatty acid concentrations were identified using gas-liquid chromatography. Samples were analyzed at the University of Pittsburgh's Heinz Laboratory. Total lipids (500µl of packed red blood cells) were extracted according to the general technique of Bligh and Dyer [300]. Briefly, the samples were homogenized in 4 ml of methanol, 2 ml of chloroform and 1.1 ml of water. Two ml of chloroform and 2 ml of water were added to the samples after 15 min. The tubes were then centrifuged at 1200 g for 30 min at 16°C and the upper phase discarded. The lower phase was dried under nitrogen and resuspended in 1.5 ml 14% boron trifluoride methanol. The samples were heated at 90°C

for 40 min and after cooling extracted with 4.0 ml pentane and 1.5 ml water. The mixtures were vortexed and the organic phase recovered [301]. The extracts were dried under nitrogen, resuspended in 50 µl heptane and 2 ml injected into a capillary column (SP-2380, 105 m x 53 mm ID, 0.20 um film thickness). Gas chromatographic analyses were carried out on a Perkin Elmer Clarus 500 equipped with a flame ionization detector. Operating conditions were as follows: the oven temperatures were 140°C for 35 min; 8°C/min to 220°C, held for 12 min; injector and detector temperatures were both at 260°C; and helium, the carrier gas, was at 15 psi. Identification of fatty acids was by comparison of retention times with those of authentic standards (Sigma). A random subset of 27 samples was analyzed for reproducibility; laboratory personnel were blinded to duplicate samples and subject identification. The inter-assay coefficients of variation (CV) for the fatty acid measures reported ranged between 1.7-15.2%. CVs's were 4.6% for LA, 3.4% for AA, and 1.7% for total n-6 fatty acids. CV's were higher for the n-3 fatty acids, with CV's of 15.2% for ALA, 5.3% for EPA, 7.5% for DHA and 5.3% for total n-3 fatty acids. The CVs for the total n-6:n-3, LA:ALA, AA:EPA, and AA:EPA+DHA ratios were 5.2%, 11.1%, 4.5% and 5.7% respectively. The individual and total n-6 and n-3 fatty acids are expressed as a percentage by weight of the total erythrocyte fatty acid content.

### 3.3.6 Measurement of Total Estradiol

Serum total estradiol was measured by radioimmunoassay (RIA) after diethyl ether extraction using a highly specific rabbit antiserum raised against an E2-6-carboxymethyloxime-BSA conjugate (EIR, Wurenlingen, Switzerland) and Third

Generation Estradiol [I125] reagent DSL 39120 (Diagnostic Systems Laboratories Inc., Texas USA). Assays were conducted at the Royal Marsden Hospital in England [302]. The lower detection limit of the assay was 3pmol/L by calculation from the 95% confidence limits of the zero standard. Twenty-seven replicate quality control samples were analyzed to assess reproducibility; the calculated CV between duplicates for estradiol was 14.5%. Laboratory personnel were blinded to quality control status.

### 3.3.7 Statistical Analyses

Statistical analyses were performed using SAS software version 9.1 (SAS Institute, Inc., Cary, North Carolina). To improve normality for statistical tests, a log transformation was applied to serum total estradiol concentrations. One participant was excluded from analyses because total estradiol concentrations were deemed unreliable by the laboratory. An additional 9 participants with estradiol values greater than 150 pmol/L were removed from analyses because such high levels likely indicated the participants were not postmenopausal or incorrectly reported current hormone use. Analyses were repeated with extreme data points included **(Appendix A)**, and because findings did not change substantially the 9 participants were not included in the final report. The final sample included 260 women.

Descriptive results for continuous variables are expressed as means and standard deviations (SD). Categorical variables are reported as frequencies and percentages (%). Correlation relationships between n-6 and n-3 fatty acid measures and serum estradiol were examined with Spearman's correlation coefficients, with no adjustments and controlling for the effects of age and BMI.

Multivariate associations for serum total estradiol according to tertile of fatty acid were assessed by analysis of covariance (ANCOVA) using the general linear models (GLM) procedure of SAS (PROC GLM). Adjusted geometric mean estradiol and 95% confidence intervals (CI) were calculated using least squares means, controlling for the effects of age (continuous), BMI (continuous), years menopausal (continuous), regular alcohol intake in the past year (0g/day, <12g/day, ≥12g/day, entered as a dummy variable), current smoking status (nonsmoker vs. smoker), and current NSAID use (non-user vs. user). The comparisions were adjusted for these variables as they were strongly related to the majority of erythrocyte fatty acid measures, and with the exception of smoking status, were also related to serum estradiol levels (**Appendix A)**. The geometric mean concentrations of estradiol were calculated by taking the anti-log of the least squares means after adjustment. NSAIDs reduce PGE2 synthesis and hence aromatase activation, thus, intake of the n-6 and n-3 PUFAs may have less of an effect on estradiol levels among NSAID users. Therefore, we performed analyses stratified by NSAID use to assess possible effect modification by this variable. To formally test whether the effects of fatty acids were altered by current NSAID use, an interaction term between fatty acid (tertile) and NSAID use was entered into the unstratified multivariate model. Tests of linear trends were performed across fatty acid measures by modeling tertiles as consecutive integers (continuous variable).

The assumptions of the models were checked by residual analysis. Plots of the residuals versus the predicted values were examined to check for heteroscedasticity. The normal probability plot of the residuals was examined to assess the normality of the error terms. Model assumptions of normality and homogeneity of variance were met for all

models presented. Tests of statistical significance were two-tailed and given the exploratory nature of this work, we reported our results at the $p<0.05$ significance level, rather than correct for multiple comparisons. Analyses were repeated excluding participants (n=13) reporting fatty acid supplementation at blood draw, but results did not differ substantially and are therefore not presented.

## 3.4 RESULTS

The characteristics of the study population are shown in **Table 6**. The mean (SD) age was 62.8 (8.4) years and the mean (SD) BMI was 28.5 (6.0) $kg/m^2$. Only 6.9% of the women were non-white and 5.8% current smokers. Close to half (47.7%) of the population indicated current use of a NSAID (aspirin, non-aspirin, and/or COX-2 inhibitor) at study enrollment. The geometric mean serum estradiol concentration for the study population was 19.5 pmol/L, with levels ranging from 3.3-140.0 pmol/L.

On average, the proportion of total n-6 fatty acids was higher than the proportion of total n-3 fatty acids in erythrocytes (**Table 7**). The average ratio of mean total n-6 fatty acids to mean total n-3 fatty acids was 5.2. N-6 AA and LA were the most abundant fatty acids, with AA composing 16.0% and LA 15.8% of total fatty acids. Of the n-3 fatty acids, DHA accounted for the greatest percentage (4.5%) of total fatty acids.

**Table 8** presents the unadjusted and adjusted correlations between fatty acid measures and serum total estradiol for the entire study population. Analyses revealed distinct differences in the relationships of the erythrocyte n-6 and n-3 fatty acids with

serum estradiol. Statistically significant positive correlations were observed for both total n-6 fatty acids (r=0.15, p=0.02) and AA (r=0.13, p=0.04) with estradiol. A nonsignificant inverse association was observed for n-6 LA (r=-0.08, p=0.21). Erythrocyte total n-3 fatty acids and all individual n-3 fatty acids were inversely related with serum total estradiol. Correlation coefficients ranged from -0.17 to –0.24 and were statistically significant ($p<0.05$) for all n-3 fatty acid measures. The strongest inverse correlation was found for EPA ($r = -0.24$, $p<0.0001$), followed closely by total n-3 fatty acids (r=-0.22, p=0.0003). Highly significant positive correlations between serum estradiol and the four 6:3 ratios (total n-6:n-3, LA:ALA, AA:EPA, and AA:EPA+DHA) were found. Spearman correlation coefficients ranged from 0.18 ($p < 0.004$) to 0.25 ($p < 0.0001$). However, adjustment for age and BMI attenuated the findings.

**Table 9** shows the estimated geometric mean of serum estradiol concentration across tertile of erythrocyte fatty acid. After adjustment for age, BMI, years menopausal, alcohol intake, current smoking status, and current NSAID use, no individual fatty acid was significantly related to estradiol concentration. However, a significant trend of increasing estradiol concentration with tertile of LA:ALA (p trend=0.03) was found. The adjusted total estradiol concentration was approximately 20.5% higher among participants in the highest tertile of LA:ALA as compared to those in the lowest tertile. Although a higher mean estradiol concentration was observed in the highest tertile as compared to the lowest tertile of all other 6:3 ratios (total n-6:n-3, AA:EPA, and AA:EPA+DHA), the findings were not statistically significant. A suggestive inverse trend for ALA tertiles (p trend =0.09) was noted.

Because NSAIDs inhibit the COX/PGE2/aromatase pathway, we next explored the associations between fatty acids and estradiol by NSAID use. **Table 9** shows the estimated geometric mean estradiol concentrations across tertile of fatty acid stratified by NSAID use. Among non-users of NSAIDs, multivariate adjusted analyses revealed several significant or borderline significant associations between fatty acid measures and estradiol. Total n-6 fatty acids was positively and significantly related to serum estradiol (p trend=0.02). The adjusted geometric mean estradiol levels also rose with increasing tertile of n-6 AA among NSAID non-users, with mean estradiol levels 24.2% higher in the topmost tertile as compared to the lowest tertile (p trend=0.09). No association was observed between LA and estradiol (p trend=0.97). On the contrary, geometric mean serum estradiol levels decreased with increasing tertile of total n-3 fatty acids (p=0.05), with mean estradiol levels 24.3% lower in the highest tertile as compared to the lowest. Mean estradiol levels were also lower in the highest tertile of all individual n-3 fatty acids (ALA, EPA, and DHA) as compared to the lowest tertile; however, the p for trend did not reach statistical significance for any of these measures. The total n-6:n-3 ratio was positively linked to estradiol and the finding approached statistical significance (p trend=0.06), with 30.1% greater mean estradiol levels in the upper tertile as compared to the lower tertile. A positive relationship was observed between the AA:EPA+DHA ratio and estradiol, with mean estradiol concentrations 40.7% higher in the highest as compared to the lowest tertile (p=0.01). The geometric mean estradiol levels were also higher in the AA:EPA and LA:ALA ratios' uppermost tertiles as compared to the lowest tertiles; however, these trends were not statistically significant.

Among women reporting current NSAID use, a positive association between the LA:ALA ratio and estradiol was noted, with mean estradiol in the highest tertile 48.6% higher than the mean estradiol of the lowest tertile (p trend=0.01). This observation was largely attributable to n-3 ALA, which was inversely related to estradiol in NSAID users (p trend=0.05). Estradiol was 26.9% lower in the highest tertile of ALA as compared to the lowest tertile. No other fatty acid measure was related to serum total estradiol levels within the NSAID user stratam.

Effect modification by NSAID use was formally tested by including interaction terms in the GLMs. Despite substantial differences in the relationships between fatty acids and estradiol between NSAID users and non-users, the only signficant interaction was between NSAID use and total n-6 fatty acids with respect to circulating estradiol ($p<0.02$). The interaction between NSAID use and the AA:EPA+DHA ratio was suggestive (p=0.12). However, this study had limited power to detect interaction effects.

## 3.5 CONCLUSIONS

In this cross-sectional investigation, we examined the relationships between erythrocyte n-6 and n-3 fatty acids and postmenopausal serum total estradiol concentrations. In a population of women not reporting current NSAID use, we found a positive association between total n-6 fatty acids and estradiol and an inverse association between total n-3 fatty acids and estradiol. We further observed positive relationships between the AA:EPA+DHA ratio and the total n-6:n-3 ratio with serum estradiol. As high postmenopausal circulating estradiol concentrations are related to increased breast cancer

risk, these findings are consistent with the hypothesis that n-6 fatty acids may increase the risk of breast cancer and n-3 fatty acids may protect against this disease. Similar associations were not noted among women who reported current NSAID use. Although, none of the significant findings observed among non-users of NSAIDs were found among current NSAID users, a significant positive relationship was noted between the LA:ALA ratio and estradiol levels. This relationship was largely attributable to the inverse association between ALA and estradiol.

A potential explanation for the null finding between n-6 fatty acids and estradiol among NSAID users is that since NSAIDs inhibit PGE2 formation, limiting the amount of substrate available (n-6 fatty acid AA) for PGE2 synthesis is not of biological importance. In addition, a relationship between total n-3 fatty acid measures and estradiol might not have been observed among NSAID users, because both n-3 consumption and NSAID use reduce PGE2 production, and therefore exposure to both anti-inflammatory agents might not offer additional benefit. The strong inverse relationship noted between ALA and estradiol in conjunction with the speculations offered above, may be suggestive that an additional pathway is involved other than ALAs ability to compete for COX enzymes (i.e. through elongation to EPA) among NSAID users. Possible mechanisms of action include ALA's ability to reduce TNF-alpha and IL-6 [303], which have also been shown to stimulate aromatase activity [304]. Although this finding may be biologically plausible, we also must acknowledge the potential role chance plays when multiple comparisons are made.

Although the majority of interactions between fatty acid measures and NSAID use were not statistically significant, the effect sizes suggest that there are differences in

the strengths of the relationships between NSAID non-users and users. Thus the lack of significance of the interaction terms may be attributable to the low statistical power of our study. However, it should also be acknowledged that given the nonsignificant interaction terms, the effect modification noted may be a result of chance findings. Nonetheless, given the biological plausibility of an interaction and because no study has previously reported on these associations, we chose to present the data stratified by NSAID use.

We are unaware of any study reporting weaker effects of the n-6 and n-3 fatty acids on breast cancer risk among NSAID users; however, long chain n-3 PUFA levels in blood were associated with decreased colorectal cancer risk among aspirin non-users, but not among aspirin users [305]. Futher a statistically significant interaction between total fat intake and NSAID use ($p=0.007$) has been noted. Among non-users of NSAIDs, decreasing fat intake was inversely related to recurrence of adenomous polyps [306]. A similar finding was also noted in relation to squamous cell carcinoma of the skin. Erythrocyte levels of n-6 AA were significantly greater among cases than controls and this relationship was more apparent among NSAID non-users [307].

To our knowledge this is the first study to report on the relationship between the essential fatty acids found in biospecimens and endogenous estradiol levels. Further, there is a paucity of data on the impact of n-6 and n-3 fatty acids, as measured via self-report, on concentrations of postmenopausal estradiol levels. Consistent with our findings, estradiol levels have been found to be significantly inversely related to n-3 fat from fish [277]. We are not aware of any epidemiological investigation that assessed the

relationship between individual or total n-6 fatty acids or n-6:n-3 ratios and postmenopausal estradiol levels.

A key limitation of this study is its cross-sectional nature, which does not allow causal inference. Hormone and erythrocyte fatty acid concentrations were measured once and a single measure may be inadequate to some degree because of variability within individuals over time; for that reason, assaying multiple samples over time might better characterize levels in these women. However, use of a single measurement of erythrocyte membranes is capable of reflecting recent n-6 and n-3 fatty acid intake [161]. We cannot rule out that perhaps n-6 and n-3 levels are simply markers of poor or healthy lifestyles. While we adjusted for BMI, alcohol intake and smoking in multivariate analyses, relations between the fatty acids and estradiol levels could be due to residual confounding by unmeasured lifestyle characteristics rather than real dietary effects. Additionally, the MAMS participants included in this analysis are a relatively homogenous sample as all are postmenopausal, not using hormone therapy, and predominantly white, thus the study results may have limited generalizability. In spite of these limitations, this study is unique in that we believe no other epidemiological study has assessed the relationship between circulating fatty acids and serum estradiol levels. Additional study strengths include the use of an objective measure of dietary fat intake and standardized assessment of participant characteristics.

In summary, this study provides modest evidence supporting a positive association between n-6 PUFAs and a negative association between n-3 PUFAs and serum estradiol levels. However, given the cross-sectional design of the study, the observed relationships should be viewed as hypothesis generating and interpreted with

caution. The answers to the role of the essential n-6 and n-3 PUFAs in breast cancer development are not definitive, the data being too insufficient to be convincing. Because of limitations in current research, chemoprotective dietary recommendations for women cannot be issued. Given this study's findings and the limitations listed above, prospective studies assessing the relationship between the n-6 and n-3 PUFAs and circulating estrogens using validated dietary assessment instruments along with repeated blood sampling for fatty acid and estradiol analysis are warranted.

**Table 6. Distribution of selected characteristics among postmenopausal women in the Mammograms and Masses Study**

| Continuous variables | mean | SD |
|---|---|---|
| Age at blood draw (years) | 62.8 | 8.4 |
| BMI (kg/m$^2$) | 28.5 | 6.0 |
| Age at menopause (years)* | 48.7 | 5.1 |
| Years menopausal* | 14.1 | 10.0 |
| **Categorical variables** | **n** | **%** |
| Race | | |
| White | 242 | 93.1 |
| Non-white | 18 | 6.9 |
| Surgical menopause * | | |
| No | 229 | 88.4 |
| Yes | 30 | 11.6 |
| Prior hormone therapy use | | |
| No | 100 | 38.5 |
| Yes | 160 | 61.5 |
| Regular alcohol intake in past year | | |
| None | 188 | 72.3 |
| < 12 g/day | 46 | 17.7 |
| ≥ 12 g/day | 26 | 10.0 |
| Current Smoker | | |
| No | 245 | 94.2 |
| Yes | 15 | 5.8 |
| Current NSAID use | | |
| No | 136 | 52.3 |
| Yes | 124 | 47.7 |

NOTE: BMI, body mass index; NSAID, nonsteroidal anti-inflammatory drug

* Mean and prevalence estimates were determined on nonmissing data; missing n=7 for age at menopause, n=7 for years menopausal, and n=1 for surgical menopause

**Table 7. Mean fatty acid composition in erythrocytes**

| Fatty Acids (wt. %) | mean (SD) |
|---|---|
| Total n-6 PUFA* | 38.3 (2.6) |
| 18:2n-6 (LA) | 15.8 (2.4) |
| 20:4n-6 (AA) | 16.0 (2.0) |
| Total n-3 PUFA † | 7.9 (2.0) |
| 18:3n-3 (ALA) | 0.2 (0.1) |
| 20:5n-3 (EPA) | 0.9 (0.4) |
| 22:6n-3 (DHA) | 4.5 (1.5) |
| 6:3 Ratios | |
| Total n-6:n-3 | 5.2 (1.5) |
| LA:ALA | 72.7 (19.3) |
| AA:EPA | 21.8 (9.0) |
| AA:EPA+DHA | 3.3 (1.2) |

NOTE: N=260. Data are expressed as mean (SD). Fatty acids are reported as a percentage by weight of the total fatty acids (weight percent, wt. %). PUFA, polyunsaturated fatty acid; LA, linoleic acid; AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; and DHA, docosahexaenoic acid.

*18:2n-6+18:3n-6+20:2n-6+20:3n-6+20:4n-6+22:4n-6+22:5n-6

†18:3n-3+20:4n-3+20:5n-3+22:5n-3+22:6n-3

**Table 8. Spearman rank order correlation coefficients between n-6 and n-3 fatty acids in erythrocytes and serum estradiol concentrations**

| Fatty Acids (wt. %) | r* | p | r† | p |
|---|---|---|---|---|
| n-6 PUFAs | | | | |
| Total n-6 PUFAs‡ | 0.15 | 0.02 | 0.09 | 0.13 |
| 18:2n-6 (LA) | -0.08 | 0.21 | -0.04 | 0.57 |
| 20:4n-6 (AA) | 0.13 | 0.04 | 0.09 | 0.13 |
| n-3 PUFAs | | | | |
| Total n-3 PUFAs§ | -0.22 | 0.0003 | -0.11 | 0.06 |
| 18:3n-3 (ALA) | -0.18 | 0.004 | -0.13 | 0.04 |
| 20:5n-3 (EPA) | -0.24 | <0.0001 | -0.15 | 0.02 |
| 22:6n-3 (DHA) | -0.17 | 0.006 | -0.06 | 0.31 |
| 6:3 Ratios | | | | |
| Total n-6:n-3 | 0.23 | 0.0002 | 0.12 | 0.05 |
| LA:ALA | 0.18 | 0.004 | 0.14 | 0.03 |
| AA:EPA | 0.25 | <0.0001 | 0.16 | 0.01 |
| AA:EPA+DHA | 0.22 | 0.0004 | 0.11 | 0.07 |

NOTE: N=260. Fatty acids are reported as a percentage by weight of the total fatty acids (weight percent, wt. %). PUFA, polyunsaturated fatty acid; LA, linoleic acid; AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; and DHA, docosahexaenoic acid

*Unadjusted Spearman correlation coefficient

†Age- and BMI- adjusted Spearman correlation coefficient

‡18:2n-6+18:3n-6+20:2n-6+20:3n-6+20:4n-6+22:4n-6+22:5n-6

§18:3n-3+20:4n-3+20:5n-3+22:5n-3+22:6n-3

**Table 9. Multivariable-adjusted geometric mean (95% confidence interval) estradiol concentrations (pmol/L) by tertile of erythrocyte fatty acid according to NSAID use**

| Fatty Acids (wt. %) | N | All (N=253) | p for trend | N | NSAID Non-user (N=135) | p for trend | N | NSAID User (N=118) | p for trend |
|---|---|---|---|---|---|---|---|---|---|
| n-6 PUFAs | | | | | | | | | |
| Total n-6 | | | 0.18 | | | 0.02 | | | 0.44 |
| ≤37.32 | 83 | 16.7 (14.4, 19.3) | | 49 | 16.0 (13.4, 18.9) | | 34 | 18.5 (14.5, 23.7) | |
| 37.33-39.49 | 86 | 22.8 (19.8, 26.1) | | 40 | 27.9 (23.2, 33.6) | | 46 | 18.8 (15.4, 23.1) | |
| ≥39.50 | 84 | 19.3 (16.7, 22.3) | | 46 | 21.8 (18.3, 26.1) | | 38 | 16.3 (13.0, 20.5) | |
| 18:2n-6 (LA) | | | 0.71 | | | 0.99 | | | 0.66 |
| ≤14.69 | 83 | 19.3 (16.6, 22.2) | | 40 | 20.1 (16.4, 24.7) | | 43 | 17.8 (14.4, 22.1) | |
| 14.70-16.84 | 86 | 20.8 (18.0, 23.9) | | 47 | 22.5 (18.7, 27.0) | | 39 | 19.3 (15.4, 24.1) | |
| ≥16.85 | 84 | 18.5 (16.0, 21.3) | | 48 | 20.2 (16.8, 24.3) | | 36 | 16.6 (13.2, 20.9) | |
| 20:4n-6 (AA) | | | 0.20 | | | 0.09 | | | 0.95 |
| ≤15.24 | 83 | 18.8(16.3, 21.7) | | 51 | 19.0 (16.0, 22.6) | | 32 | 18.8 (14.7, 24.0) | |
| 15.25-16.57 | 86 | 18.3 (15.9, 21.0) | | 38 | 20.7 (16.8, 25.4) | | 48 | 16.6 (13.6, 20.2) | |
| ≥16.58 | 84 | 21.5 (18.6, 24.9) | | 46 | 23.6 (19.6, 28.5) | | 38 | 18.9 (15.0, 23.8) | |
| n-3 PUFAs | | | | | | | | | |
| Total n-3 | | | 0.17 | | | 0.05 | | | 0.89 |
| ≤6.68 | 85 | 21.3 (18.5, 24.6) | | 46 | 24.3 (20.1, 29.3) | | 39 | 18.7 (14.9, 23.6) | |
| 6.69-8.36 | 83 | 18.7 (16.2, 21.6) | | 45 | 20.4 (17.0, 24.6) | | 38 | 16.7 (13.3, 20.8) | |
| ≥8.37 | 85 | 18.4 (16.0, 21.3) | | 44 | 18.4 (15.2, 22.3) | | 41 | 18.3 (14.6, 22.9) | |

**Table 9 (continued)**

| Fatty Acids (wt. %) | N | All (N=253) | p for trend | N | NSAID Non-user (N=135) | p for trend | N | NSAID User (N=118) | p for trend |
|---|---|---|---|---|---|---|---|---|---|
| 18:3n-3(ALA) | | | 0.09 | | | 0.57 | | | 0.05 |
| ≤0.19 | 84 | 21.2 (18.4, 24.5) | | 42 | 21.5 (17.7, 26.2) | | 42 | 20.8 (16.8, 25.8) | |
| 0.20-0.25 | 86 | 19.5 (17.0, 22.4) | | 43 | 21.5 (17.7, 26.0) | | 43 | 17.5 (14.2, 21.5) | |
| ≥0.26 | 83 | 17.8 (15.4, 20.5) | | 50 | 20.0 (16.8, 23.9) | | 33 | 15.2 (12.0, 19.3) | |
| 20:5n-3 (EPA) | | | 0.39 | | | 0.31 | | | 0.89 |
| ≤0.64 | 83 | 19.4 (16.8, 22.5) | | 48 | 20.9 (17.5, 25.1) | | 35 | 17.6 (13.8, 22.4) | |
| 0.65-0.90 | 85 | 21.4 (18.6, 24.6) | | 43 | 24.3 (20.0, 29.3) | | 42 | 18.9 (15.2, 23.3) | |
| ≥0.91 | 85 | 17.7 (15.4, 20.5) | | 44 | 18.2 (15.1, 21.9) | | 41 | 17.2 (13.8, 21.5) | |
| 22:6n-3 (DHA) | | | 0.35 | | | 0.14 | | | 0.92 |
| ≤3.69 | 84 | 20.8 (18.0, 24.0) | | 48 | 23.1 (19.2, 27.8) | | 36 | 18.3 (14.4, 23.2) | |
| 3.70-4.91 | 84 | 18.9 (16.4, 22.0) | | 40 | 21.1 (17.4, 25.8) | | 44 | 17.0 (13.8, 20.9) | |
| ≥4.92 | 85 | 18.8 (16.3, 21.7) | | 47 | 18.8 (15.7, 22.7) | | 38 | 18.6 (14.7, 23.5) | |
| 6:3 Ratios | | | | | | | | | |
| Total n-6:n-3 | | | 0.21 | | | 0.06 | | | 0.98 |
| ≤4.48 | 84 | 17.7 (15.3, 20.3) | | 46 | 17.6 (14.6, 21.2) | | 38 | 17.7 (14.0, 22.4) | |
| 4.49-5.72 | 84 | 20.5 (17.8, 23.6) | | 43 | 22.9 (19.0, 27.7) | | 41 | 18.2 (14.6, 22.5) | |
| ≥5.73 | 85 | 20.3 (17.5, 23.4) | | 46 | 22.9 (19.0, 27.8) | | 39 | 17.8 (14.2, 22.3) | |

**Table 9 (continued)**

| Fatty acids (wt. %) | N | All (N=253) | p for trend | N | NSAID Non-user (N=135) | p for trend | N | NSAID User (N=118) | p for trend |
|---|---|---|---|---|---|---|---|---|---|
| LA:ALA | | | 0.03 | | | 0.51 | | | 0.01 |
| ≤64.00 | 85 | 17.6 (15.3, 20.3) | | 51 | 20.4 (17.0, 24.3) | | 34 | 14.2 (11.3, 17.9) | |
| 64.01-78.05 | 85 | 19.0 (16.5, 21.8) | | 46 | 20.5 (17.0, 24.7) | | 39 | 18.1 (14.6, 22.5) | |
| ≥78.06 | 83 | 22.1 (19.2, 25.5) | | 38 | 22.4 (18.2, 27.4) | | 45 | 21.1 (17.3, 25.8) | |
| AA:EPA | | | 0.11 | | | 0.10 | | | 0.59 |
| ≤16.92 | 84 | 17.3 (15.0, 20.0) | | 47 | 17.6 (14.7, 21.1) | | 37 | 17.1 (13.5,21.8) | |
| 16.93-25.89 | 84 | 20.7 (18.0, 23.9) | | 41 | 24.5 (20.1, 29.7) | | 43 | 17.7 (14.4,21.8) | |
| ≥25.90 | 85 | 20.5 (17.7, 23.7) | | 47 | 21.7 (18.1, 26.1) | | 38 | 18.9 (14.9,23.9) | |
| AA:EPA+DHA | | | 0.18 | | | 0.01 | | | 0.63 |
| ≤2.68 | 84 | 18.6 (16.1, 21.5) | | 50 | 17.7 (14.8, 21.1) | | 34 | 19.9 (15.6, 25.5) | |
| 2.69-3.77 | 84 | 18.5 (16.1, 21.3) | | 41 | 21.5 (17.8, 26.0) | | 43 | 16.2 (13.2, 20.0) | |
| ≥3.78 | 85 | 21.4 (18.6, 24.7) | | 44 | 24.9 (20.6, 30.0) | | 41 | 18.1 (14.5, 22.7) | |

NOTE: Fatty acids are expressed as a percentage by weight of the total fatty acids (weight, percent, wt.%). Tertile cutpoints were determined from entire study population (n=260). NSAID, nonsteroidal anti-inflammatory drug; PUFA, polyunsaturated fatty acid; LA, linoleic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; and DHA, docosahexaenoic acid. Values were adjusted for age (continuous), BMI (continuous), years menopausal (continuous), alcohol intake (none, <12g/day, ≥12g/day; indicator variable), and current smoker (nonsmoker vs. smoker). The unstratified analysis was also adjusted for current NSAID use (yes vs. no). 7 participants were excluded because years since menopause were undeterminable. p for interaction (fatty acid tertile x NSAID use) was significant for total n-6 ($p<0.02$) and suggestive for AA:EPA+DHA ($p<0.12$). Linear trend tests were performed by treating the fatty acid tertile groups as continuous variables

# 4.0 ARTICLE TWO: NONSTEROIDAL ANTI-INFLAMMATORY DRUG USE AND SERUM TOTAL ESTRADIOL IN POSTMENOPAUSAL WOMEN

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## 4.1 ABSTRACT

Laboratory and epidemiologic evidence suggest that nonsteroidal anti-inflammatory drug (NSAID) use may be inversely related to the risk of breast cancer; however, the mechanism by which NSAIDs may protect against the development of this disease is uncertain. The objective of this observational study was to assess the relationship between current NSAID use and endogenous estradiol levels, an established breast cancer risk factor. To evaluate this aim, we conducted a cross-sectional investigation among 260 postmenopausal women who were not recently exposed to exogenous hormones. Information on current NSAID use (aspirin, COX-2 inhibitors and other NSAIDs combined) was collected using a questionnaire at the time of blood draw. Estradiol was quantified in serum by radioimmunoassay. General linear models were used to evaluate the association between NSAID use and serum total estradiol. The age- and BMI-adjusted geometric mean serum estradiol concentration among NSAID users (N=124) was significantly lower than non-users of NSAIDs (N=136) (17.8 pmol/L vs. 21.3 pmol/L; p=0.03). Further adjustment for additional potential confounding factors did not substantially alter estimates (17.7 pmol/L vs. 21.2 pmol/L; p=0.03). To our knowledge, this report is the first to examine the relationship between NSAID use and serum estradiol in postmenopausal women. These cross-sectional findings suggest that NSAID use may be associated with lower circulating estradiol levels, potentially representing one mechanism through which NSAIDs exert protective effects on breast cancer.

## 4.2 INTRODUCTION

Although breast cancer is a major public health problem, little is known about preventing this disease. Experimental studies have reported a protective effect of nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin and ibuprofen, against mammary carcinogenesis [308-310] and accumulating evidence from both case-control and cohort studies suggests that use of NSAIDs may be associated with a modest decreased risk of breast cancer in women [193, 194, 196, 311-314]. However, findings are mixed [315-321]. Clarifying the association between NSAID use and the development of breast cancer is potentially of great importance clinically. NSAIDs are widely used, readily available and inexpensive agents. If they were shown to be chemopreventive, they could have a substantial impact on public health.

Although the mechanisms by which NSAIDs may protect against breast cancer are not fully understood, data suggest that the protective effect may be attributed, in part, to NSAIDs' ability to decrease the formation of prostaglandin E2 (PGE2) by blocking cyclooxygenase (COX)-1 and/or COX-2 activity. One possible mechanism by which the COX/PGE2 cascade promotes breast cancer is via increasing estrogen production, as exposure to endogenous estrogens has been shown to play a causal role in the development of some breast cancers [41].

PGE2 upregulates aromatase activity [200], the enzyme that converts androgens to estrogens, and leads to increased estrogen synthesis. In postmenopausal women, aromatatic conversion of androgens is the primary source of circulating estrogens, and suppression of this enzyme has been shown to have a profound effect on both circulating estrogen levels [322] and breast cancer recurrence [213]. Recently, dose dependent

decreases in aromatase activity were observed in breast cancer cells following treatment with NSAIDs, a COX-1 selective inhibitor, and COX-2 selective inhibitors [201]. Therefore, NSAIDs may offer protection against breast cancer by reducing a woman's exposure to estrogen via the inhibition of aromatase activity. Indeed, laboratory results have shown that estradiol production is decreased in breast cells that are exposed to the selective COX-2 inhibitor Celecoxib [202].

While the above-mentioned pathway through which NSAIDs may decrease the development of breast cancer has previously been highlighted [323, 324], the association between NSAID use and circulating estradiol in women is currently unknown. Therefore, in this cross-sectional investigation, we asked whether differences in serum estradiol levels could be observed between self-reported NSAID users and nonusers in a population of postmenopausal women not taking hormone therapy (HT).

## 4.3 MATERIALS AND METHODS

### 4.3.1 Study Population

We used data from controls drawn from the Mammograms and Masses Study (MAMS), a case-control study of estrogen metabolites, mammographic breast density and breast cancer risk. Details of the study methodologies have been presented elsewhere [296]. In brief, 869 cancer-free women and 264 recently diagnosed breast cancer cases were recruited into the MAMS through the Magee Womens Hospital Mammographic Screening and Diagnostic Imaging Program in the greater Pittsburgh area (Pennsylvania,

USA) between September 2001 and May 2005. Women who were 18 years or older, who reported no previous personal history of cancer, with the exception of nonmelanoma skin cancer, and who could provide written informed consent were eligible for study enrollment. Participants in the MAMS include; 1) breast cancer cases who were recruited from the Magee-Womens Surgical Clinic for an initial evaluation after newly diagnosed primary breast cancer (n=264); 2) controls who were undergoing outpatient needle breast biopsy through the Breast Biopsy Service at Magee-Womens Hospital (Pittsburgh, PA), but who were not subsequently diagnosed with breast cancer (n=313); 3) "healthy" controls who received screening mammography through Magee-Womens Hospital or through Pittsburgh Magee Womancare Centers (n=538) and; 4) an additional 18 participants whose blood was dedicated solely to an ancillary study of intra-individual cytokine and hormone level reproducibility. To increase recruitment of the "healthy" control group, study flyers were attached to screened negative mammogram reports mailed to patients between November 2003 and April 2005. The MAMS is approved by the University of Pittsburgh's Institutional Review Board and all participants provided written informed consent at the time of study entry.

### 4.3.2 Subsample Selection

Participants were selected for the present study if they met the following eligibility criteria: 1) healthy controls recruited only via study flyers through Magee-Womens Hospital or through Pittsburgh Magee Womancare Centers between 2003-2005 (n=453), because these participants completed a self-administered questionnaire on the day of blood draw; 2) postmenopausal, defined as having no menstrual bleeding during the year

prior to enrollment, having undergone a bilateral oophorectomy, or having a hysterectomy without bilateral oophorectomy and aged 50 years or older. We measured follicle-stimulating hormone (FSH) for women under 55 years of age at blood draw who had a hysterectomy without bilateral oophorectomy (n=5); all five participants had FSH levels above 40mIU/ml (range: 49.1-185.2), consistent with FSH elevation in the postmenopausal range; 3) not using HT within three months of enrollment; and 4) did not report using vaginal estrogen creams, oral contraceptives, selective estrogen receptor modulators (SERMs) or corticosteroids on the day of blood draw. Ninety-eight premenopausal women, 55 postmenopausal women using exogenous hormones, 24 women using SERMs, 5 participants on corticosteroids, and 1 participant later found to have a personal history of breast cancer were excluded from the study. Two hundred and seventy participants met the above-mentioned criteria.

### 4.3.3 Covariate Information

A standardized, self-administered questionnaire was used to gather exposure information. Participants in the subsample completed the questionnaire at study enrollment on the day of blood draw. Information collected included demographic data, current use of medication and supplements, reproductive history, family medical history, past exogenous hormone use, and lifestyle factors such as smoking status and alcohol intake. Alcohol use (grams/day) in the past year was calculated as previously reported [298]. Age of onset of menopause was defined according to the methods formerly described by the Women's Health Initiative [299], where age at menopause corresponded to the age of a woman's last natural menstrual bleeding, bilateral oophorectomy, or age a woman

began using HT. For a hysterectomized woman without a bilateral oophorectomy, age at menopause was the earliest age at which she began using HT or first had menopausal symptoms. If neither occurred and her age at hysterectomy was 50 years or older, then age at menopause was her age at hysterectomy. Age at menopause was undeterminable in 7 participants. Years since menopause were calculated by subtracting the age at menopause from the age at enrollment.

### 4.3.4 Assessment of NSAID Use

The primary exposure variable "current NSAID use" was collected on the day of blood draw. On the self-administered questionnaire, participants were asked to report all prescribed and over-the-counter medications that were currently being used. The question asked, "Are you CURRENTLY taking any medications (prescription or over the counter, including aspirin and ibuprofen)?" If a participant responded affirmatively, she was prompted to "please list them in this table." Dosage data were collected, but not analyzed as many participants knew only the number of tablets taken rather than the actual dose. The questionnaire was reviewed for completeness by a trained research nurse (study coordinator), who queried participants if further clarification was needed. Each medication reported in the table was subsequently assigned a code using a therapeutic classification system as indexed in the Nurse Practitioners' Prescribing Reference, which is updated quarterly [325]. Participants who listed aspirin, COX-2 inhibitor, or other non-aspirin NSAID use on the questionnaire were considered "current NSAID users." Participants who did not list using a NSAID were considered "current NSAID non-users." Because acetaminophen is generally reported to be a poor inhibitor of the COX -

1/COX-2 enzymes [297] and its mechanism of action has yet to be resolved, we classified acetaminophen users as non-users of NSAIDs (n=12) unless they also reported taking a NSAID (n=6).

Two additional NSAID exposure variables were considered in relation to estradiol levels, a secondary exposure variable and a NSAID variable constructed from the primary and secondary variables. The secondary NSAID exposure variable was from the participant's yes-or-no response to the study phlebotomist's question at blood draw, "Have you taken any aspirin or anti-inflammatory agents in the last 48 hours?" No effort was undertaken to determine the specific agent the participant had used. Therefore, this variable is more subjective in that responses were based solely upon each individual's perception of what constitutes an anti-inflammatory agent and aspirin. The secondary exposure variable was used in conjunction with the primary NSAID exposure variable to construct a third variable labeled "consistent NSAID use." "Consistent NSAID users" listed on the questionnaire that they were currently taking a medication that was an aspirin, COX-2 inhibitor, or non-aspirin NSAID and also verbally reported that they took an aspirin or other anti-inflammatory agent in the past 48 hours. "Consistent NSAID non-users" did not list using any NSAID nor did they state having taken an aspirin or anti-inflammatory agent in the past 48 hours. This latter variable was created as an attempt to reduce potential NSAID use/non-use misclassification. None of the participants in this analysis were missing any of the NSAID exposure variable data. Exposure data were collected and coded without knowledge of estradiol levels.

### 4.3.5 Clinical Measures

The study coordinator obtained physical measurements (height and weight) and recorded information on a standardized form. After the participant removed her shoes and heavy clothing, weight was measured at a standing position to the nearest 0.1 kg using a standard balance beam; standing height was measured at full inspiration to the nearest 0.1 cm. All anthropometric measurements were taken twice and were repeated if the first two measurements differed by more than 0.5 cm or 0.5 kg. The mean of the measurements was used in the analysis. Body mass index (BMI) was calculated as weight (kg) divided by the square of height in meters ($m^2$).

Forty milliliters (mL) of peripheral non-fasting blood was collected from the participants at study enrollment. All samples were processed on site at the Magee Womens Hospital Satellite General Clinical Research Center according to standard protocols. After processing, the samples were aliquotted into 1 mL cryovials in which red blood cells, serum, plasma and buffy coat were separated. Samples were stored at or below -70°C prior to laboratory analyses.

### 4.3.6 Laboratory Analyses

Serum samples were used for the quantification of total estradiol (Sex hormone binding globulin and albumin-bound plus unbound estradiol) and were assayed at the Royal Marsden Hospital in England. Estradiol concentrations were measured by radioimmunoassay after ether extraction, using a highly specific rabbit antiserum raised against an estradiol-6-carboxymethyloxime-bovine serum albumin conjugate (EIR,

Wurenlingen, Switzerland) and Third Generation Estradiol [I125] reagent DSL 39120 (Diagnostic Systems Laboratories Inc., Texas USA) [302]. The assay detection limit was 3pmol/L by calculation from the 95% confidence limits of the zero standard. A random subset of 27 replicate quality control samples was included to assess reproducibility; the calculated coefficient of variation between duplicates for estradiol was 14.5%. Laboratory personnel were masked to both subject identification and quality control status.

### 4.3.7 Statistical Analyses

Wilcoxon's rank sum test was used to compare selected continuous characteristics between current users and non-users of NSAIDs and the chi-square test or the Fisher's exact test were used to assess differences in categorical variables. The Kruskal-Wallis test was used to test for significant differences in continuous characteristics across estradiol tertile categories. A log transformation was applied to serum estradiol concentrations to obtain homoscedacity and an approximately normal distribution for linear model residuals. One participant was excluded from analyses because her total estradiol level was deemed unreliable by the laboratory. An additional 9 participants with estradiol levels greater than 150 pmol/L, were removed from analyses because such high levels likely indicated the women were not postmenopausal or did not correctly report current hormone use. All analyses were replicated with the 9 data points included; however, findings did not change appreciably and therefore the 9 participants were not included in the reported results (**Appendix B**). Thus, the final sample included 260 women.

Cohen's kappa statistic was calculated as a measure of agreement between the primary and secondary NSAID exposure variables. Differences in mean log estradiol levels between users and nonusers of NSAIDs were tested by Student's t-test. The general linear model (GLM) approach was performed to calculate multivariable-adjusted estradiol levels and to assess differences in levels between NSAID users and non-users. Adjusted means and confidence intervals for each NSAID category were quantified using the least squares mean option of PROC GLM. Two adjusted models are presented. The first model was adjusted for age and BMI, which were deemed necessary covariates given their previously reported associations with both NSAID use [326] and estradiol levels [327, 328]. The second model was further adjusted for variables found to be associated with NSAID use or estradiol levels within the study population (univariate association $p<0.15$). The final multivariable model was adjusted for age (continuous), BMI (continuous), years since menopause (continuous), race (white vs. non-white), and regular alcohol intake in the past year (none, <12g/day, ≥ 12g/day, entered as an indicator variable). The geometric mean concentrations were calculated by taking the anti-log of the least squares means after adjustment. Additional adjustment for family history of breast cancer, past HT use, smoking status, and various reproductive factors yielded similar results, and are not presented.

For each model, a plot of the studentized residuals versus the predicted values was examined to check whether the equality of variance assumption was met. A normal probability plot of the residuals was examined to assess normality. Assumptions of normality and homogeneity of variance were met for all models presented. Tests of statistical significance were two-tailed and, given the exploratory nature of this work, we

reported our results at the $p<0.05$ significance level, rather than correct for multiple comparisons. All analyses were performed using SAS software version 9.1 (SAS Institute, Inc., Cary, North Carolina).

## 4.4 RESULTS

Characteristics of the study population by current NSAID use are shown in **Table 10**. The majority of participants (66.9%) were overweight or obese (BMI $\geq 25 kg/m^2$), white (93.1%), and non-smokers (94.2%). Overall, 124 (47.7%) participants reported current NSAID use at the time of blood draw (**Table 11**). In this study, 25.0%, 12.3%, and 2.3% participants reported using only aspirin, non-aspirin NSAIDs and COX-2 selective inhibitors, respectively, whereas 8.1% reported using at least two different types of NSAIDs (data not shown). One hundred forty (53.8%) women reported that they took aspirin or another anti-inflammatory agent within 48 hours of blood draw. One hundred (38.5%) participants listed current use of a NSAID on the baseline questionnaire and verbally reported aspirin or anti-inflammatory use within 48 hour of blood draw, and 96 (37.0%) reported no use of NSAIDs in both settings. The agreement between the primary and secondary exposure variables was moderate with a kappa value of 0.51.

With the exception of race, NSAID users and nonusers were statistically similar with regard to all other demographic characteristics (**Table 10**). Current users of NSAIDs were more likely to be white than non-users (96.8% vs. 89.7%; $p=0.03$). Demographic differences between users and nonusers for all NSAID exposure variables (primary, secondary and constructed) were similar, with the exception of BMI. Participants who

reported aspirin or anti-inflammatory drug use within the past 48 hours and those who were consistent users were heavier than participants who reported no use of NSAIDs (**Appendix B**).

The geometric mean serum estradiol concentration for the study population was 19.5 pmol/L, with levels ranging from 3.3-140.0 pmol/L. As illustrated in **Table 12**, higher serum estradiol levels were associated with increasing BMI ($p<0.0001$) and negatively associated with alcohol intake ($p=0.003$). Although not statistically significant, it was observed that women with higher circulating estradiol levels were on average fewer years from menopause ($p=0.11$). With the exception of alcohol intake, all associations persisted after controlling for BMI (data not shown). The association between alcohol intake and estradiol diminished after controlling for BMI.

After adjustment for age and BMI, current NSAID use was significantly inversely associated with serum estradiol concentrations (17.8 pmol/L vs. 21.3 pmol/L; $p=0.03$) (**Table 13)**, with approximately 16.4% lower levels in users than nonusers of NSAIDs. The age-and BMI-adjusted association between use of the secondary NSAID exposure variable (aspirin or anti-inflammatory agent in the past 48 hours) and estradiol was suggestive of an inverse effect, but this finding was not statistically significant (18.5 pmol/L vs. 20.9 pmol/L; $p=0.14$). A slightly stronger association between NSAID use and estradiol levels was observed when comparing consistent users to consistent nonusers (17.5 pmol/L vs. 21.5 pmol/L; $p=0.03$). Further adjustment for race, alcohol intake and years menopausal only slightly increased the strength of association observed in the age- and BMI- adjusted analyses. The effects were similar across BMI subgroups (**Appendix B)**.

**Figure 5** presents the adjusted geometric mean serum estradiol concentration by sub-category of NSAID use as defined by the cross-tabulation of the primary and secondary NSAID exposure variables. Three categories were defined, the two concordant groups (i.e. No NSAIDs on medication list/ No NSAIDs verbally; Yes NSAIDs on medication list/ Yes NSAIDs verbally) remained as separate exposure categories, whereas the two discordant groups (i.e. No NSAIDs on medication list/ Yes NSAIDs verbally; Yes NSAIDs on medication list/ No NSAIDs verbally) were collapsed into a single category. The three groups had significantly different adjusted geometric mean estradiol levels (p trend = 0.02). As was expected, mean estradiol was lowest for participants who reported NSAID use for both measures, and highest for participants who did not report use for either measure.

To assess the possible effects of acetaminophen use on the findings, all analyses were repeated excluding acetaminophen users from the NSAID non-user groups (n=12). Results did not differ substantially (**Appendix B**).

## 4.5 DISCUSSION

In this cross-sectional investigation we observed lower circulating estradiol levels among postmenopausal women reporting NSAID use. Specifically, we observed approximately 16% lower estradiol levels among current users than non-users. Decreased estradiol levels were consistent regardless of how NSAID use was assessed (i.e. self-reported current NSAID use on questionnaire, verbal reporting of use in past 48 hours, and the agreement between these two variables). Further, the strength of association was slightly

stronger when comparing participants who reported NSAID use at both the time of blood draw and within 48 hours of blood draw to those who reported no use of NSAIDs for both measures. Associations were independent of age, BMI, and other potential confounding variables. As elevated serum estradiol levels have been linked to breast cancer risk, these results provide support to the growing body of evidence linking NSAID use to decreased breast cancer incidence.

Although findings in the literature are not completely consistent, results of several epidemiologic studies suggest that use of aspirin, non-aspirin NSAIDs and COX-2 inhibitors may reduce the risk of breast cancer (reviewed in [329]). The inconsistent findings among studies may be explained, in part, by differences in the definition of NSAID use, dosage and frequency data, and NSAID assessment periods. Notably, some studies suggest the decreased risk is stronger among estrogen receptor positive (ER+) breast cancers [192, 330] and, if true, would strengthen the hypothesis of an estrogen modulatory effect by NSAIDs. However, this relationship is not consistently observed [314, 331].

The mechanisms underlying the protective effects of these anti-inflammatory agents have been extensively investigated in the laboratory environment but have been less commonly explored in an epidemiological setting. Establishing the relationship between NSAID use and the various biochemical markers (i.e. steroid hormones, growth factors, and cytokines) involved in the pathogenesis of breast cancer in humans is essential in order to determine the effects these agents have on the development of breast cancer. The reduced risk of breast cancer observed among NSAID users in prior studies may, in part, be mediated through NSAIDs favorable effects on PGE2 production.

Decreased PGE2 synthesis may result in suppressed estradiol production in postmenopausal women and subsequently reduced breast cancer risk. In accordance with this biologic paradigm, we observed that postmenopausal participants reporting NSAID use had lower estradiol levels. We know of no other epidemiological study that has reported this relationship. As NSAID use is modifiable, a chemoprotective action attributed to its use could have a considerable public health impact. However, the risk-to-benefit ratio would need to be considered since NSAIDs have potentially serious side effects [332, 333].

The present study has limitations that deserve attention and that should be considered when evaluating the study findings. First, as this is a cross-sectional investigation, we cannot ascertain the temporal relationship between NSAID use and serum estradiol, meaning that causal conclusions cannot be made. Multiple measurements of NSAID use and serum estradiol would have resulted in more precise estimates. Additional limitations of this study include our inability to assess duration of NSAID use or dosage information, as duration of NSAID use was not collected and dosage data were deemed unreliable as many participants listed number of tablets taken rather than the actual dose. Women exposed to a longer duration of NSAID use or larger doses may have more pronounced effects on circulating estradiol levels than occasional NSAID users (i.e. as-needed) or those consuming smaller doses (i.e. low-dose aspirin). The sample size was not large enough to assess the effect of the different types of NSAIDs (e.g. aspirin, non-aspirin NSAIDs, and selective COX-2 inhibitors) on serum estradiol. Further, we cannot rule out exposure misclassification. The result of nondifferential misclassification of our exposure variable (NSAID use vs. NSAID non-use) would most likely bias the findings

toward the null hypothesis and possibly underestimate the true association between NSAID use and serum estradiol. We attempted to reduce misclassification by repeating analyses limiting the sample to women who consistently reported NSAID use or nonuse. Further, although we attempted to control for potential confounders in the statistical analyses, we cannot rule out the possibility that women who are users of NSAIDs had a factor in common that we did not measure that is related to lower serum estradiol levels. Finally, the lack of ethnic diversity and exclusion of premenopausal women in our sample limits the generalizability of the results.

Strengths of our study include the use of standardized instruments, reproducible measures of total estradiol, and the assessment of NSAID use on the same day as blood draw. The last strength is important, because the effect of NSAIDs on the inhibition of COX enzymes and PGE2 formation occurs rapidly [334]. Finally, the observed distribution of postmenopausal total estradiol levels and the self-reported prevalence of NSAID use in this population were similar to previous reports [328, 335]. Thus, study findings may be generalizable to similar populations. In our study, 33% MAMS participants (aged 42-85) reported aspirin use and 19% other NSAID use at study enrollment. The third National Health and Nutrition Survey (NHANES III) described a similar prevalence of aspirin and NSAID use [335]. In this population, women aged 45-64 reported 36% and 25% monthly use of aspirin and other NSAIDs, respectively and women aged 65-74 reported 42% aspirin and 16% other NSAID use.

In summary, we believe that we are the first to report on the association between NSAID use and postmenopausal estradiol levels. We found NSAID users to have significantly lower serum estradiol concentrations than non-users which may account for

the protective effect NSAID use has been observed to exhibit on breast cancer development. However, continued research efforts are needed to verify our findings.

**Table 10. Distribution of selected characteristics by NSAID use among postmenopausal women in the Mammograms and Masses Study (MAMS)**

| Characteristic | NSAID user (N=124) | NSAID non-user (N=136) | p |
|---|---|---|---|
| Age at blood draw (years), mean (SD) | 62.6 (8.1) | 62.9 (8.7) | 0.91 |
| BMI (kg/m2), mean (SD) | 28.6 (6.0) | 28.3 (6.1) | 0.63 |
| Age at menopause (years), mean (SD)* | 48.7 (4.4) | 48.7 (5.7) | 0.57 |
| Years menopausal, mean (SD)* | 14.1 (9.8) | 14.2 (10.3) | 0.94 |
| Surgical menopause, %* | | | 0.38 |
| No | 90.2 | 86.8 | |
| Yes | 9.8 | 13.2 | |
| Age at menarche, %* | | | 0.88 |
| <12 years | 19.4 | 17.8 | |
| 12-13 years | 57.3 | 56.3 | |
| ≥14 years | 23.4 | 25.9 | |
| Race, % | | | 0.03 |
| White | 96.8 | 89.7 | |
| Non-white | 3.2 | 10.3 | |
| Family history of breast cancer, %*† | | | 0.98 |
| No | 86.9 | 86.8 | |
| Yes | 13.1 | 13.2 | |
| Prior hormone therapy use, % | | | 0.86 |
| No | 37.9 | 39.0 | |
| Yes | 62.1 | 61.0 | |
| Previous breast biopsy, % | | | 0.83 |
| No | 85.5 | 84.6 | |
| Yes | 14.5 | 15.4 | |

**Table 10 (continued)**

| Characteristic | NSAID user (N=124) | NSAID non-user (N=136) | p |
|---|---|---|---|
| Regular alcohol intake in past year, % | | | 0.10 |
| None | 66.1 | 77.9 | |
| < 12 g/day | 21.8 | 14.0 | |
| ≥ 12 g/day | 12.1 | 8.1 | |
| Smoking status, % | | | 0.88 |
| Never | 59.7 | 61.8 | |
| Former | 33.9 | 33.1 | |
| Current | 6.5 | 5.2 | |
| Parous, % | | | 0.50 |
| No | 21.0 | 17.7 | |
| Yes | 79.0 | 82.4 | |
| Age at first full-term pregnancy, % ‡ | | | 0.94 |
| < 30 years | 81.6 | 81.3 | |
| ≥ 30 years | 18.4 | 18.8 | |
| Ever breast fed for > 1 month, % ‡ | | | 0.52 |
| No | 50.0 | 54.5 | |
| Yes | 50.0 | 45.5 | |

NOTE: Percentages may not add up to 100% due to rounding. NSAID, nonsteroidal anti-inflammatory drug; BMI, body mass index

*missing n=7 for age at menopause; n=7 for years menopausal; n=1 for surgical menopause; n =1 for age at menarche; n=2 for family history of breast cancer

† family history of breast cancer in mother or sister

‡ among parous women

**Table 11. Self-reported NSAID use in the Mammograms and Masses Study (MAMS)**

| NSAID use | N (%) |
|---|---|
| Primary exposure variable | |
| Current use* | |
| Non-user | 136 (52.3) |
| User | 124 (47.7) |
| Secondary exposure variable | |
| Past 48 hour use† | |
| Non-user | 120 (46.2) |
| User | 140 (53.8) |
| Constructed exposure variable | |
| Consistent use‡ | |
| Non-user | 96 (36.9) |
| User | 100 (38.5) |

NOTE: NSAID, nonsteroidal anti-inflammatory drug

*Current use: Based on participant's self-reported current medication list

†Past 48 hour use: Based on participant's verbal response to the question "Have you taken an aspirin or other anti-inflammatory drug in the past 48 hours?"

‡Consistent use: The agreement between current NSAID use and past 48 hour use. Non-user=Participant's current medication list did not indicate use of a NSAID and the participant verbally responded that she did not consume an aspirin or anti-inflammatory agent within 48 hours of blood draw. User= Participant's current medication list indicated use of a NSAID, and the participant verbally responded that she consumed an aspirin or anti-inflammatory agent within 48 hours of blood draw.

**Table 12. Distribution of selected characteristics by tertile of serum estradiol levels among postmenopausal women in the Mammograms and Masses Study (MAMS)**

| | Estradiol concentrations | | | |
|---|---|---|---|---|
| Characteristic | Tertile 1 (n=91) | Tertile 2 (n=81) | Tertile 3 (n=88) | p |
| Age at blood draw (years), mean (SD) | 63.8 (8.5) | 62.6 (8.5) | 61.9 (8.2) | 0.38 |
| BMI (kg/m2), mean (SD) | 25.4 (4.41) | 27.3 (4.9) | 32.6 (6.2) | <0.0001 |
| Age at menopause (years), mean (SD)* | 48.3 (4.53) | 49.0 (5.0) | 48.8 (5.7) | 0.27 |
| Years menopausal, mean (SD)* | 15.6 (9.74) | 13.5 (10.2) | 13.1 (10.1) | 0.11 |
| Surgical menopause, %* | | | | 0.64 |
| No | 87.8 | 86.4 | 90.9 | |
| Yes | 12.2 | 13.6 | 9.1 | |
| Age at menarche, %* | | | | 0.49 |
| <12 years | 14.3 | 17.3 | 24.1 | |
| 12-13 years | 60.4 | 59.3 | 50.6 | |
| ≥14 years | 25.3 | 23.5 | 25.3 | |
| Race, % | | | | 0.46 |
| White | 95.6 | 92.6 | 90.9 | |
| Other | 4.4 | 7.4 | 9.1 | |
| Family history of breast cancer, %*† | | | | 0.95 |
| No | 86.8 | 87.7 | 86.1 | |
| Yes | 13.2 | 12.4 | 14.0 | |
| Prior hormone therapy use, % | | | | 0.30 |
| No | 33.0 | 38.3 | 44.3 | |
| Yes | 67.0 | 61.7 | 55.7 | |
| Previous breast biopsy, % | | | | 0.48 |
| No | 82.4 | 84.0 | 88.6 | |
| Yes | 17.6 | 16.1 | 11.4 | |

**Table 12 (continued)**

| Characteristic | Estradiol concentrations Tertile (n=91) | Tertile 2 (n=81) | Tertile (n=88) | p |
|---|---|---|---|---|
| Regular alcohol intake in past year, % | | | | 0.003 |
| None | 60.4 | 72.8 | 84.1 | |
| < 12 g/day | 28.6 | 13.6 | 10.2 | |
| ≥ 12 g/day | 11.0 | 13.6 | 5.7 | |
| Smoking status | | | | 0.46 |
| Never | 58.2 | 56.8 | 67.1 | |
| Former | 37.4 | 37.0 | 26.1 | |
| Current | 4.4 | 6.2 | 6.8 | |
| Parous, % | | | | 0.47 |
| No | 23.1 | 18.5 | 15.9 | |
| Yes | 76.9 | 81.5 | 84.1 | |
| Age at first full-term pregnancy, % ‡ | | | | 0.93 |
| < 30 years | 80.0 | 81.8 | 82.4 | |
| ≥ 30 years | 20.0 | 18.2 | 17.6 | |
| Ever breast fed for > 1month, % ‡ | | | | 0.75 |
| No | 54.3 | 48.5 | 54.1 | |
| Yes | 45.7 | 51.5 | 46.0 | |

NOTE: Percentages may not add up to 100% due to rounding. BMI, body mass index

*missing n=7 for age at menopause; n=7 for years menopausal; n=1 for surgical menopause; n =1 for age at menarche; n=2 for family history of breast cancer

† family history of breast cancer in mother or sister

‡ among parous women

**Table 13. Unadjusted and adjusted geometric mean estradiol levels (95% confidence interval) according to NSAID use**

| | Serum estradiol concentrations (pmol/L) | | | | | |
|---|---|---|---|---|---|---|
| All Participants | Model 1* | p | Model 2† | p | Model 3‡ | p |
| Primary exposure variable | | | | | | |
| Current use | | 0.11 | | 0.03 | | 0.03 |
| NSAID Non-user (N=136) | 21.0 (18.4, 24.0) | | 21.3 (19.0, 23.7) | | 21.2 (18.9, 23.7) | |
| NSAID User (N=124) | 18.0 (15.7, 20.7) | | 17.8 (15.9, 20.0) | | 17.7 (15.7, 19.9) | |
| Secondary exposure variable | | | | | | |
| Past 48 hour use | | 0.94 | | 0.14 | | 0.07 |
| NSAID Non-user (N=120) | 19.5 (16.9, 22.4) | | 20.9 (18.5, 23.5) | | 21.1 (18.7, 23.8) | |
| NSAID User (N=140) | 19.6 (17.2, 22.3) | | 18.5 (16.5, 20.6) | | 18.1 (16.2, 20.3) | |
| Constructed exposure variable | | | | | | |
| Consistent use | | 0.39 | | 0.03 | | 0.02 |
| NSAID Non-user (N=96) | 20.3 (17.3, 23.8) | | 21.5 (18.9, 24.4) | | 21.4 (18.8, 24.4) | |
| NSAID User (N=100) | 18.4 (15.8, 21.5) | | 17.5 (15.4, 19.8) | | 17.2 (15.1, 19.6) | |

NOTE: NSAID, nonsteroidal anti-inflammatory drug; BMI, body mass index. Current use: Based on participant's medication list. Past 48 hour use: Participant's verbal response to the question, "Have you taken an aspirin or other anti-inflammatory drug in the past 48 hours?" Consistent NSAID use: The agreement between current NSAID and past 48 hours use.

*Unadjusted model

†Adjusted for age at blood draw (continuous) and BMI (continuous)

‡Missing N=7; Adjusted for age at blood draw (continuous), BMI (continuous), race (white, nonwhite), years menopausal (continuous), and current alcohol intake (none, <12 g, ≥12 g, indicator variable)

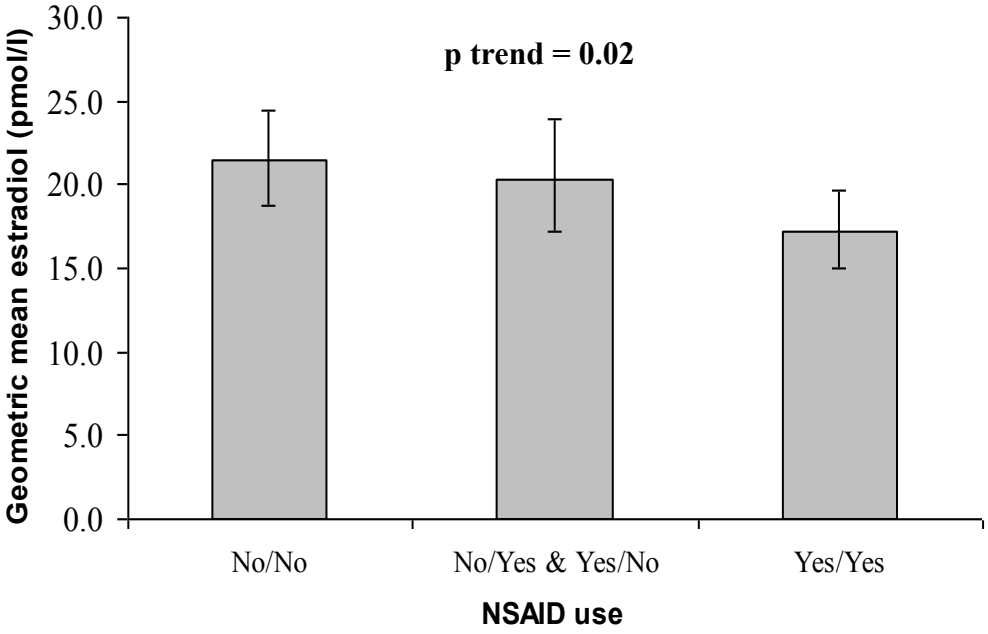


**Figure 5. Adjusted geometrc mean estradol according to self-reported NSAID use**

Serum total estradiol was adjusted for age at blood draw, BMI, race, years menopausal, and current alcohol intake in a general linear model (n=7 missing data). **No/No**=Participant's current medication list did not indicate use of a NSAID and the participant verbally responded that she did not take aspirin or an anti-inflammatory agent within 48 hours of blood draw (n=96). **No/Yes**=Participant's current medication list did not indicate use of a NSAID, but participant verbally responded that she took aspirin or an anti-inflammatory agent within 48 hours of blood draw (n=40). **Yes/No**=Participant's current medication list indicated use of a NSAID, but the participant verbally responded that she did not take aspirin or an anti-inflammatory agent within 48 hours of blood draw (n=24). **Yes/Yes**=Participant's current medication list indicated use of a NSAID, and the participant verbally responded that she did not take aspirin or an anti-inflammatory agent within 48 hours of blood draw (n=100).

# 5.0 ARTICLE THREE: ERYTHROCYTE OMEGA-6 AND OMEGA-3 FATTY ACIDS AND MAMMOGRAPHIC BREAST DENSITY

To be submitted for publication

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## 5.1 ABSTRACT

Diets low in omega-6 (n-6) fatty acids and/or rich in omega-3 (n-3) fatty acids may protect against breast cancer development. Mammographic breast density is one of the strongest risk factors for breast cancer, and may be affected by dietary intake. Therefore, the aim of this study was to examine the association between the essential n-6 and n-3 fatty acids and mammographic breast features, specifically percent breast density, dense breast area, and nondense area of the breast. Data were included from 248 breast cancer free, postmenopausal women who were not using hormone therapy. Mammographic breast density, dense area of the breast and nondense areas of the breast were assessed by planimetry. Fatty acids in erythrocytes were measured by gas-liquid chromatography. Spearman's rank order correlation coefficients were used to evaluate the relationships between fatty acid measures and mammographic characteristics. Erythrocyte n-6 fatty acids, n-3 fatty acids, and the n-6:n-3 ratios were not associated with percent breast density or dense area of the breast before or after adjustment for age and body mass index (BMI). Several fatty acid measures were associated with the nondense area of the breast; however, the associations did not persist after controlling for the effects of age and BMI. This is the first study to report on the relationship between erythrocyte fatty acids and mammographic features. These results suggest that if n-6 and n-3 fatty acids influence breast cancer development then the effect may not be through influencing mammographic breast density.

## 5.2 INTRODUCTION

Over the past two decades, mammographic breast density has become acknowledged as one of the strongest, independent risk factors for breast cancer. The majority of epidemiological studies report approximately a four- to six-fold increased risk of breast cancer when comparing women with extensive areas of density to women whose breasts are composed primarily of fatty tissue [110, 23]. Moreover, increased density also reduces the sensitivity of screening mammography [336, 337], thus it is important to identify factors that can reduce dense breast tissue. Several characteristics that have been related with increased density have also been linked to increased breast cancer risk, such as nulliparity, late age at first full term birth, late age of menopause, and hormone therapy (HT) use [70, 65, 72]. Data also exist suggesting that dietary habits can influence breast density [278, 280], though this area of research has not been sufficiently explored.

Experimental and epidemiological studies have found a positive association between n-6 fatty acids and an inverse association between n-3 fatty acids with the risk of breast cancer [252, 253, 264, 266]. Additionally metabolic byproducts of the n-6 and n-3 fatty acids have also been linked with breast cancer. Both n-6 and n-3 fatty acids serve as substrates for prostaglandins. Prostaglandin E2 (PGE2), a metabolic product of n-6 arachidonic acid (AA; 20:4n-6) metabolism, is unregulated in breast tumors [185-187]. On the contrary, the prostaglandin product of n-3 eicosapentaenoic acid (EPA; 20:5n-3) metabolism is of the 3 series (PGE3) and is not a potent stimulator of breast cancer cell growth [176].

One potential mechanism through which these essential fatty acids could influence breast density is via increased estrogen production within the breast. PGE2 stimulates the CYP19 gene [200] which is transcribed and translated into aromatase, the key enzyme in the biosynthesis of estrogen in postmenopausal women [203]. Although circulating levels of estrogen in postmenopausal women drop substantially after the menopause, tissue levels of estrogen are considerably greater than even premenopausal tissue levels, which is likely the result of aromatase activity [328]. Dense areas of the breast are believed to represent proliferation of breast epithelial and/or stromal tissue [339]. Estrogen, a steroid hormone with known mitogenic effects, drives cellular division in breast epithelial cells [340]. Thus, excessive intake of n-6 fatty acids may increase breast cell proliferation via localized estrogen exposure which may be reflected on a mammogram by areas of density. On the other hand, consumption of n-3 fatty acids results in reduced PGE2 synthesis, and therefore may not amplify estrogen production or cellular division within the breast.

There are few studies that have assessed the role of the n-6 and n-3 fatty acids on mammographic breast density. N-6 intake has been associated with increased density [281], and n-3 intake with reduced density [282], however findings are not consistent [279, 281-283]. All studies, thus far, have measured dietary fat intake via self-report dietary assessment instruments, and limitations of these assessment tools and nutrient composition tables are well-known. Biomarkers of the essential n-6 and n-3 fatty acid intake have the advantage of being free of error due to human memory and can reflect recent intake of individual fatty acids [161]. Therefore, in the present study, we

examined the associations between erythrocyte n-6 and n-3 fatty acids with percent mammographic breast density and other mammographic characteristics.

## 5.3 MATERIALS AND METHODS

### 5.3.1 Study Population

The data presented were collected as part of the Mammograms and Masses Study (MAMS), the methods of which have previously been reported [296]. The MAMS is a case-control study of estrogen metabolites, mammographic breast density and breast cancer risk. MAMS recruited a total of 869 cancer-free women and 264 recently diagnosed breast cancer cases through the Magee Womens Hospital Mammographic Screening and Diagnostic Imaging Program in the Pittsburgh area (Pennsylvania, USA) during 2001-2005. Study participants were women aged 18 years or older who reported no previous personal history of cancer, with the exception of nonmelanoma skin cancer. Only MAMS controls who were recruited via study flyers (n=453) attached to screened negative mammogram reports (2003-2005) were included in the present analysis, as these participants completed a self-administered questionnaire on the day of blood draw. Prior to enrollment, written informed consent and a signed mammogram release form were obtained from each woman. The MAMS protocol was approved by the Institutional Review Board of the University of Pittsburgh.

### 5.3.2 Subsample Selection

In addition to the above eligibility criteria of the parent study, the participants were only selected for this ancillary study if they met the following entry criteria: postmenopausal (having had no menstrual bleeding during prior year or having undergone a bilateral oophorectomy); no use of hormone therapy (HT) within 3 months of study enrollment; and not using vaginal estrogen creams, oral contraceptives, corticosteroids or selective estrogen receptor modulators (SERMs) at blood sampling. Additionally, because MAMS utilized routine mammograms, the timing of the film does not coincide with the timing of the baseline blood draw, therefore participants whose time between film date and blood date was greater than 120 days (~4 months), the lifetime of a red blood cell, were also excluded. Of those who were excluded, 98 were premenopausal, 84 were using exogenous hormones, corticosteroids or SERMs, 1 control was later diagnosed with breast cancer, and 13 participants did not have an available mammogram taken within 120 days of blood draw. Additionally, we excluded 9 participants with estradiol levels greater than 150pmol/L as it indicated they might be pre- or peri-menopasual, or misreported current hormone use. Two hundred forty-eight women met the inclusion criteria for the present analysis.

### 5.3.3 Data Collection

A standardized, self-administered questionnaire was administered at blood sampling, and collected information on participant demographics, current use of medications and supplements, reproductive history, family history of breast cancer, past hormone therapy

use, and various lifestyle habits (smoking status and alcohol intake). Participants who reported using aspirin, COX-2 inhibitors, or other non-aspirin NSAIDs were considered "current NSAID users." Participants who did not list using any of these agents on the questionnaire were considered “current NSAID non-users.” Regular alcohol use (grams/day) in the past year was calculated as previously reported [298]. Age was defined as a participant’s age at the time of blood draw, rather than her age at mammogram. Age of menopause was calculated as the age at last natural menstrual bleeding or bilateral oophorectomy. However, for a hysterectomized woman without a bilateral oophorectomy, age at menopause corresponded to the earliest age at which she began using HT or first had menopausal symptoms. If neither occurred and her age at hysterectomy was 50 years or older, then age at menopause was her age at hysterectomy [299]. Years since menopause were calculated by subtracting a woman’s age at menopause from her age at study enrollment.

Height and weight were measured by trained clinical staff, after participants removed shoes and heavy clothing. Weight was measured to the nearest 0.1 kg using a standard balance beam. Standing height was measured at full inspiration to the nearest 0.1 cm. The measurements were repeated, and the average of the values was used in the analyses. Weight and height measurements were used to calculate body mass index (BMI, weight in kg divided by height in meters squared ($m^2$)).

A 40 mL non-fasting blood sample was donated by each participant. Samples were processed immediately on site according to a standardized protocol. After processing, the samples were fractioned into 1 mL cryovials of red blood cell, serum, plasma and buffy coat aliquots. Samples were stored at or below -70°C until assayed.

### 5.3.4 Erythrocyte Fatty Acid Analysis

Fatty acids in erythrocytes were analyzed by gas-liquid chromatography at the University of Pittsburgh's Heinz Laboratory. Total lipids (500µl of packed red blood cells) were extracted as previously described [300]. In brief, the samples and an internal standard were homogenized in 4 ml of methanol. Two ml of chloroform and 1.1 ml of water were added to the samples after 15 min. The samples were centrifuged at 1200 g for 30 min at 16°C and the upper phase of the sample was discarded. The lower phase was dried under nitrogen and resuspended in 1.5 ml 14% boron trifluoride methanol. Samples were heated at 90°C for 40 min and extracted after cooling with 4.0 ml pentane and 1.5 ml water. The samples were vortexed and the organic phase recovered [301]. The extracts were dried under nitrogen, resuspended in 50 µl heptane and 2 ul injected into a capillary column (SP-2380, 105 m x 53 mm ID, 0.20 um film thickness). Gas chromatographic analyses were performed with a Perkin Elmer Clarus 500 equipped with a flame ionization detector. The operating conditions were: oven temperatures programmed at 140°C for 35 min, 8°C/min to 220°C, and held for 12 min; the injector and detector temperatures were both at 260°C; and helium, the carrier gas, was at 15 psi. Fatty acid identification was based on the retention time data obtained for the authentic standards (Sigma). Erythrocyte fatty acids are expressed as a percentage by weight of total fatty acids. Inter-assay coefficients of variations (CV) were calculated from 27 masked duplicate samples. The inter-assay coefficients of variation for the erythrocyte fatty acid measures reported ranged from 1.7-15.2%. CVs's were 1.7% for linoleic acid (LA; 18:2n-6), 3.4 % for AA, and 1.7% for total n-6 fatty acids. CV's were 15.2% for alpha-linolenic acid (ALA; 18:3n-3), 5.3% for EPA, 7.5% for docosahexaenoic acid (DHA;

22:6n-3) and 5.3% for total n-3 fatty acids. The CVs for the total n-6: n-3, LA:ALA, AA:EPA, and AA:EPA+DHA ratios were 5.2%, 11.1%, 4.5% and 5.7% respectively.

### 5.3.5 Mammographic Breast Density Assessment

The craniocaudal view of the mammographic film corresponding closest to the participant's date of blood draw was obtained and copied after radiologic evaluation had ruled out malignancy. The film copies were deidentified and sent to a single expert reviewer (Ms. Martine Salane) for assessment of breast density. A transparent overlay was placed on top of the film, and using a wax pencil, the reviewer outlined the total area of the breast and areas of dense tissue. Biopsy scars, Cooper's ligaments and breast masses were not considered in the reading. Next, using a compensating planimeter (LASICO, Los Angeles, CA), the reviewer traced the outlined areas to compute total area of the breast ($cm^2$) and dense breast area ($cm^2$). Percent breast density was calculated by dividing the dense breast area by the total area of the breast multiplied by 100. Nondense area ($cm^2$) of the breast was determined by subtracting dense breast area from the total area of the breast. To determine the reproducibility of the mammographic readings twenty-one randomly selected mammograms (7 from each tertile of density) were read blindly a second time by the reader and the intraclass correlation coefficient (ICC) was calculated. The ICC was calculated from an F value that was derived by dividing the mean square error terms for the between-participant variance by that of the within-participant variance. Calculated ICC values were excellent at $\rho$=0.92, $\rho$=0.99 and $\rho$=0.96 for area of density, total area of the breast, and percent breast density, respectively.

Mammograms were also visually assessed for quality of film (excellent, good, fair, and poor).

### 5.3.6 Statistical Analyses

Percent breast density, dense breast area, and nondense area of the breast were examined as continuous variables. Descriptive results for selected, participant characteristics are expressed as a mean and standard deviation (SD) or as a frequency and percentage (%). Medians and interquartile ranges were determined for the mammographic features. Differences in continuous variables between tertiles of percent density were determined using Kruskal-Wallis. Differences in categorical variables between tertiles of breast density were compared using the chi-square analysis, unless expected cell sizes were less than 5, in which case the Fisher's exact test was used. Pearson's correlation coefficients were calculated to determine the relationship between percent breast density, dense breast area, and nondense area of the breast; a square root transformation was applied to the three mammographic features to normalize distributions for this statistical test. Correlation relationships between erythrocyte n-6 and n-3 fatty acid measures and mammographic characteristics were examined with Spearman's correlation coefficients, with no adjustments and adjusting for the effects of age and BMI. Data were analyzed using SAS statistical program version 9.1 (SAS Institute, Cary, NC). All statistical tests were two-sided, and an alpha level of 0.05 was set *a priori* in order to determine statistical significance.

## 5.4 RESULTS

As shown in **Table 14**, the mean (SD) age of the study population was 63.0 (8.4) years, mean BMI was 28.6 (6.1) $kg/m^2$, the mean time since menopause was 14.3 (10.1) years, and 93.6% were white. The average time interval between the mammogram and blood collection was 35 days, with a range of 8-114 days (data not shown). The median (interquartile range) percent breast density was 23.7 (13.4 to 39.7)%, dense area was 35.8 (21.7 to 54.6) $cm^2$, and nondense breast area was 104.2 (65.2 to 169.2) $cm^2$. Percent mammographic density was strongly correlated with both dense breast area ($r=0.80$; $p<0.0001$) and nondense area of the breast ($r=-0.77$; $p<0.0001$) (**Table 15**).

The characteristics of the study participants, by tertiles of percent breast density, are shown in **Table 16**. Women with denser breasts were more likely to have reported prior HT use, regular use of alcohol in the past year, and had a breast biopsy. Contrary to prior reports, parous women in our population who breastfed for more than 1 month had greater percent breast density. BMI, time since menopause, and current use of cigarettes were inversely related with percent breast density. Although, a significant difference was noted for age at menopause across tertiles of density, the relationship between the two variables was not clear.

Mean erythrocyte fatty acid compositions for the participants are described in **Table 17**. Study participants had a greater concentration of n-6 fatty acids as compared to n-3 fatty acids incorporated into their erythroctytes. The mean (SD) total n-6 fatty acids was 38.3 (2.6)% and total n-3 was 7.9 (2.1)%. N-6 AA and LA were the most abundant fatty acids, with AA composing 16.1 (1.9)% of the total erythrocyte fatty acid

content and LA 15.8 (2.4)%. Of the n-3 fatty acids, DHA accounted for the greatest percentage of the total fatty acids at 4.5 (1.5)%.

Unadjusted and adjusted Spearman rank correlation coefficients were determined and presented in **Table 18**. No association was observed between any one of the erythrocyte fatty acid measures and percent breast density or dense breast area in either the unadjusted or adjusted analyses. N-6 LA, total n-3 and all individual n-3 fatty acids were inversely correlated with nondense area of the breast before adjustments were made for age and BMI. However, none of these associations persisted after adjusting for these variables. The n-6:n-3 ratios were all positively and significantly correlated with nondense area of the breast, but associations diminished after correcting for covariates. Adjustment for additional factors in a general linear model did not produce any statistically significant findings (**Appendix C**).

In a secondary analysis, we excluded participants (n=128) whose date of mammogram was more than 30 days before blood sampling as well as participants whose mammographic films were rated as poor quality. Findings did not differ substantially (**Appendix C**).

## 5.5 CONCLUSIONS

In this cross-sectional investigation, we assessed the association between the n-6 and n-3 fatty acids in erythrocytes and percent breast density and other mammographic features. To our knowledge, we are the first to report on these relationships. In contrast with what we had predicted, no association was found between any fatty acid measure and percent

breast density. Further, a relationship was not observed between the n-6 or the n-3 fatty acids and dense breast area. These null associations remained after limiting the study population to women whose mammograms were taken within 30 days of blood draw. Although several fatty acid measures were significantly related to nondense area of the breast, these associations disappeared after controlling for the confounding effects of age and BMI.

A few epidemiological studies have reported on the association between dietary intake of the n-6 and n-3 fatty acids and mammographic breast density, and all measured diet via self-report. Women with the highest mammographic density pattern reported significantly higher consumption of n-6 PUFAs when compared to women with the lowest mammographic pattern [281]; however, another study reported no association between the n-6 fatty acid LA and breast density [282]. No difference in breast density was observed when comparing quartiles of total n-3 intake or long chain n-3 fatty acids [279, 281, 283]. Consumption of fish, which is typically high in the long chain n-3 fatty acids, has not been linked to mammographic density in postmenopausal women [282-284]. Only one study has investigated the effects of the n-3 fatty acid, ALA, with breast density and a significant inverse association was observed [282].

Several limitations of this study should be mentioned. First, blood was not drawn on the same day that the mammogram was taken (range 8-114 days); however, erythrocyte n-3 and n-6 fatty acids reflect dietary intake over the preceding weeks or months [161]. Further, the dietary intake within our study population may have been too similar to detect a difference in mammographic features. Study results have reduced generalizibility due to the study population being entirely postmenopausal and

predominately white. The cross-sectional nature of this study does not allow temporal relationships between fatty acid intake and mammographic characteristics to be established. Finally, as information on NSAID use at the time of mammogram was not collected, we were unable to accurately determine if the relationship between erythrocyte fatty acid measures and breast density is modified by NSAID use (**Appendix C**). This may be of some concern given that differences in the strengths of the relationships between fatty acid measures and estradiol concentrations differed by NSAID use in this population (Research article 1, unpublished data). Study strengths include the use of a single expert reader with excellent reproducibility for breast density assesment, the use of a validated biochemical marker of dietary n-6 and n-3 fatty acids which is capable of reflecting recent dietary intake [161], and standardized measurement of participant characteristics.

In conclusion, in the present study we found no evidence of an association between n-6 or n-3 fatty acids and mammographic breast density in postmenopausal women. Thus our results suggest that if the n-6 and n-3 fatty acids affect the risk of breast cancer, it may not be through altering mammographic breast density. However, considering the biological plausibility of an association between the essential n-6 and n-3 fatty acids and breast density and the study limitations noted above, further studies are necessary to confirm these findings. Understanding the possible influences of dietary intake on mammographic breast density may contribute understanding to the etiology of breast cancer, and could aid in improving the sensitivity of mammograms, which are hindered by dense breast tissue [341].

**Table 14. Selected demographic, anthropometric, and lifestyle characteristics of the study population**

| Characteristics (N=248) | |
|---|---|
| *Means (SD)* | |
| Age at blood sampling (years) | 63.0 (8.4) |
| Age at menopause (years)* | 48.7 (5.2) |
| Years menopausal* | 14.3 (10.1) |
| BMI (kg/m$^2$) | 28.6 (6.1) |
| | |
| *Frequencies (%)* | |
| Race | |
| White | 232 (93.6) |
| Non-white | 16 (6.5) |
| Age at menarche (years)* | |
| <12 | 47 (19.0) |
| 12-13 | 139 (56.3) |
| ≥13 | 61 (24.7) |
| Family history of breast cancer* † | |
| No | 214 (87.0) |
| Yes | 32 (13.0) |
| Surgical Menopause* | |
| No | 221 (89.5) |
| Yes | 26 (10.5) |
| Past hormone therapy use | |
| No | 97 (39.1) |
| Yes | 151 (60.9) |
| Past year regular alcohol intake (g/day) | |
| None | 179 (72.2) |
| <12 | 45 (18.2) |
| ≥12 | 24 (9.7) |
| Current Smoker | |
| No | 234 (94.4) |
| Yes | 14 (5.7) |
| Current NSAID use | |
| No | 129 (52.2) |
| Yes | 119 (47.8) |
| Ever pregnant | |
| No | 41 (16.5) |
| Yes | 207 (83.5) |

**Table 14 (continued)**

| Characteristics | |
|---|---|
| Number of live births‡ | |
| 1 | 28 (14.1) |
| 2 | 83 (41.7) |
| ≥3 | 88 (44.2) |
| Age at first full term pregnancy‡ | |
| <30 | 163 (81.9) |
| ≥30 | 36 (18.1) |
| Ever breastfed >1 month‡ | |
| No | 103 (51.8) |
| Yes | 96 (48.2) |
| Ever breast biopsy | |
| No | 212 (85.5) |
| Yes | 36 (14.5) |
| | |
| *Median (IQR)* | |
| Percent breast density | 23.7 (13.4 – 39.7) |
| Dense breast area ($cm^2$) | 35.8 (21.7 – 54.6) |
| Nondense breast area ($cm^2$) | 104.2 (65.2 – 169.2) |

NOTE: Percentages (%) may not add up to 100% due to rounding. BMI, body mass index; NSAID, nonsteroidal anti-inflammatory drug; IQR, interquartile range

*missing n=6 for age at menopause, n=6 for years menopausal, n=1 for age at menarche n=2 for family history of breast cancer, and n=1 for surgical menopause

†family history of breast cancer in mother of sister

‡ among parous women

**Table 15. Pearson's correlation coefficients between mammographic features**

| | Dense breast area ($cm^2$) | Nondense breast area ($cm^2$) |
|---|---|---|
| Percent breast density | 0.80 (<0.0001) | -0.77 (<0.0001) |
| Dense breast area ($cm^2$) | | -0.28 (<0.0001) |

Note: A square root transformation was applied to percent breast density, dense breast area, and nondense breast area

**Table 16. Characteristics of participants by tertile of percent breast density**

| Characteristics (n=248) | Tertile 1 (<16.46) | Tertile 2 (16.47-34.24) | Tertile 3 (≥34.25) | p |
|---|---|---|---|---|
| *Means (SD)* | | | | |
| Age at blood sampling (years) | 64.1 (8.7) | 62.7 (8.2) | 62.2 (8.1) | 0.31 |
| Age at menopause (years)* | 49.3 (4.7) | 47.8 (5.3) | 49.1 (5.4) | 0.03 |
| Years menopausal* | 14.9 (9.6) | 14.9 (9.8) | 13.1 (10.9) | 0.15 |
| BMI (kg/m$^2$) | 31.1 (6.6) | 29.3 (5.7) | 25.2 (4.2) | <0.0001 |
| | | | | |
| *Frequencies (%)* | | | | |
| Race | | | | 0.73 |
| White | 77 (91.7) | 77 (93.9) | 78 (95.1) | |
| Non-white | 7 (8.3) | 5 (6.1) | 4 (4.9) | |
| Age at menarche (years)* | | | | 0.90 |
| <12 | 18 (21.7) | 16 (19.5) | 13 (15.9) | |
| 12-13 | 46 (55.4) | 46 (56.1) | 47 (57.3) | |
| ≥13 | 19 (22.9) | 20 (24.4) | 22 (26.8) | |
| Family history of breast cancer* † | | | | 0.44 |
| No | 69 (83.1) | 72 (88.9) | 73 (89.0) | |
| Yes | 14 (16.9) | 9 (11.1) | 9 (11.0) | |
| Surgical Menopause* | | | | 0.29 |
| No | 77 (92.8) | 74 (90.2) | 70 (85.4) | |
| Yes | 6 (7.2) | 8 (9.8) | 12 (14.6) | |
| Past hormone therapy use | | | | 0.05 |
| No | 40 (47.6) | 24 (29.3) | 33 (40.2) | |
| Yes | 44 (52.4) | 58 (70.7) | 49 (59.8) | |
| Past year regular alcohol intake (g/day) | | | | 0.05 |
| None | 68 (81.0) | 61 (74.4) | 50 (61.0) | |
| <12 g/day | 11 (13.1) | 15 (18.3) | 19 (23.2) | |
| ≥12 g/day | 5 (6.0) | 6 (7.3) | 13 (15.9) | |
| Current Smoker | | | | 0.13 |
| No | 76 (90.5) | 80 (97.6) | 78 (95.1) | |
| Yes | 8 (9.5) | 2 (2.4) | 4 (4.9) | |
| Current NSAID use | | | | 0.77 |
| No | 45 (53.6) | 40 (48.8) | 44 (53.7) | |
| Yes | 39 (46.4) | 42 (51.2) | 38 (46.3) | |
| Ever pregnant | | | | 0.31 |
| No | 10 (11.9) | 14 (17.1) | 17 (20.7) | |
| Yes | 74 (88.1) | 68 (82.9) | 65 (79.3) | |

**Table 16 (continued)**

| Characteristics | Tertile 1 (<16.46) | Tertile 2 (16.47-34.24) | Tertile 3 (≥34.25) | p |
|---|---|---|---|---|
| Number of live births‡ | | | | 0.93 |
| 1 | 11 (15.3) | 8 (12.3) | 9 (14.5) | |
| 2 | 29 (40.3) | 26 (40.0) | 28 (45.2) | |
| ≥3 | 32 (44.4) | 31 (47.7) | 25 (40.3) | |
| Age at first full term pregnancy‡ | | | | 0.32 |
| <30 | 61 (84.7) | 55 (84.6) | 47 (75.8) | |
| ≥30 | 11(15.3) | 10 (15.4) | 15 (24.2) | |
| Ever breastfed >1 month‡ | | | | 0.07 |
| No | 43 (59.7) | 35 (53.9) | 25 (40.3) | |
| Yes | 29 (40.3) | 30 (46.2) | 37 (59.7) | |
| Ever breast biopsy | | | | 0.02 |
| No | 79 (94.1) | 68 (82.9) | 65 (79.3) | |
| Yes | 5 (6.0) | 14 (17.1) | 17 (20.7) | |

NOTE: BMI, body mass index; NSAID, nonsteroidal anti-inflammatory drug. Percentages (%) may not add up to 100% due to rounding.

*missing n=6 for age at menopause, n=6 for years menopausal, n=1 for age at menarche, n=2 for family history of breast cancer, and n=1 for surgical menopause

†family history of breast cancer in mother of sister

‡ among parous women

**Table 17. Mean fatty acid composition in erythrocytes**

| Fatty acids (wt. %) | mean (SD) |
|---|---|
| n-6 PUFAs* | |
| Total n-6 | 38.3 (2.6) |
| 18:2n-6 (LA) | 15.8 (2.4) |
| 20:4n-6 (AA) | 16.1 (1.9) |
| n-3 PUFAs† | |
| Total n-3 | 7.9 (2.1) |
| 18:3n-3 (ALA) | 0.2 (0.1) |
| 20:5n-3 (EPA) | 0.9 (0.4) |
| 22:6n-3 (DHA) | 4.5 (1.5) |
| 6:3 Ratios | |
| Total n-6:n-3 | 5.2 (1.5) |
| LA:ALA | 72.8 (19.5) |
| AA:EPA | 21.7 (8.9) |
| AA:EPA+DHA | 3.3 (1.2) |

NOTE: N=248. Fatty acids are reported as a percentage by weight of the total fatty acids (weight percent, wt.%). PUFA, polyunsaturated fatty acid; LA, linoleic acid; AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; and DHA, docosahexaenoic acid

*18:2n-6+18:n-6+20:2n-6+20:3n-6+20:4n-6+22:4n-6+22:5n-6

†18:3n-3+20:4n-3+20:5n-3+22:5n-3+22:6n-3

**Table 18. Unadjusted and adjusted Spearman correlation coefficients between erythrocyte n-6 and n-3 fatty acids and mammographic characteristics**

| Fatty acids (n=248) | % breast density | dense area | nondense area |
|---|---|---|---|
| N-6 PUFAs | | | |
| Total n-6* | -0.006 (0.93) | 0.001 (0.99) | 0.03 (0.69) |
| | 0.02 (0.75) | -0.01 (0.87) | -0.04 (0.51) |
| 18:2n-6 (LA) | 0.08 (0.22) | -0.04 (0.51) | -0.17 (0.006) |
| | -0.02 (0.78) | -0.06 (0.34) | -0.08 (0.22) |
| 20:4n-6 (AA) | 0.01 (0.85) | 0.07 (0.25) | 0.08 (0.23) |
| | 0.08 (0.20) | 0.08 (0.22) | -0.02 (0.79) |
| N-3 PUFAs | | | |
| Total n-3† | 0.10 (0.11) | 0.01 (0.88) | -0.18 (0.004) |
| | 0.02 (0.77) | 0.02 (0.77) | -0.04 (0.51) |
| 18:3n-3 (ALA) | 0.04 (0.52) | -0.05 (0.46) | -0.12 (0.06) |
| | -0.04 (0.55) | -0.06 (0.39) | -0.02 (0.76) |
| 20:5n-3 (EPA) | 0.07 (0.28) | -0.04 (0.48) | -0.18 (0.004) |
| | -0.02 (0.70) | -0.04 (0.51) | -0.05 (0.45) |
| 22:6n-3 (DHA) | 0.10 (0.12) | 0.04 (0.57) | -0.14 (0.03) |
| | 0.03 (0.64) | 0.05 (0.47) | -0.02 (0.80) |
| 6:3 Ratios | | | |
| Total n-6:n-3 | -0.09 (0.15) | -0.01 (0.91) | 0.16 (0.009) |
| | -0.01 (0.84) | -0.02 (0.78) | 0.02 (0.65) |
| LA:ALA | -0.02 (0.75) | 0.03 (0.61) | 0.07 (0.29) |
| | 0.02 (0.74) | 0.03 (0.63) | 0.004 (0.95) |
| AA:EPA | -0.06 (0.32) | 0.05 (0.39) | 0.19 (0.003) |
| | 0.04 (0.51) | 0.05 (0.40) | 0.04 (0.52) |
| AA:EPA+DHA | -0.08 (0.18) | 0.003 (0.96) | 0.17 (0.008) |
| | 0.01 (0.88) | -0.003 (0.96) | 0.01 (0.83) |

NOTE: PUFA, polyunsaturated fatty acid; LA, linoleic acid; AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; and DHA, docosahexaenoic acid. First line is unadjusted estimates. Partial correlations estimates adjusted for age and BMI, and appear immediately below the unadjusted correlations. P between parentheses.

*18:2n-6+18:n-6+20:2n-6+20:3n-6+20:4n-6+22:4n-6+22:5n-6

†18:3n-3+20:4n-3+20:5n-3+22:5n-3+22:6n-3

# 6.0 GENERAL DISCUSSION

Breast cancer incidence rates in the United States are among the highest in the world [1]. This year alone, it is estimated that approximately 178,480 new cases of invasive and 62,030 new cases of in situ breast cancer will be diagnosed in women residing in the United States [2]. Despite substantial advances in treatment for this disease, breast cancer still ranks second in female cancer mortality [2], and therefore, it is important to identify modifiable factors associated with this disease.

One of the most investigated nutritional associations with breast cancer risk is dietary fat, yet despite decades of intensive experimental and epidemiological research, our understanding on the role of fat consumption in the etiology of breast cancer is deficient. Cross-national and migrant studies of breast cancer rates indicate that dietary fat may be partially responsible for the large population differences in breast cancer risk [6-8], yet case-control and cohort findings repeatedly produce inconsistent results [29]. It has been implicated that type of fat consumed may be more important than total fat intake, and if specific fatty acids were found to possess unique breast cancer chemopreventive properties it would help to determine the ideal proportions of each type of fat to be consumed and potentially reduce the burden of this disease. Yet, in spite of its clear public health importance, no consensus exists as to which fats are harmful and which fats are beneficial.

Research has shown circulating estradiol concentrations and mammographic breast density to be strong risk factors for postmenopausal breast cancer [41, 61]. Women with the highest levels of estradiol or highest percentage of breast density are at increased risk of developing breast cancer [41, 61]. Unlike the majority of other well-established risk factors, both circulating estradiol and breast density have been shown to be modifiable [270, 278]. Dietary habits that alter circulating estradiol levels and/or mammographic breast density may also alter breast cancer risk, although this has yet to be proven. Nonetheless, determining factors that influence these breast cancer risk factors may lead to a greater understanding of breast cancer pathogenesis.

Prostaglandin E2 (PGE2), the metabolic product of omega-6 (n-6) arachidonic acid (AA), stimulates biosynthesis of estrogen by upregulation of the enzyme aromatase [200]. Consumption of omega-3 (n-3) fatty acids inhibits the synthesis of PGE2 [176, 179]. Therefore, in theory, a diet rich in n-3 fatty acids and/or low in n-6 fatty acids should decrease estradiol production by suppressing PGE2 activation of aromatase. Such an event may ultimately result in a reduction of estrogen dependent breast cancer occurrence. For that reason, we sought to determine the relationship between n-6 and n-3 fatty acids and two hormonally influenced breast cancer risk factors, specifically, serum total estradiol concentrations and percent mammographic breast density. Given that nonsteroidal anti-inflammatory drugs (NSAIDs) also reduce the synthesis of PGE2 [334], we further assessed the relationship between current NSAID use and serum total estradiol concentrations. NSAID use data was not available at the time of mammogram; therefore, the relationship between NSAID use and mammographic density could not accurately be assessed.

The Mammogram and Masses Study (MAMS), a case-control study on the hormonal determinants of breast density, provided a unique opportunity to assess the relationship between fatty acids, NSAID use, and modifiable breast cancer risk factors. Our first study tested the association between the essential n-6 and n-3 fatty acids in erythrocytes and serum total estradiol concentrations. The second study assessed the relationship between current NSAID use and serum total estradiol levels. Finally, the third study investigated the association between the erythrocyte n-6 and n-3 fatty acids and mammographic features. The three research projects utilized a population of breast cancer-free, postmenopausal women not using hormone therapy (HT). We are unaware of any studies that have previously reported on any one of these study aims.

## 6.1 RESEARCH ARTICLE 1

The aim of the first research article was to investigate the relationship between erythrocyte n-6 and n-3 fatty acids and serum total estradiol concentrations. Exposure to endogenous estrogens has been consistently linked to increased postmenopausal breast cancer risk [41]; therefore, lifestyle factors related to reducing a woman's exposure to estrogens may lead to the prevention of breast cancer. We believe this to be the first study to report on the association between fatty acids in biological specimens with circulating endogenous estradiol levels.

The study population consisted of 260 breast cancer free, postmenopausal women not using exogenous hormones who were recruited between 2003 and 2005 in Pittsburgh, Pennsylvania. Study results revealed a statistically significant inverse association

between total n-3 fatty acids and serum estradiol concentrations ($p<0.05$). Further, total n-6 fatty acids ($p=0.02$), the total n-6:n-3 ratio ($p=0.06$) and the ratio of n-6 arachidonic acid (AA) to n-3 fatty acids eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA) ($p<0.01$) were positively associated with total estradiol. These statistically significant relationships were only noted among women not reporting current use of NSAIDs. Among NSAID users, the ratio of n-6 linolenic acid (LA) to n-3 alpha-linolenic acid (ALA) was positively related to estradiol concentrations ($p=0.01$), and was primarily a result of the inverse relationship between ALA and estradiol ($p=0.05$). Because circulating postmenopausal estradiol concentrations are positively related to breast cancer risk, our results provide a mechanism through which the essential n-6 and n-3 PUFAs may impact breast cancer development.

## 6.2 RESEARCH ARTICLE 2

Research article 2 assessed the relationship between current NSAID use and serum total estradiol concentrations in a population of postmenopausal women. Our interest in evaluating this association stemmed from our previous finding that n-3 fatty acids were inversely related with circulating estradiol levels. Similarly to n-3 fatty acid intake, NSAID use decreases the production of PGE2 [334], thus reducing upreguation of aromatase and hence biosynthesis of estrogen [200]. Therefore, if it is through PGE2 inhibition that n-3 fatty acids lower estradiol levels, then we would also expect to observe reduced estradiol levels among users of NSAIDs. Discovering an inverse association

between NSAID use and circulating estradiol levels would provide additional support to the mechanism proposed by which the n-6 and n-3 fatty acids are related to breast cancer.

Participants recruited between 2003 and 2005 into the MAMS study who were breast cancer free, postmenopausal, and not using exogenous hormones were selected for this ancillary study (n=260). As hypothesized, the geometric mean serum estradiol concentration among NSAID users (N=124) was significantly lower than non-users of NSAIDs (N=136) (17.8 pmol/L vs. 21.3 pmol/L; p=0.03). Lower estradiol levels were noted regardless of how NSAID use was defined (i.e. self-reported current NSAID use on questionnaire, verbal reporting of use in past 48 hours, and the agreement between these two variables).

Many studies have assessed the relationship between NSAID use and breast cancer [329], but we unaware of any published reports that document the relationship between NSAID use and circulating estradiol concentrations. The detection of lower estradiol levels among NSAID users is consistent with the notion that NSAIDs are protective against breast cancer, via reducing estrogen exposure.

## 6.3 RESEARCH ARTICLE 3

Research article 3 evaluated the association between the n-6 and n-3 fatty acids in erythrocytes and mammographic breast density, a hormonally responsive breast cancer risk factor [72]. Breast density is believed to represent cellular division of mammary epithelial cells [339], and estrogen has been shown to drive cellular division in breast epithelial cells [340]. Therefore dense tissue, as reflected on a mammogram, may

represent the stimulatory effect of estrogen on epithelial cell proliferation. Since n-6 and n-3 fatty acids may be capable of altering estrogen synthesis, via influencing PGE2 production, these fatty acids may also influence breast density. For instance, consuming n-6 fatty acids may increase mammographic breast density by encouraging cellular proliferation via increasing estrogen production. On the contrary, intake of n-3 fatty acids may result in lower breast density, as estrogen synthesis is not stimulated and cellular division not amplified. To test the hypotheses that erythrocyte n-6 fatty acid content is positively related and erythrocyte n-3 fatty acid content is inversely related to mammographic breast density we undertook an observational study in 248 breast cancer-free women enrolled in the MAMS study. All women selected for this ancillary study were postmenopausal, reported no use of HT within 3 months of study entry, and had a mammographic exam within 120 days (the lifetime of an erythrocyte) of blood draw.

Contrary to our hypotheses, the results of this study do not support the premise that n-6 and n-3 fatty acids influence mammographic breast density. No statistically significant relationship was found between any of the fatty acids measures (n-6 fatty acids, n-3 fatty acids, and 6:3 ratios) and measurements of breast density (percent density or dense breast area). These findings suggest that if n-6 and n-3 fatty acids influence breast cancer development then the effect may not be through affecting mammographic breast density. Future studies are needed to gain a better understanding of the true relationship between the essential fatty acids and mammographic breast features.

## 6.4 SUMMARY

In summary, we attempted to clarify the relationship between the n-6 and n-3 fatty acids and NSAIDs with two well-established breast cancer risk factors, specifically, serum total estradiol concentrations and mammographic breast density. As hypothesized, we observed a positive relationship between n-6 fatty acids and serum estradiol concentrations and inverse associations between both the n-3 fatty acids and NSAID use and serum estradiol. These findings are consistent with extensive experimental data and a growing body of epidemiological evidence. Interestingly, the majority of associations observed between the essential fatty acids and estradiol concentrations were observed in NSAID nonusers, but not among current NSAID users. Contrary to our hypotheses, we did not observe an association between any one of the fatty acid measures with mammographic density (percentage or absolute). Therefore, if the n-6 and n-3 fatty acids influence breast cancer risk, it may not be through affecting breast density. To our knowledge, none of the aforementioned relationships have previously been explored.

## 6.5 STRENGTHS AND LIMITATIONS

Some important limitations of this study must be acknowledged. The primary weakness of this study is that it is cross-sectional in nature and does not allow us to determine temporal associations. The use of multiple measurements of fatty acids, NSAID use, serum estradiol and mammographic breast density over time might better characterize these women. Our biological samples were stored at -70°C, and reliability of erythrocyte

fatty acids at this temperature has only been reported in one study. Correlation was high for all PUFAs (r >0.90), but analyses were reported only after a 12 month time frame [342]. Some of our samples were stored for longer than one year before analysis (8 months-25 months) and we are unsure of the effects of longer storage at -70°C on the individual fatty acid levels; however, storage at -80°C for up to 48 months does not result in significant decreases in any of the n-6 or n-3 PUFAs [343]. A final weakness of this study is the homogeneity of the study population, thus potentially limiting the generalizibility of the study findings.

Regardless of the aforementioned limitations, study strengths should also be noted. Strengths of our study include the use of standardized instruments; reproducible measures of fatty acids, total estradiol and mammographic breast density; assessment of NSAID use on the same day as blood draw and; the use of a biochemical marker of dietary intake. Above all, we believe no other epidemiological study has previously explored these study aims.

## 6.6 FUTURE DIRECTIONS

The findings from this project, could spawn research in multiple areas with potential research aims including, but not limited to the following: 1) establish the relationship between NSAID use and breast density; 2) investigate the relationships between fatty acids and NSAID use on modifiable breast cancer risk factors (estradiol and breast density) in premenopausal women and minority populations; 3) assess the relationship between circulating PGE2 levels and both estradiol concentrations and mammographic

breast density; 4) determine if an interaction exists between the n-6 and n-3 PUFAs, and genetic polymorphisms in the COX-2 and CYP19 (aromatase) genes with serum estradiol and breast density; 5) determine if an interaction exists between NSAID use and genetic polymorphisms in the COX-2 gene with serum estradiol and breast density; 6) test the association between fatty acids in breast adipose tissue, a long-term marker of fatty acid intake, and mammographic breast density and; 7) conduct a clinical trial assessing fish oil supplementation and NSAID use on modifiable breast cancer risk factors (estradiol and breast density).

# 7.0 PUBLIC HEALTH SIGNFICANCE

The public health burden of breast cancer is substantial, with approximately 178,480 incident invasive cases and 40,460 deaths from breast cancer expected to occur among women in 2007. Age is a major determinant of breast cancer and with a rapidly aging population the affliction of breast cancer will likely worsen. Therefore, primary prevention of this disease is a much desired and sought after public health goal.

The relationships between circulating levels of estradiol, mammographic density, and breast cancer risk are greatly documented; hence, agents capable of altering these well-established risk factors could have a substantial impact on public health. In this body of research, we observed a positive relationship between erythrocyte n-6 fatty acids and serum estradiol. We further observed inverse associations between n-3 fatty acids and NSAID use with circulating estradiol concentrations. To date, there has been no epidemiological study to investigate these relationships and the discovery of modifiable behaviors that favorably alter breast cancer risk factors is needed. If confirmed, these findings could aid in the development of chemopreventive guidelines, and ultimately prevent the development of estrogen-dependent breast cancer.

# APPENDIX A: ERYTHROCYTE OMEGA-6 AND OMEGA-3 FATTY ACIDS AND POSTMENOPAUSAL SERUM ESTRADIOL ADDITIONAL ANALYSES

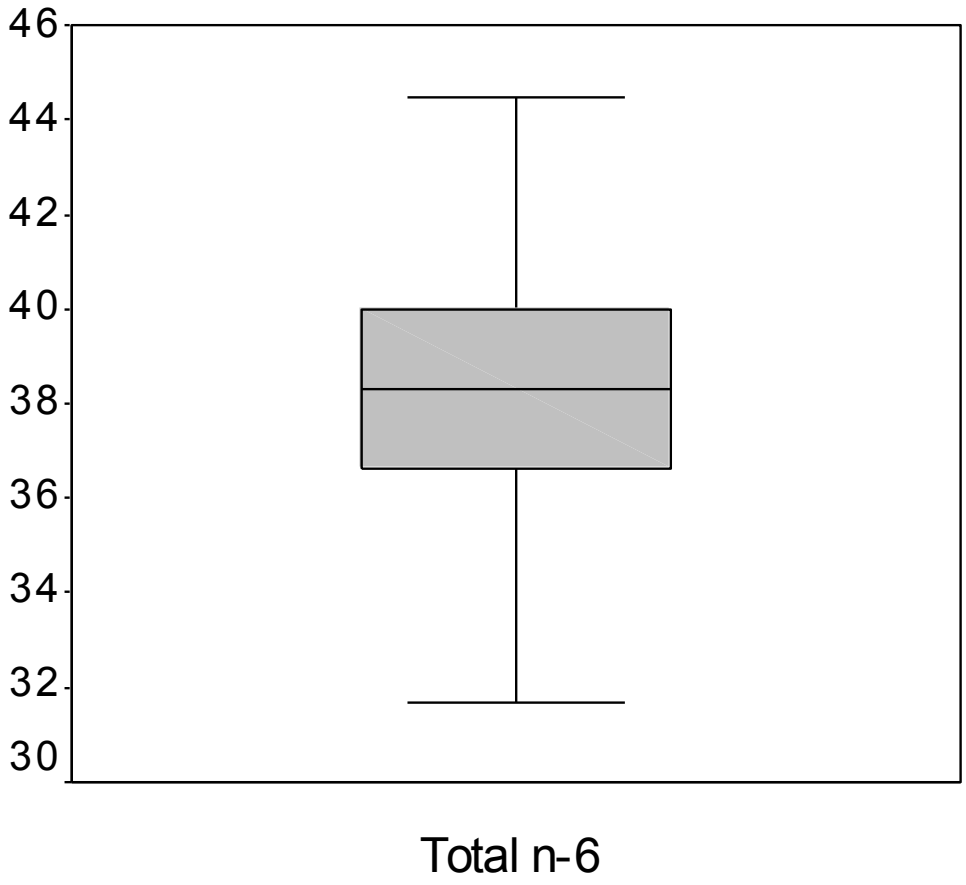


**Figure A6. Distribution of erythrocyte total n-6 fatty acids in the study population.**

In this boxplot, the edges of the box correspond to 25th and 75th percentiles. The horizontal line in the middle of the box corresponds to the median. The vertical bars above and below the box correspond to percentiles 10 and 90.

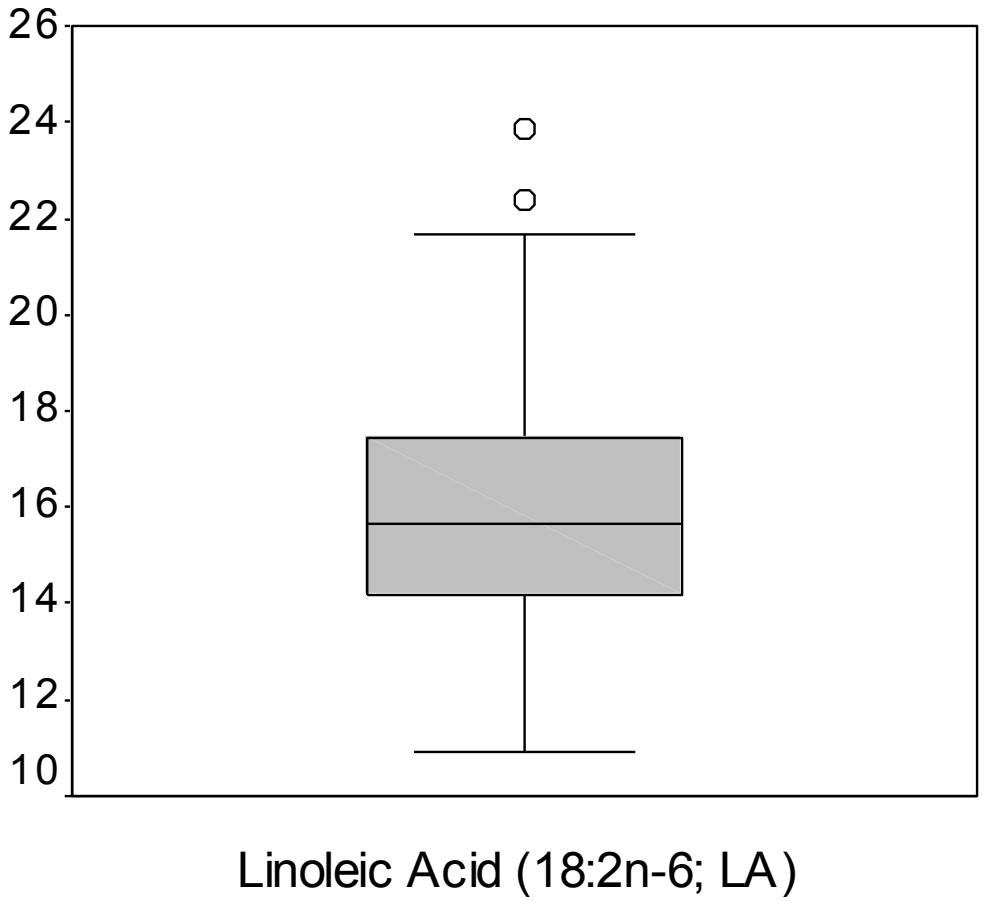


**Figure A7. Distribution of erythrocyte LA in the study population.**

In this boxplot, the edges of the box correspond to 25th and 75th percentiles. The horizontal line in the middle of the box corresponds to the median. The vertical bars above and below the box correspond to percentiles 10 and 90. Open circles beyond the box correspond to outliers (values between 1.5 and 3 box lengths from the upper or lower edge of the box).

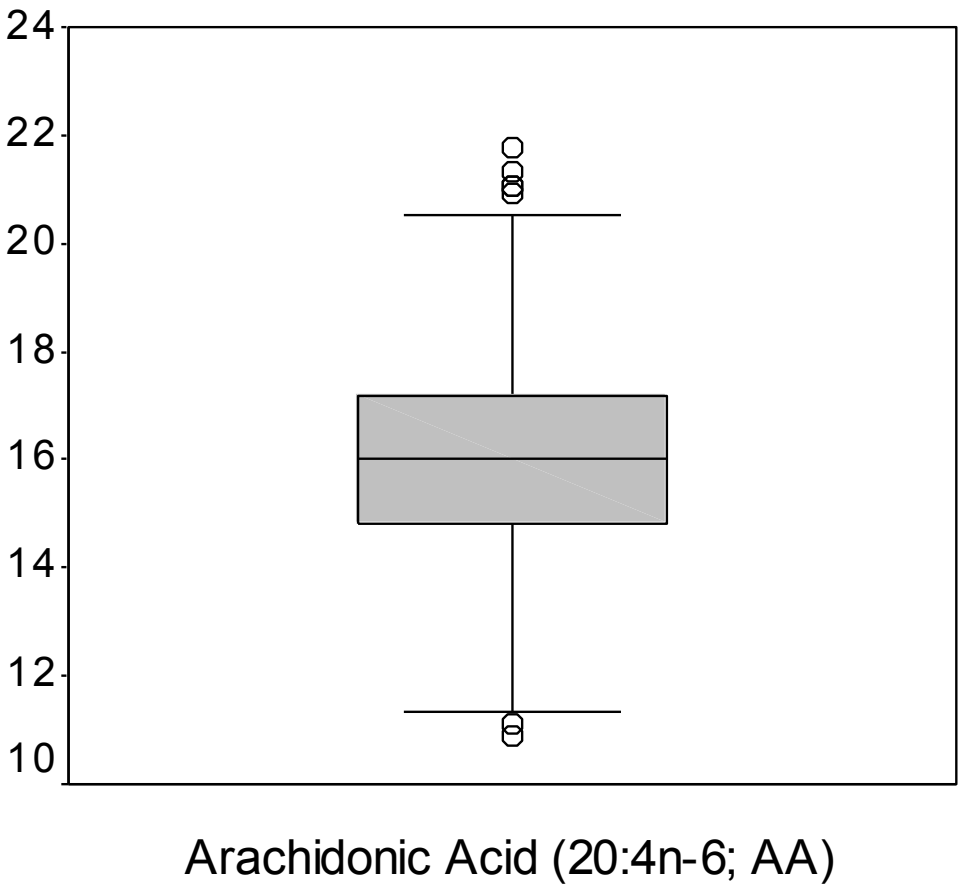


**Figure A8. Distribution of erythrocyte AA in the study population.**

In this boxplot, the edges of the box correspond to 25th and 75th percentiles. The horizontal line in the middle of the box corresponds to the median. The vertical bars above and below the box correspond to percentiles 10 and 90. Open circles beyond the box correspond to outliers (values between 1.5 and 3 box lengths from the upper or lower edge of the box).

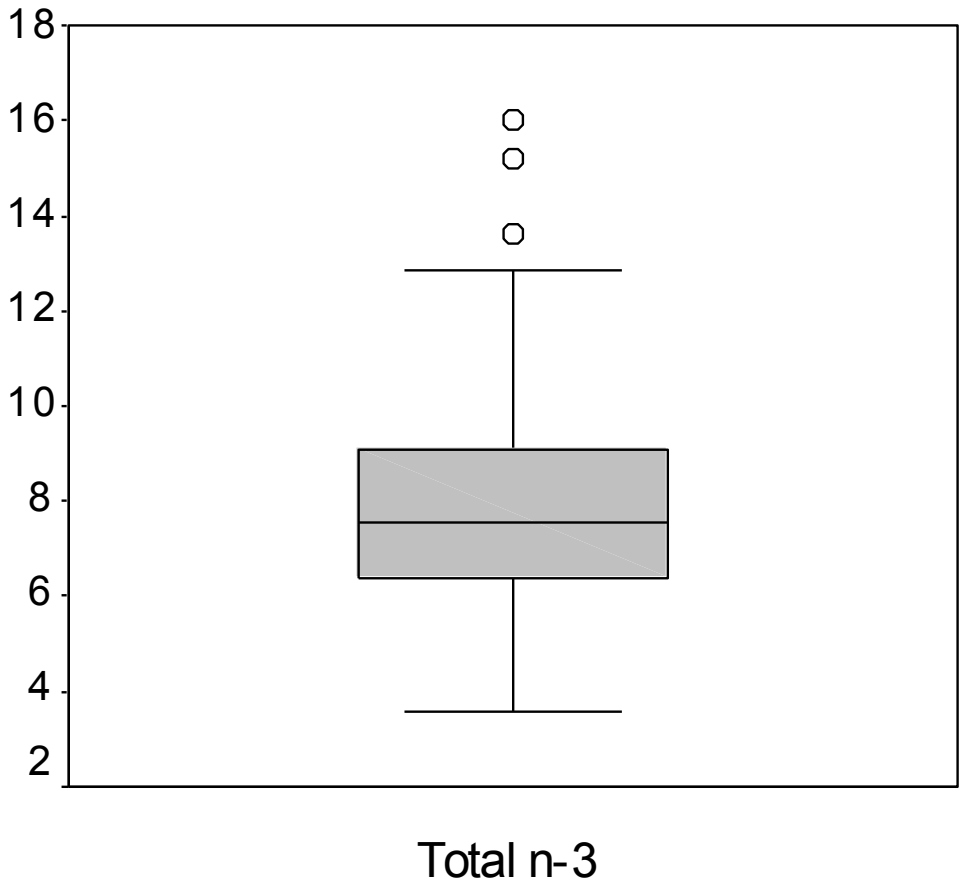


**Figure A9. Distribution of erythcoyte total n-3 fatty acids in the study population.**

In this boxplot, the edges of the box correspond to 25th and 75th percentiles. The horizontal line in the middle of the box corresponds to the median. The vertical bars above and below the box correspond to percentiles 10 and 90. Open circles beyond the box correspond to outliers (values between 1.5 and 3 box lengths from the upper or lower edge of the box).

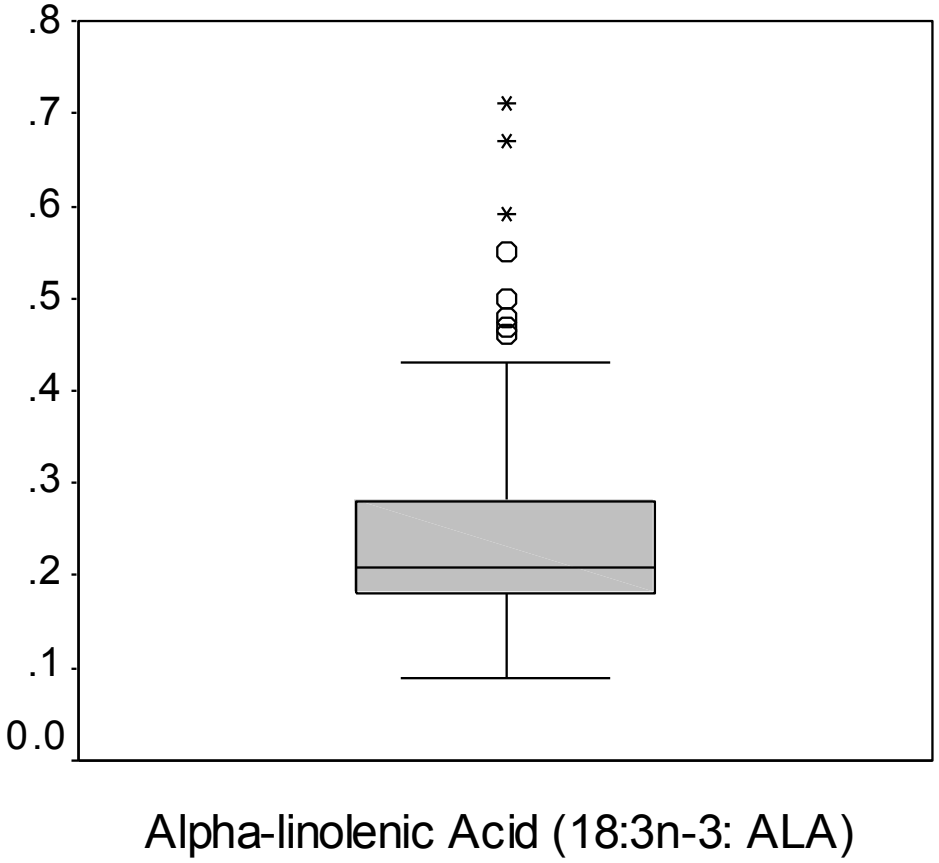


**Figure A10. Distribution of erythrocyte ALA in the study population.**

In this boxplot, the edges of the box correspond to 25th and 75th percentiles. The horizontal line in the middle of the box corresponds to the median. The vertical bars above and below the box correspond to percentiles 10 and 90. Open circles beyond the box correspond to outliers (values between 1.5 and 3 box lengths from the upper or lower edge of the box) and stars correspond to extremes (values beyond 3 box lengths from the edge of the box).

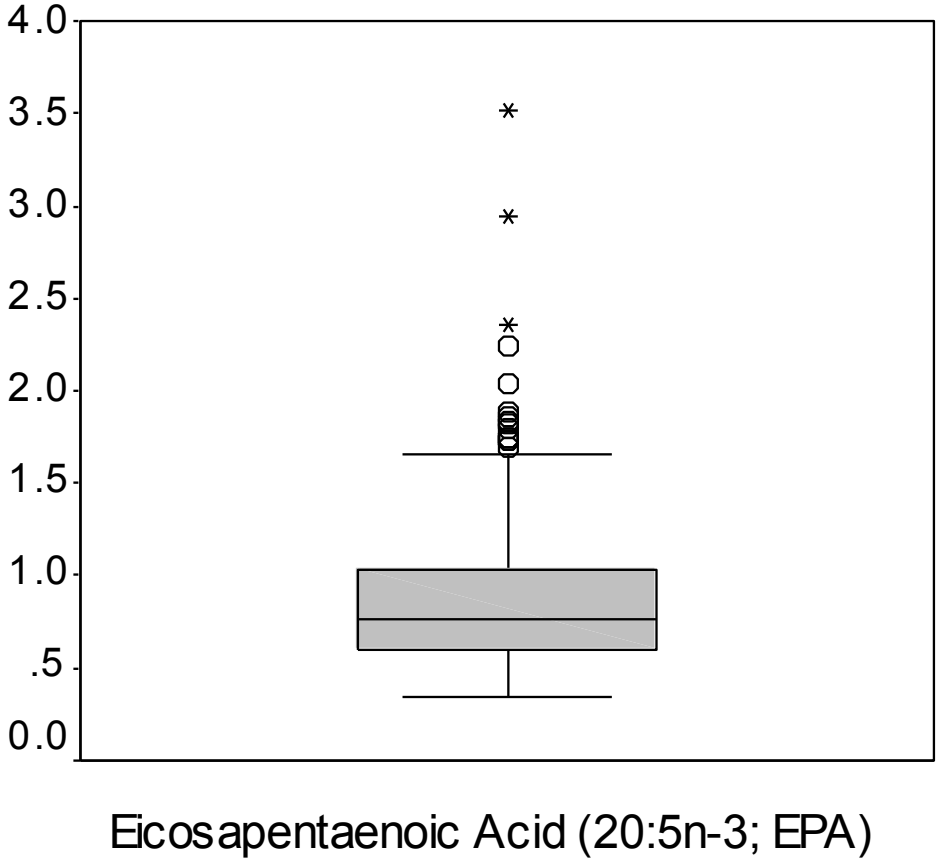


**Figure A11. Distribution of erythrocyte EPA in the study population.**

In this boxplot, the edges of the box correspond to 25th and 75th percentiles. The horizontal line in the middle of the box corresponds to the median. The vertical bars above and below the box correspond to percentiles 10 and 90. Open circles beyond the box correspond to outliers (values between 1.5 and 3 box lengths from the upper or lower edge of the box) and stars correspond to extremes (values beyond 3 box lengths from the edge of the box).

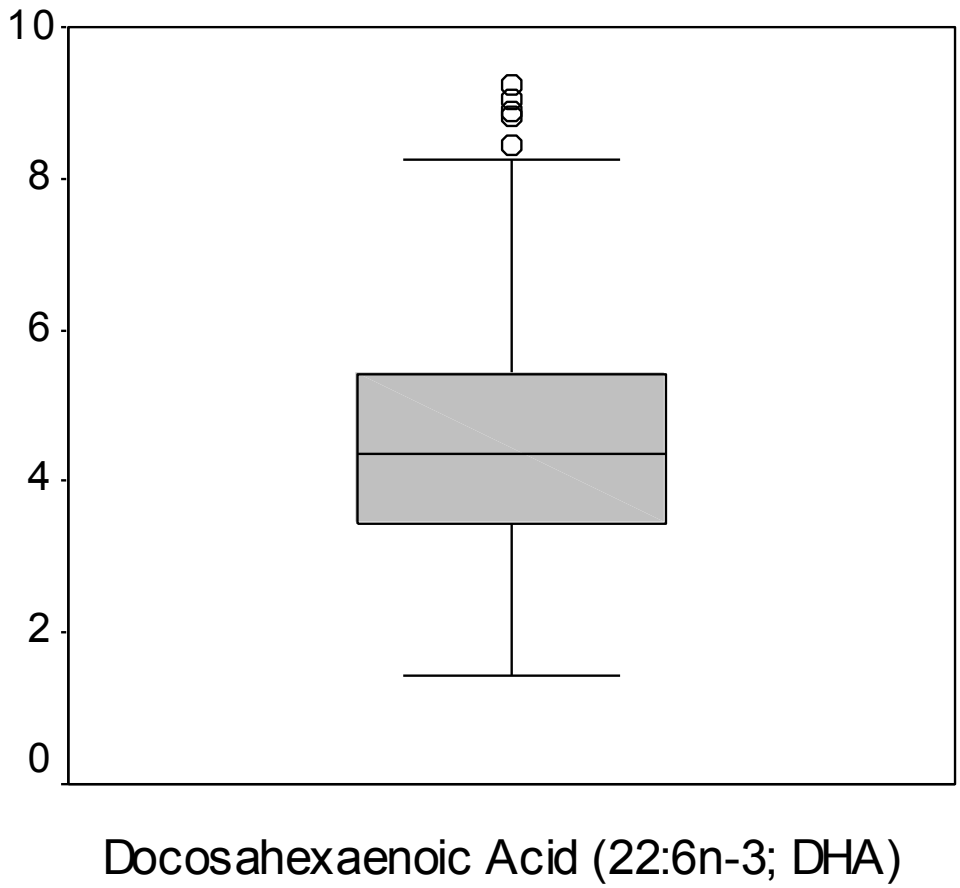


**Figure A12. Distribution of erythrocyte DHA in the study population.**

In this boxplot, the edges of the box correspond to 25th and 75th percentiles. The horizontal line in the middle of the box corresponds to the median. The vertical bars above and below the box correspond to percentiles 10 and 90. Open circles beyond the box correspond to outliers (values between 1.5 and 3 box lengths from the upper or lower edge of the box).

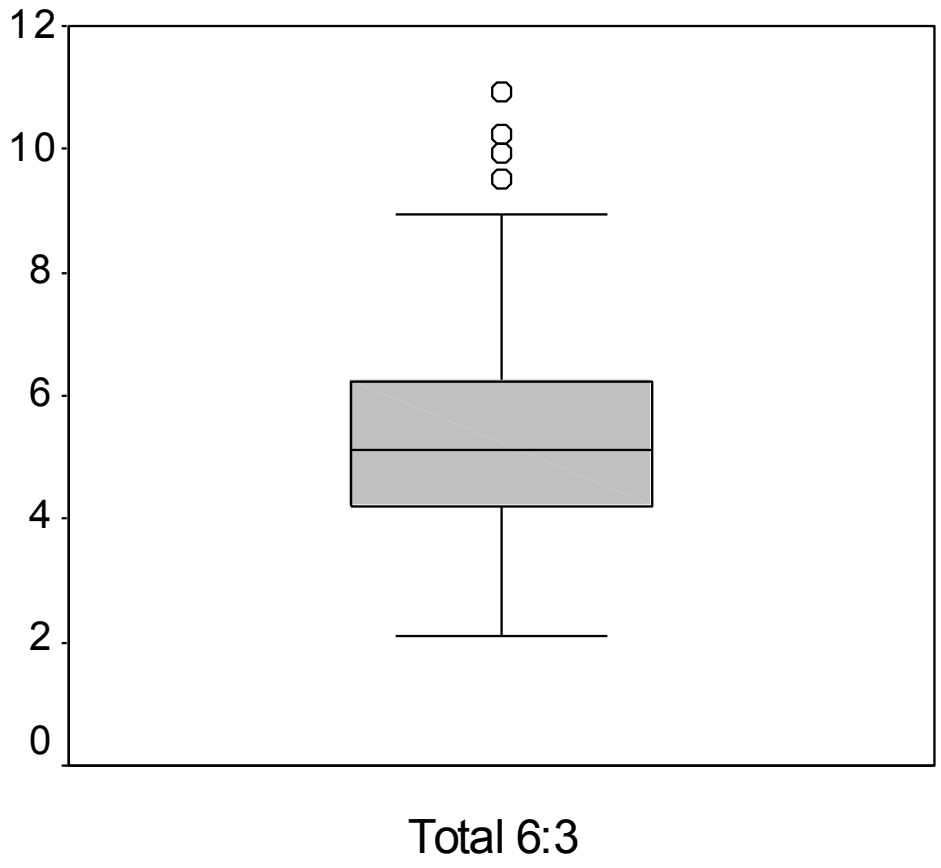


**Figure A13. Distribution of the erythrocyte total 6:3 ratio in the study population.**

In this boxplot, the edges of the box correspond to 25th and 75th percentiles. The horizontal line in the middle of the box corresponds to the median. The vertical bars above and below the box correspond to percentiles 10 and 90. Open circles beyond the box correspond to outliers (values between 1.5 and 3 box lengths from the upper or lower edge of the box).

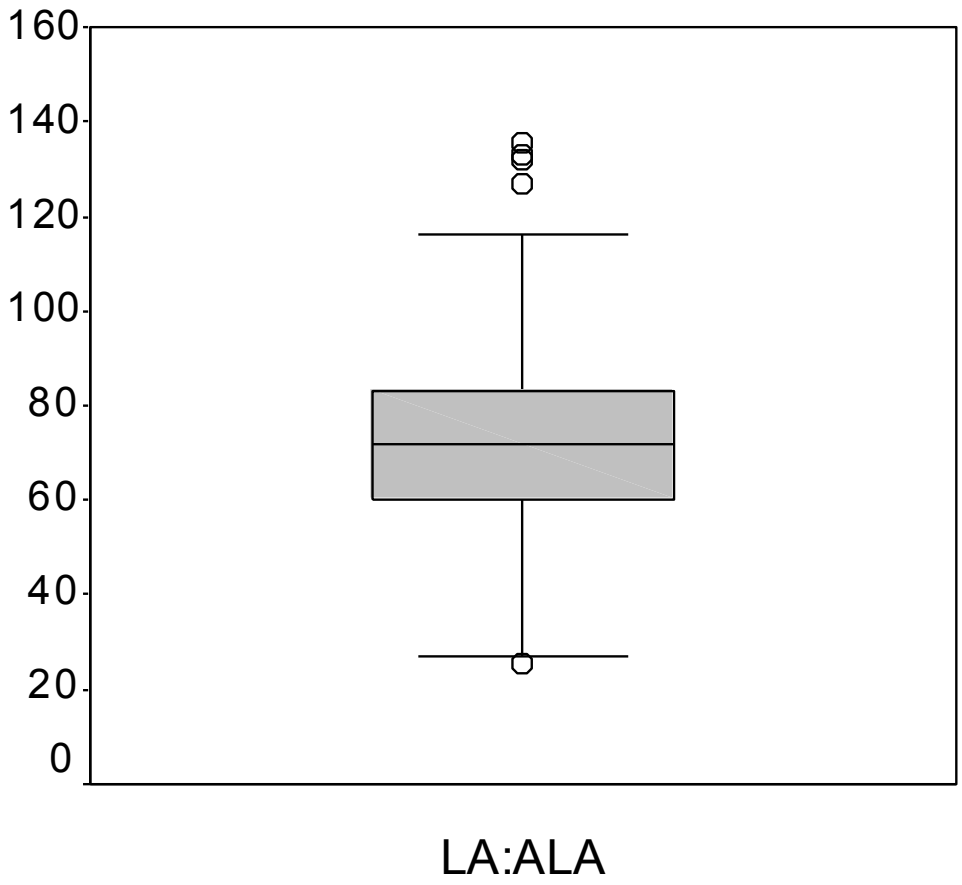


**Figure A14. Distrubution of the erythrocyte LA:ALA ratio in the study population.**

In this boxplot, the edges of the box correspond to 25th and 75th percentiles. The horizontal line in the middle of the box corresponds to the median. The vertical bars above and below the box correspond to percentiles 10 and 90. Open circles beyond the box correspond to outliers (values between 1.5 and 3 box lengths from the upper or lower edge of the box).

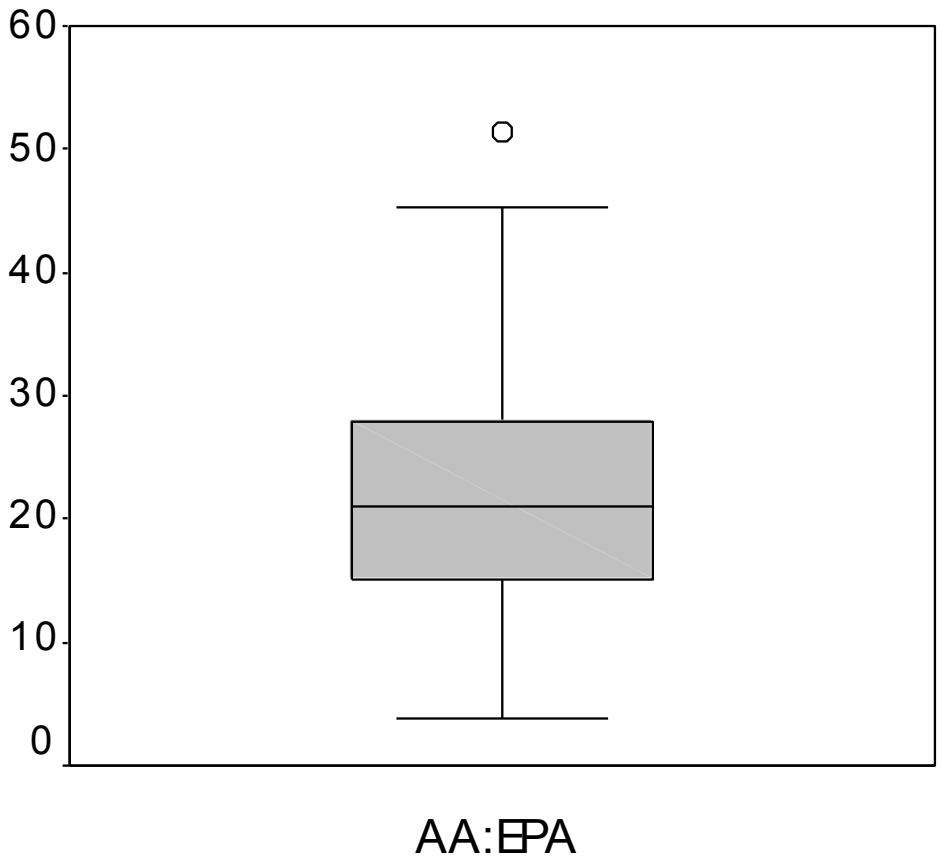


**Figure A15. Distribution of the erythrocyte AA:EPA ratio in the study population.**

In this boxplot, the edges of the box correspond to 25th and 75th percentiles. The horizontal line in the middle of the box corresponds to the median. The vertical bars above and below the box correspond to percentiles 10 and 90. Open circles beyond the box correspond to outliers (values between 1.5 and 3 box lengths from the upper or lower edge of the box).

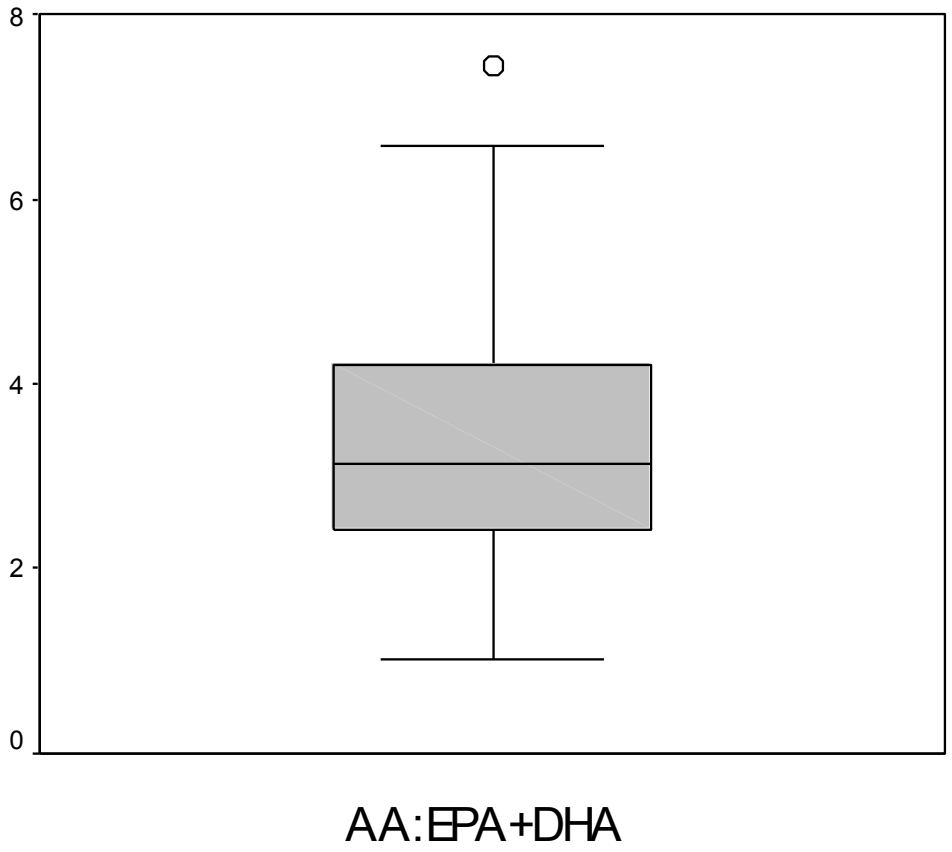


**Figure A16. Distribution of the erythrocyte AA:EPA+DHA ratio in the study population.**

In this boxplot, the edges of the box correspond to 25th and 75th percentiles. The horizontal line in the middle of the box corresponds to the median. The vertical bars above and below the box correspond to percentiles 10 and 90. Open circles beyond the box correspond to outliers (values between 1.5 and 3 box lengths from the upper or lower edge of the box).

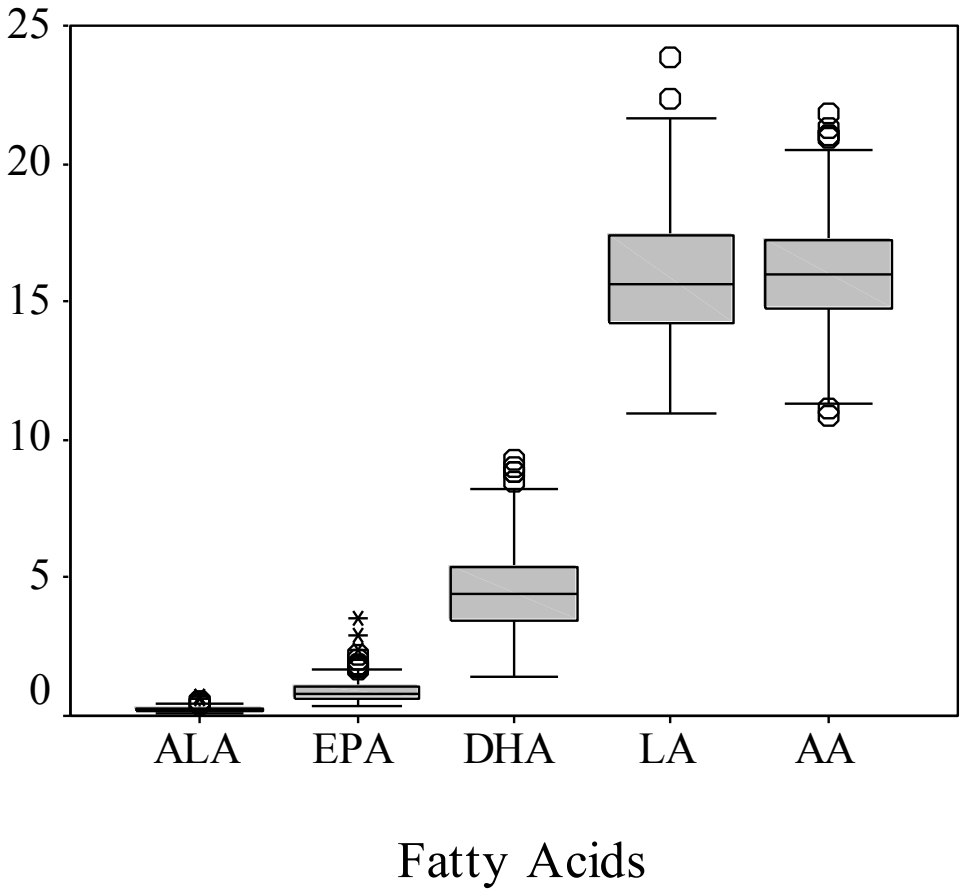


**Figure A17. Distribution of erythrocyte individual n-6 and n-3 fatty acids in the study population.**

In this boxplot, the edges of the box correspond to 25th and 75th percentiles. The horizontal line in the middle of the box corresponds to the median. The vertical bars above and below the box correspond to percentiles 10 and 90. Open circles beyond the box correspond to outliers (values between 1.5 and 3 box lengths from the upper or lower edge of the box) and stars correspond to extremes (values beyond 3 box lengths from the edge of the box).

**Table A19. Coefficients of variation for erythrocyte n-6 and n-3 fatty acids**

| Fatty Acid (wt. %) | Mean (SD) | Coefficient of Variation (%) |
|---|---|---|
| n-6 PUFAs | | |
| Total n-6 PUFAs* | 38.3 (2.6) | 1.7 |
| 18:2n-6 (LA) | 15.8 (2.4) | 4.6 |
| 20:4n-6 (AA) | 16.0 (2.0) | 3.4 |
| n-3 PUFAs | | |
| Total n-3 PUFAs† | 7.9 (2.0) | 5.3 |
| 18:3n-3 (ALA) | 0.2 (0.1) | 15.2 |
| 20:5n-3 (EPA) | 0.9 (0.4) | 5.3 |
| 22:6n-3 (DHA) | 4.5 (1.5) | 7.5 |
| 6:3 Ratios | | |
| Total n-6:n-3 | 5.2 (1.5) | 5.2 |
| LA:ALA | 72.7 (19.3) | 11.1 |
| AA:EPA | 21.8 (9.0) | 4.5 |
| AA:EPA+DHA | 3.3 (1.2) | 5.7 |

NOTE: Fatty acids are reported as a percentage by weight of the total fatty acids (weight percent, wt. %). PUFA, polyunsaturated fatty acid; LA, linoleic acid; AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; and DHA, docosahexaenoic acid

*18:2n-6+18:3n-6+20:2n-6+20:3n-6+20:4n-6+22:4n-6+ 22:5n-6

†18:3n-3+20:4n-3+20:5n-3+22:5n-3+22:6n-3

**Table A20. Spearman correlation coefficients among erythrocyte n-6 and n-3 fatty acids**

| | 18:3 n-6 | 20:2 n-6 | 20:3 n-6 | 20:4 n-6 | 22:4 n-6 | 22:5 n-6 | 18:3 n-3 | 20:4 n-3 | 20:5 n-3 | 22:5 n-3 | 22:6 n-3 | Total n-6* | Total n-3† |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 18:2n-6 | 0.16 | 0.29 | 0.14 | -0.45 | -0.36 | -0.25 | 0.60 | 0.10 | -0.06 | -0.34 | -0.24 | 0.46 | -0.24 |
| 18:3n-6 | | -0.27 | 0.17 | -0.10 | -0.15 | -0.08 | 0.28 | 0.06 | 0.03 | -0.21 | -0.29 | -0.25 | -0.06 |
| 20:2n-6 | | | 0.05 | -0.22 | 0.01 | -0.01 | 0.16 | 0.16 | -0.17 | -0.12 | -0.05 | 0.12 | -0.08 |
| 20:3n-6 | | | | -0.27 | -0.01 | 0.19 | 0.04 | 0.47 | -0.19 | -0.10 | -0.31 | 0.09 | -0.28 |
| 20:4n-6 | | | | | 0.51 | 0.37 | -0.38 | -0.39 | -0.15 | 0.09 | -0.08 | 0.45 | -0.09 |
| 22:4n-6 | | | | | | 0.72 | -0.36 | -0.32 | -0.51 | -0.01 | -0.44 | 0.42 | -0.45 |
| 22:5n-6 | | | | | | | -0.31 | -0.19 | -0.70 | -0.22 | -0.54 | 0.39 | -0.59 |
| 18:3n-3 | | | | | | | | 0.31 | 0.17 | -0.09 | -0.10 | 0.15 | -0.01 |
| 20:4n-3 | | | | | | | | | 0.21 | 0.02 | 0.06 | -0.25 | 0.13 |
| 20:5n-3 | | | | | | | | | | 0.55 | 0.70 | -0.42 | 0.82 |
| 22:5n-3 | | | | | | | | | | | 0.38 | -0.29 | 0.57 |
| 22:6n-3 | | | | | | | | | | | | -0.49 | 0.96 |
| Total n-6* | | | | | | | | | | | | | -0.51 |

NOTE: Correlations coefficients calculated on 260 postmenopausal participants in the Mammograms and Masses Study (MAMS)

*18:2n-6+18:3n-6+20:2n-6+20:3n-6+20:4n-6+22:4n-6+22:5n-6

†18:3n-3+20:4n-3+20:5n-3+22:5n-3+22:6n-3

**Table A21. Relationship between erythrocyte n-6 and n-3 fatty acids and family history of breast cancer**

| | Family history of breast cancer | | |
|---|---|---|---|
| Fatty Acids (wt. %) | yes (n=34) | no (n=224) | p |
| Total n-6 PUFA* | 38.4 (2.4) | 38.3 (2.7) | 0.83 |
| 18:2n-6 (LA) | 15.7 (2.3) | 15.8 (2.5) | 0.78 |
| 20:4n-6 (AA) | 16.1 (1.9) | 16.0 (2.0) | 0.90 |
| Total n-3 PUFA† | 8.0 (1.7) | 7.9 (2.1) | 0.37 |
| 18:3n-3 (ALA) | 0.3 (0.1) | 0.2 (0.1) | 0.30 |
| 20:5n-3 (EPA) | 0.9 (0.3) | 0.9 (0.5) | 0.16 |
| 22:6n-3 (DHA) | 4.5 (1.3) | 4.5 (1.5) | 0.83 |
| Total n-6:n-3 | 5.0 (1.2) | 5.3 (1.6) | 0.44 |
| LA:ALA | 68.1 (16.1) | 73.4 (19.8) | 0.12 |
| AA:EPA | 20.0 (7.7) | 22.1 (9.1) | 0.20 |
| AA:EPA+DHA | 3.2 (1.0) | 3.3 (1.2) | 0.63 |

NOTE: Data are expressed as mean (SD). Fatty acids are reported as a percentage by weight of the total fatty acids (weight percent, wt. %). PUFA, polyunsaturated fatty acid; LA, linoleic acid; AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid

*18:2n-6+18:3n-6+20:2n-6+20:3n-6+20:4n-6+22:4n-6+22:5n-6

†18:3n-3+20:4n-3+20:5n-3+22:5n-3+22:6n-3

**Table A22. Relationship between erythrocyte n-6 and n-3 fatty acids and postmenopausal hormone therapy use**

| | Ever hormone therapy use | | |
|---|---|---|---|
| Fatty Acids (wt. %) | yes (n=160) | no (n=100) | p |
| Total n-6 PUFA* | 38.2 (2.7) | 38.4 (2.6) | 0.63 |
| 18:2n-6 (LA) | 15.9 (2.5) | 15.6 (2.3) | 0.36 |
| 20:4n-6 (AA) | 15.9 (2.0) | 16.3 (2.0) | 0.20 |
| Total n-3 PUFA† | 7.9 (2.1) | 7.8 (1.9) | 0.74 |
| 18:3n-3 (ALA) | 0.2 (0.1) | 0.2 (0.1) | 0.27 |
| 20:5n-3 (EPA) | 0.9 (0.4) | 0.9 (0.4) | 0.35 |
| 22:6n-3 (DHA) | 4.5 (1.6) | 4.5 (1.5) | 0.69 |
| Total n-6:n-3 | 5.2 (1.5) | 5.3 (1.5) | 0.86 |
| LA:ALA | 71.8 (19.0) | 74.0 (20.0) | 0.47 |
| AA:EPA | 21.2 (8.9) | 22.8 (9.0) | 0.13 |
| AA:EPA+DHA | 3.3 (1.1) | 3.4 (1.3) | 0.71 |

NOTE: Data are expressed as mean (SD). Fatty acids are reported as a percentage by weight of the total fatty acids (weight percent, wt. %). PUFA, polyunsaturated fatty acid; LA, linoleic acid; AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid

*18:2n-6+18:3n-6+20:2n-6+20:3n-6+20:4n-6+22:4n-6+22:5n-6

†18:3n-3+20:4n-3+20:5n-3+22:5n-3+22:6n-3

**Table A23. Relationship between erythrocyte n-6 and n-3 fatty acids and current smoking status**

| | Current smoker | | |
|---|---|---|---|
| Fatty Acids (wt. %) | yes (n=15) | no (n=245) | p |
| Total n-6 PUFA* | 39.9 (2.1) | 38.2 (2.6) | 0.02 |
| 18:2n-6 (LA) | 16.2 (1.8) | 15.8 (2.5) | 0.36 |
| 20:4n-6 (AA) | 16.6 (2.5) | 16.0 (2.0) | 0.29 |
| Total n-3 PUFA† | 6.5 (1.4) | 8.0 (2.1) | 0.007 |
| 18:3n-3 (ALA) | 0.2 (0.1) | 0.2 (0.1) | 0.64 |
| 20:5n-3 (EPA) | 0.6 (0.2) | 0.9 (0.4) | 0.006 |
| 22:6n-3 (DHA) | 3.6 (1.2) | 4.6 (1.5) | 0.02 |
| Total n-6:n-3 | 6.5 (1.8) | 5.2 (1.5) | 0.005 |
| LA:ALA | 81.3 (23.7) | 72.1 (19.0) | 0.16 |
| AA:EPA | 29.1 (9.0) | 21.4 (8.8) | 0.003 |
| AA:EPA+DHA | 4.3 (1.5) | 3.3 (1.1) | 0.004 |

NOTE: Data are expressed as mean (SD). Fatty acids are reported as a percentage by weight of the total fatty acids (weight percent, wt. %). PUFA, polyunsaturated fatty acid; LA, linoleic acid; AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid

*18:2n-6+18:3n-6+20:2n-6+20:3n-6+20:4n-6+22:4n-6+22:5n-6

†18:3n-3+20:4n-3+20:5n-3+22:5n-3+22:6n-3

**Table A24. Relationship between erythrocyte n-6 and n-3 fatty acids and race**

| | Race | | |
|---|---|---|---|
| Fatty Acids (wt. %) | white (n=242) | non-white (n=18) | p |
| Total n-6 PUFA* | 38.2 (2.6) | 39.6 (2.5) | 0.03 |
| 18:2n-6 (LA) | 15.8 (2.5) | 16.0 (2.0) | 0.54 |
| 20:4n-6 (AA) | 15.9 (1.9) | 17.1 (2.7) | 0.08 |
| Total n-3 PUFA† | 7.8 (2.0) | 8.4 (2.5) | 0.39 |
| 18:3n-3 (ALA) | 0.2 (0.1) | 0.3 (0.1) | 0.99 |
| 20:5n-3 (EPA) | 0.9 (0.4) | 0.9 (0.7) | 0.53 |
| 22:6n-3 (DHA) | 4.5 (1.5) | 5.0 (1.6) | 0.17 |
| Total n-6:n-3 | 5.2 (1.5) | 5.1 (1.4) | 0.80 |
| LA:ALA | 72.6 (18.8) | 73.8 (26.3) | 0.97 |
| AA:EPA | 21.6 (8.7) | 24.9 (11.5) | 0.22 |
| AA:EPA+DHA | 3.3 (1.2) | 3.2 (1.1) | 0.86 |

NOTE: Data are expressed as mean (SD). Fatty acids are reported as a percentage by weight of the total fatty acids (weight percent, wt. %). PUFA, polyunsaturated fatty acid; LA, linoleic acid; AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid

*18:2n-6+18:3n-6+20:2n-6+20:3n-6+20:4n-6+22:4n-6+22:5n-6

†18:3n-3+20:4n-3+20:5n-3+22:5n-3+22:6n-3

**Table A25. Relationship between erythrocyte n-6 and n-3 fatty acids and alcohol intake**

| | Past year alcohol intake | | | |
|---|---|---|---|---|
| Fatty Acids (wt. %) | none (n=188) | <12g/day (n=46) | ≥12g/day (n=26) | p |
| Total n-6 PUFA* | 38.5 (2.6) | 38.1 (2.3) | 37.0 (3.0) | 0.05 |
| 18:2n-6 (LA) | 15.6 (2.5) | 16.7 (2.1) | 15.9 (2.2) | 0.01 |
| 20:4n-6 (AA) | 16.3 (2.0) | 15.4 (1.8) | 15.2 (1.9) | 0.007 |
| Total n-3 PUFA† | 7.7 (2.0) | 8.2 (2.2) | 8.5 (2.0) | 0.07 |
| 18:3n-3 (ALA) | 0.2 (0.1) | 0.2 (0.1) | 0.3 (0.1) | 0.17 |
| 20:5n-3 (EPA) | 0.8 (0.4) | 1.0 (0.5) | 1.1 (0.5) | <0.001 |
| 22:6n-3 (DHA) | 4.4 (1.5) | 4.7 (1.6) | 4.8 (1.4) | 0.22 |
| Total n-6:n-3 | 5.4 (1.5) | 5.0 (1.5) | 4.6 (1.3) | 0.04 |
| LA:ALA | 73.2 (19.2) | 72.5 (19.2) | 69.0 (20.8) | 0.73 |
| AA:EPA | 23.3 (8.9) | 18.9 (8.4) | 16.3 (6.7) | <0.0001 |
| AA:EPA+DHA | 3.5 (1.2) | 3.0 (1.1) | 2.8 (1.0) | 0.01 |

NOTE: Data are expressed as mean (SD). Fatty acids are reported as a percentage by weight of the total fatty acids (weight percent, wt. %). PUFA, polyunsaturated fatty acid; LA, linoleic acid; AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid

*18:2n-6+18:3n-6+20:2n-6+20:3n-6+20:4n-6+22:4n-6+22:5n-6

†18:3n-3+20:4n-3+20:5n-3+22:5n-3+22:6n-3

**Table A26. Relationship between erythrocyte n-6 and n-3 fatty acids and age**

| Fatty Acids (wt. %) | r | p |
|---|---|---|
| Total n-6 PUFA* | -0.24 | 0.0001 |
| 18:2n-6 (LA) | -0.25 | <0.0001 |
| 20:4n-6 (AA) | 0.05 | 0.38 |
| Total n-3 PUFA† | 0.19 | 0.003 |
| 18:3n-3 (ALA) | -0.10 | 0.10 |
| 20:5n-3 (EPA) | 0.12 | 0.06 |
| 22:6n-3 (DHA) | 0.19 | 0.002 |
| Total n-6:n-3 | -0.22 | 0.0003 |
| LA:ALA | -0.05 | 0.42 |
| AA:EPA | -0.10 | 0.12 |
| AA:EPA+DHA | -0.16 | 0.01 |

NOTE: Fatty acids are reported as a percentage by weight of the total fatty acids (weight percent, wt. %). r=Spearman correlation coefficient. PUFA, polyunsaturated fatty acid; LA, linoleic acid; AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid

*18:2n-6+18:3n-6+20:2n-6+20:3n-6+20:4n-6+22:4n-6+22:5n-6

†18:3n-3+20:4n-3+20:5n-3+22:5n-3+22:6n-3

**Table A27. Relationship between erythrocyte n-6 and n-3 fatty acids and BMI**

| Fatty Acids (wt. %) | r | p |
|---|---|---|
| Total n-6 PUFA* | 0.12 | 0.05 |
| 18:2n-6 (LA) | -0.11 | 0.08 |
| 20:4n-6 (AA) | 0.10 | 0.12 |
| Total n-3 PUFA† | -0.24 | <0.0001 |
| 18:3n-3 (ALA) | -0.14 | 0.03 |
| 20:5n-3 (EPA) | -0.21 | 0.0005 |
| 22:6n-3 (DHA) | -0.21 | 0.0006 |
| Total n-6:n-3 | 0.24 | 0.0001 |
| LA:ALA | 0.12 | 0.05 |
| AA:EPA | 0.22 | 0.0003 |
| AA:EPA+DHA | 0.24 | 0.0001 |

NOTE: Fatty acids are reported as a percentage by weight of the total fatty acids (weight percent, wt. %). r=Spearman correlation coefficient. BMI, body mass index; PUFA, polyunsaturated fatty acid; LA, linoleic acid; AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid

*18:2n-6+18:3n-6+20:2n-6+20:3n-6+20:4n-6+22:4n-6+22:5n-6

†18:3n-3+20:4n-3+20:5n-3+22:5n-3+22:6n-3

**Table A28. Relationship between erythrocyte n-6 and n-3 fatty acids and years since onset of menopause**

| Fatty Acids (wt. %) | r | p |
|---|---|---|
| Total n-6 PUFA* | -0.20 | 0.001 |
| 18:2n-6 (LA) | -0.22 | 0.0004 |
| 20:4n-6 (AA) | 0.04 | 0.50 |
| Total n-3 PUFA† | 0.17 | 0.006 |
| 18:3n-3 (ALA) | -0.09 | 0.14 |
| 20:5n-3 (EPA) | 0.09 | 0.13 |
| 22:6n-3 (DHA) | 0.18 | 0.004 |
| Total n-6:n-3 | -0.20 | 0.002 |
| LA:ALA | -0.03 | 0.65 |
| AA:EPA | -0.08 | 0.19 |
| AA:EPA+DHA | -0.15 | 0.02 |

NOTE: Fatty acids are reported as a percentage by weight of the total fatty acids (weight percent, wt. %). r=Spearman correlation coefficient. PUFA, polyunsaturated fatty acid; LA, linoleic acid; AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid

*18:2n-6+18:3n-6+20:2n-6+20:3n-6+20:4n-6+22:4n-6+22:5n-6

†18:3n-3+20:4n-3+20:5n-3+22:5n-3+22:6n-3

**Table A29. Mean fatty acid composition in erythrocytes according to NSAID use**

| Fatty Acids (wt. %) | Mean fatty acid (SD) NSAID non-users (N=136) | NSAID users (N=124) | p |
|---|---|---|---|
| Total n-6 PUFA* | 38.3 (2.8) | 38.4 (2.5) | 0.79 |
| 18:2n-6 (LA) | 16.0 (2.5) | 15.6 (2.4) | 0.27 |
| 20:4n-6 (AA) | 15.8 (2.1) | 16.2 (1.8) | 0.11 |
| Total n-3 PUFA† | 7.8 (2.0) | 8.0 (2.1) | 0.51 |
| 18:3n-3 (ALA) | 0.3 (0.1) | 0.2 (0.1) | 0.04 |
| 20:5n-3 (EPA) | 0.9 (0.4) | 0.9 (0.5) | 0.26 |
| 22:6n-3 (DHA) | 4.5 (1.5) | 4.5 (1.5) | 0.98 |
| Total n-6:n-3 | 5.3 (1.5) | 5.2 (1.5) | 0.73 |
| LA:ALA | 70.6 (19.1) | 75.0 (19.4) | 0.07 |
| AA:EPA | 22.2 (9.1) | 21.4 (8.9) | 0.46 |
| AA:EPA+DHA | 3.3 (1.2) | 3.4 (1.2) | 0.65 |

NOTE: Data are expressed as mean (SD). Fatty acids are reported as a percentage by weight of the total fatty acids (weight percent, wt. %). NSAID, nonsteroidal anti-inflammatory drug; PUFA, polyunsaturated fatty acid; LA, linoleic acid; AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid

*18:2n-6+18:3n-6+20:2n-6+20:3n-6+20:4n-6+22:4n-6+22:5n-6

†18:3n-3+20:4n-3+20:5n-3+22:5n-3+22:6n-3

**Table A30. Spearman correlation coefficients between n-6 and n-3 fatty acids in erythrocytes and serum estradiol concentrations**

| | NSAID non-users (N=136) | | NSAID users (N=124) | |
|---|---|---|---|---|
| Fatty acids (wt. %) | r | p | r | p |
| n-6 PUFAs | | | | |
| Total n-6 PUFAs* | 0.27 | 0.001 | 0.04 | 0.67 |
| 18:2n-6 (LA) | -0.04 | 0.65 | -0.13 | 0.14 |
| 20:4n-6 (AA) | 0.18 | 0.04 | 0.11 | 0.21 |
| n-3 PUFAs | | | | |
| Total n-3 PUFAs† | -0.25 | 0.003 | -0.18 | 0.04 |
| 18:3n-3 (ALA) | -0.14 | 0.10 | -0.24 | 0.01 |
| 20:5n-3 (EPA) | -0.22 | 0.01 | -0.22 | 0.01 |
| 22:6n-3 (DHA) | -0.22 | 0.009 | -0.11 | 0.22 |
| 6:3 Ratios | | | | |
| Total n-6:n-3 | 0.27 | 0.001 | 0.18 | 0.05 |
| LA:ALA | 0.16 | 0.07 | 0.23 | 0.01 |
| AA:EPA | 0.24 | 0.004 | 0.24 | 0.01 |
| AA:EPA+DHA | 0.28 | 0.0008 | 0.16 | 0.07 |

NOTE:). Fatty acids are reported as a percentage by weight of the total fatty acids (weight percent, wt. %). NSAID, nonsteroidal anti-inflammatory drug; PUFA, polyunsaturated fatty acid; LA, linoleic acid; AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid

*18:2n-6+18:3n-6+20:2n-6+20:3n-6+20:4n-6+22:4n-6+22:5n-6

†18:3n-3+20:4n-3+20:5n-3+22:5n-3+22:6n-3

**Table A31. Correlations between fatty acids and estradiol**

| | Serum estradiol (pmol/L) | |
|---|---|---|
| Fatty Acid (wt. %) | r | p |
| 14:0 | -0.05 | 0.43 |
| 15:0 | 0.14 | 0.03 |
| 16:0 | -0.05 | 0.45 |
| 17:0 | -0.01 | 0.89 |
| 18:0 | 0.07 | 0.24 |
| 16:1t | -0.01 | 0.82 |
| 18:1 t-1 | -0.09 | 0.13 |
| 18:1 t-2 | 0.02 | 0.74 |
| 18:1 t-3 | 0.02 | 0.80 |
| 18:1 t-4 | -0.02 | 0.79 |
| 18:1 t-5 | -0.09 | 0.14 |
| 18:2 tt | -0.04 | 0.53 |
| 16:1n7c | -0.03 | 0.66 |
| 18:1n9c | -0.05 | 0.41 |
| 18:1n7c | -0.11 | 0.08 |
| 20:1n9 | 0.10 | 0.11 |
| 24:1n9 | 0.12 | 0.05 |

Note: Fatty acids are reported as a percentage by weight of the total fatty acids (weight percent, wt. %). r= Spearman correlation coefficient. Adjusted for age (continuous) and BMI (continuous)

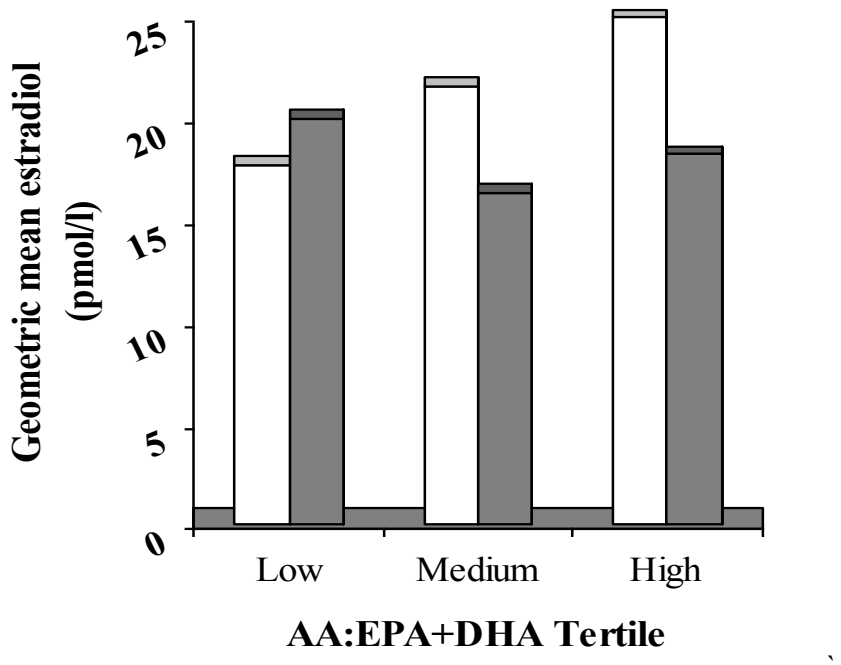


**Figure A18**. **Geometric mean serum estradiol concentrations (pmol/L) by tertile of the AA:EPA+DHA ratio according to NSAID use**

NOTE: AA:EPA+DHA= Arachidonic acid to Eicosapentaenoic acid+Docosahexaenoic acid ratio. A log transformation was applied to estradiol concentrations. Adjusted for age (continuous), BMI (continuous), years menopausal (continuous), alcohol intake (none, <12g/day, ≥12g/day) and current smoker (yes vs. no). □ Geometric means (95% CI) of estradiol for increasing tertile of the AA:EPA+DHA ratio among participants not taking NSAIDs [17.7 (14.8, 21.1); 21.5 (17.8, 26.0); and 24.9 (20.6, 20.0)].■ Geometric means (95% CI) of estradiol for increasing tertile of the AA:EPA+DHA ratio among participants taking NSAIDs [19.9 (15.7, 25.5); 16.2 (13.2, 20.0); and 18.1 (14.5, 22.7)].

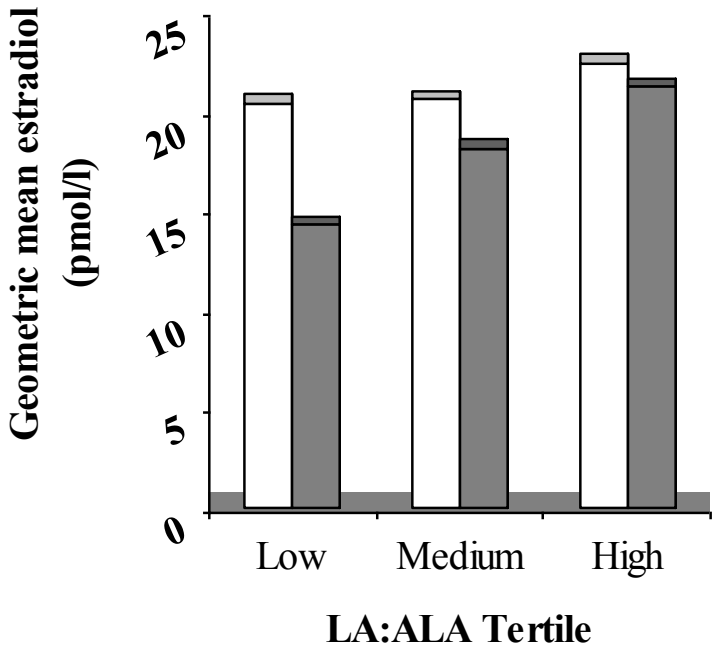


**Figure A19**. **Geometric mean serum estradiol concentrations (pmol/L) by tertile of the LA:ALA ratio according to NSAID use**

NOTE: LA:ALA=linoleic acid to alpha-linolenic acid ratio. log transformation was applied to estradiol concentrations. Adjusted for age (continuous), BMI (continuous), years menopausal (continuous), alcohol intake (none, <12g/day, ≥12g/day) and current smoker (yes vs. no). □ Geometric means (95% CI) of estradiol for increasing tertile of the LA:ALA ratio among participants not taking NSAIDs [20.4 (17.0, 24.3); 20.5 (17.0, 24.7); and 22.4 (18.2, 27.4)].■ Geometric means (95% CI) of estradiol for increasing tertile of the AA:EPA+DHA ratio among participants taking NSAIDs [14.2 (11.3, 17.9); 18.1 (14.5, 22.5); and 21.1 (17.3, 25.8)].

**Table A32. Spearman correlation coefficients between erythroyte n-6 and n-3 fatty acids and serum estradiol concentrations (pmol/L) including 9 participants with estradiol levels >150pmol/L**

| Fatty Acid (wt. %) | r | p |
|---|---|---|
| n-6 PUFAs | | |
| Total n-6 PUFAs* | 0.15 | 0.01 |
| 18:2n-6 (LA) | -0.06 | 0.35 |
| 20:4n-6 (AA) | 0.13 | 0.03 |
| n-3 PUFAs | | |
| Total n-3 PUFAs† | -0.21 | 0.0004 |
| 18:3n-3 (ALA) | -0.18 | 0.003 |
| 20:5n-3 (EPA) | -0.23 | 0.002 |
| 22:6n-3 (DHA) | -0.17 | 0.007 |
| 6:3 Ratios | | |
| Total n-6:n-3 | 0.22 | 0.0003 |
| LA:ALA | 0.19 | 0.002 |
| AA:EPA | 0.24 | <0.0001 |
| AA:EPA+DHA | 0.21 | 0.0005 |

NOTE: N=269. Fatty acids are reported as a percentage by weight of the total fatty acids (weight percent, wt. %). PUFA, polyunsaturated fatty acid; LA, linoleic acid; AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; and DHA, docosahexaenoic acid

*18:2n-6+18:3n-6+20:2n-6+20:3n-6+20:4n-6+22:4n-6+22:5n-6

†18:3n-3+20:4n-3+20:5n-3+22:5n-3+22:6n-3

# APPENDIX B: NSAID USE AND SERUM TOTAL ESTRADIOL IN POSTMENOPAUSAL WOMEN ADDITIONAL ANALYSES

**Table B33. Distribution of selected characteristics by categories of NSAID use among postmenopausal women**

| | Past 48 hour use | | | Consistent use | | |
|---|---|---|---|---|---|---|
| Characteristic | User (N=140) | Non-user (N=120) | p | User (N=100) | Non-user (N=96) | p |
| Age at blood draw (years), mean (SD) | 63.2 (8.6) | 62.3 ( 8.1) | 0.38 | 62.3 (8.1) | 61.9 (8.1) | 0.53 |
| BMI (kg/m2), mean (SD) | 29.3 (6.4) | 27.5 (5.4 | 0.03 | 29.2 (6.3) | 27.8 (5.7) | 0.13 |
| Age at menopause (years), mean (SD)* | 48.8 (4.5) | 48.6 (5.7) | 0.73 | 48.5 (4.6) | 48.3 (6.1) | 0.65 |
| Years menopausal, mean (SD)* | 14.4 (9.7) | 13.7 (10.4) | 0.37 | 14.0 (10.0) | 13.6 (10.7) | 0.55 |
| Surgical menopause, %* | | | 0.23 | | | 0.26 |
| No | 90.7 | 85.8 | | 88.9 | 83.3 | |
| Yes | 9.4 | 14.2 | | 11.1 | 16.7 | |
| Age at menarche, %* | | | 0.56 | | | 0.63 |
| <12 years | 20.1 | 16.7 | | 18.0 | 14.6 | |
| 12-13 years | 57.6 | 55.8 | | 62.0 | 60.4 | |
| ≥14 years | 22.3 | 27.5 | | 20.0 | 25.0 | |
| Race, % | | | 0.22 | | | 0.06 |
| White | 95.0 | 90.8 | | 96.0 | 88.5 | |
| Non-white | 5.0 | 9.2 | | 4.0 | 11.5 | |
| Family history of breast cancer, %*† | | | 0.16 | | | 0.41 |
| No | 84.1 | 90.0 | | 85.7 | 89.6 | |
| Yes | 15.9 | 10.0 | | 14.3 | 10.4 | |
| Prior hormone therapy use, % | | | 0.83 | | | 0.82 |
| No | 37.9 | 39.2 | | 37.0 | 38.5 | |
| Yes | 62.1 | 60.8 | | 63.0 | 61.5 | |
| Previous breast biopsy, % | | | 1.00 | | | 0.90 |
| No | 85.0 | 85.0 | | 85.0 | 84.4 | |
| Yes | 15.0 | 15.0 | | 15.0 | 15.6 | |

**Table B33 (continued)**

| Characteristic | Past 48 hour use | | | Consistent use | | |
|---|---|---|---|---|---|---|
| | User (N=140) | Non-user (N=120) | p | User (N=100) | Non-user (N=96) | p |
| Regular alcohol intake in past year, % | | | 0.76 | | | 0.28 |
| None | 70.7 | 74.2 | | 68.0 | 78.1 | |
| < 12 g/day | 19.3 | 15.8 | | 22.0 | 14.6 | |
| ≥ 12 g/day | 10.0 | 10.0 | | 10.0 | 7.3 | |
| Smoking status, % | | | 0.54 | | | 0.91 |
| Never | 61.4 | 60.0 | | 63.0 | 63.5 | |
| Former | 34.3 | 32.5 | | 32.0 | 30.2 | |
| Current | 4.3 | 7.5 | | 5.0 | 6.3 | |
| Parous, % | | | 0.33 | | | 0.38 |
| No | 21.4 | 16.7 | | 25.0 | 19.8 | |
| Yes | 78.6 | 83.3 | | 75.0 | 80.2 | |
| Age at first full-term pregnancy, % ‡ | | | 0.39 | | | 0.60 |
| < 30 years | 83.6 | 79.0 | | 81.3 | 77.9 | |
| ≥ 30 years | 16.4 | 21.0 | | 18.7 | 22.1 | |
| Ever breast fed for > 1month, % ‡ | | | 0.32 | | | 0.33 |
| No | 49.1 | 56.0 | | 44.0 | 52.0 | |
| Yes | 50.9 | 44.0 | | 56.0 | 48.1 | |

NOTE: BMI=body mass index; NSAID=nonsteroidal anti-inflammatory drug. Percentages's may not add up to 100% due to rounding

*missing n=7 for age at menopause; n=7 for years menopausal; n=1 for surgical menopause; n =1 for age at menarche; n=2 for family history of breast cancer

† family history of breast cancer in mother or sister

‡ among parous women

**Table B34. Geometric mean estradiol concentration (95% confidence interval) according to NSAID use, excluding participants in non-user group that reported taking acetaminophen and/or prescription narcotic analgesics at blood draw**

| | Serum estradiol concentrations (pmol/L) | | | | | |
|---|---|---|---|---|---|---|
| Participants (n=248) | Model 1* | p | Model 2† | p | Model 3‡ | p |
| Primary exposure variable | | | | | | |
| Current use | | 0.11 | | 0.03 | | 0.03 |
| NSAID Non-user (N=124) | 21.1 (18.4, 24.2) | | 21.3 (19.0, 23.9) | | 21.2 (18.9, 23.9) | |
| NSAID User (N=124) | 18.0 (15.7, 20.7) | | 17.8 (15.9, 20.0) | | 17.7 (15.7, 19.9) | |
| Constructed exposure variable | | | | | | |
| Consistent use | | 0.50 | | 0.05 | | 0.04 |
| NSAID Non-user (N=88) | 19.9 (16.9, 23.5) | | 21.1 (18.4, 24.1) | | 21.1 (18.4, 24.1) | |
| NSAID User (N=100) | 18.4 (15.8, 21.5) | | 17.5 (15.5, 19.9) | | 17.2 (15.1, 19.6) | |

NOTE: BMI=body mass index; NSAID=nonsteroidal anti-inflammatory drug. Current use: Based on participant's medication list. Past 48 hour use: Participant's verbal response to the question, "Have you taken an aspirin or other anti-inflammatory drug in the past 48 hours?" Consistent NSAID use: The agreement between current NSAID and past 48 hours use.

*Unadjusted model

†Adjusted for age at blood draw (continuous) and BMI(continuous)

‡ Missing N=7; Adjusted for age at blood draw (continuous), BMI (continuous), race (white, nonwhite), years menopausal (continuous), and current alcohol intake (none, <12 g/day, ≥12 g/day, indicator variable)

**Table B35. Geometric mean estradiol concentration (95% confidence interval) according to NSAID use including 9 participants with estradiol levels >150pmol/L**

| | Serum estradiol concentrations (pmol/L) | | | | | |
|---|---|---|---|---|---|---|
| Participants (n=269) | Model 1* | p | Model 2† | p | Model 3‡ | p |
| Primary exposure variable | | | | | | |
| Current use | | 0.06 | | 0.03 | | 0.03 |
| NSAID Non-user (N=142) | 23.8 (20.4, 27.7) | | 24.1 (20.9, 27.7) | | 24.0 (20.8, 27.7) | |
| NSAID User (N=127) | 19.2 (16.3, 22.6) | | 19.0 (16.4, 22.0) | | 18.9 (16.2, 22.0) | |
| Secondary exposure variable | | | | | | |
| Past 48 hour use | | 0.48 | | 0.10 | | 0.06 |
| NSAID Non-user (N=126) | 22.5 (19.1, 26.4) | | 23.6 (20.3, 27.4) | | 23.9 (20.6, 27.8) | |
| NSAID User (N=143) | 20.7 (17.8, 24.2) | | 19.8 (17.2, 22.8) | | 19.5 (16.9, 22.5) | |
| Constructed exposure variable | | | | | | |
| Consistent use | | 0.16 | | 0.03 | | 0.03 |
| NSAID Non-user (N=102) | 24.2 (20.0, 29.2) | | 25.1 (21.1, 29.9) | | 25.1 (21.0, 30.0) | |
| NSAID User (N=103) | 19.9 (16.5, 24.1) | | 19.2 (16.1, 22.8) | | 18.9 (15.8, 22.7) | |

NOTE: BMI=body mass index; NSAID=nonsteroidal anti-inflammatory drug. Current use: Based on participant's medication list. Past 48 hour use: Participant's verbal response to the question, "Have you taken an aspirin or other anti-inflammatory drug in the past 48 hours?" Consistent NSAID use: The agreement between current NSAID and past 48 hours use.

*Unadjusted model

†Adjusted for age at blood draw (continuous) and BMI(continuous)

‡Missing N=7; Adjusted for age at blood draw (continuous), BMI (continuous), race (white, nonwhite), years menopausal (continuous), and current alcohol intake (none, <12 g/day, ≥12 g/day, indicator variable)

**Table B36. Geometric mean estradiol concentration (95% confidence interval) according to type of NSAID use**

| | Serum estradiol concentrations (pmol/L) | | | | | |
|---|---|---|---|---|---|---|
| NSAID use | Model 1* | p | Model 2† | p | Model 3‡ | p |
| Aspirin | | 0.19 | | 0.05 | | 0.04 |
| Non-user (N=136) | 20.8 (18.6, 23.3) | | 21.3 (19.2, 23.7) | | 21.4 (19.2, 23.8) | |
| User (N=58) | 18.8 (14.8, 23.8) | | 17.6 (15.1, 20.5) | | 17.5 (14.9, 20.5) | |
| Non-Aspirin NSAIDs | | 0.42 | | 0.20 | | 0.45 |
| Non-user (N=136) | 21.0 (18.5, 24.0) | | 21.2 (19.0, 23.7) | | 20.8 (18.6, 23.3) | |
| User (N=32) | 18.6 (14.2, 24.4) | | 18.0 (14.3, 22.6) | | 18.8 (14.8, 23.8) | |

NOTE: BMI=body mass index; NSAID=nonsteroidal anti-inflammatory drug.

*Unadjusted model

†Adjusted for age at blood draw (continuous) and BMI(continuous)

‡Missing n=5 for aspirin user/nonuser analysis and N=2 for non-aspirin user/nonuser analysis; Adjusted for age at blood draw (continuous), BMI (continuous), race (white, nonwhite), years menopausal (continuous), and current alcohol intake (none, <12 g/day, ≥12 g/day, indicator variable)

**Table B37. Adjusted geometric mean estradiol concentrations (95% confidence interval) stratified by BMI subgroup according to categories of NSAID use**

| NSAID exposure category | Adjusted geometric mean serum estradiol (pmol/L)* | | | |
|---|---|---|---|---|
| | BMI <27.03 (n=125) | p | BMI ≥ 27.03 (n=128) | p |
| Current NSAID use | | 0.22 | | 0.07 |
| NSAID Non-user | 14.5 (12.5, 16.8) | | 30.9 (26.0, 36.6) | |
| NSAID User | 12.6 (10.7, 14.8) | | 24.6 (20.6, 29.3) | |
| 48 hour NSAID use | | 0.10 | | 0.27 |
| NSAID Non-user | 14.8 (12.8, 17.2) | | 30.1 (24.8, 36.5) | |
| NSAID User | 12.3 (10.5, 14.4) | | 26.1 (22.2, 30.6) | |
| Consistent NSAID use | | 0.07 | | 0.10 |
| NSAID Non-user | 15.0 (12.5, 17.8) | | 30.4 (24.8, 37.2) | |
| NSAID User | 11.7 (9.6, 14.2) | | 24.2 (20.1, 29.0) | |

NOTE: BMI=body mass index; NSAID=nonsteroidal anti-inflammatory drug. Median BMI based on entire population (n=260). Interaction terms between NSAID use and BMI were not statistically significant for any of the NSAID categories (all p-values ≥ 0.17)
*Missing N=7; Adjusted for age at blood draw (continuous), BMI (continuous), race (white, nonwhite), years menopausal (continuous), and current alcohol intake (none, <12g/day, ≥12g/day, indicator variable).

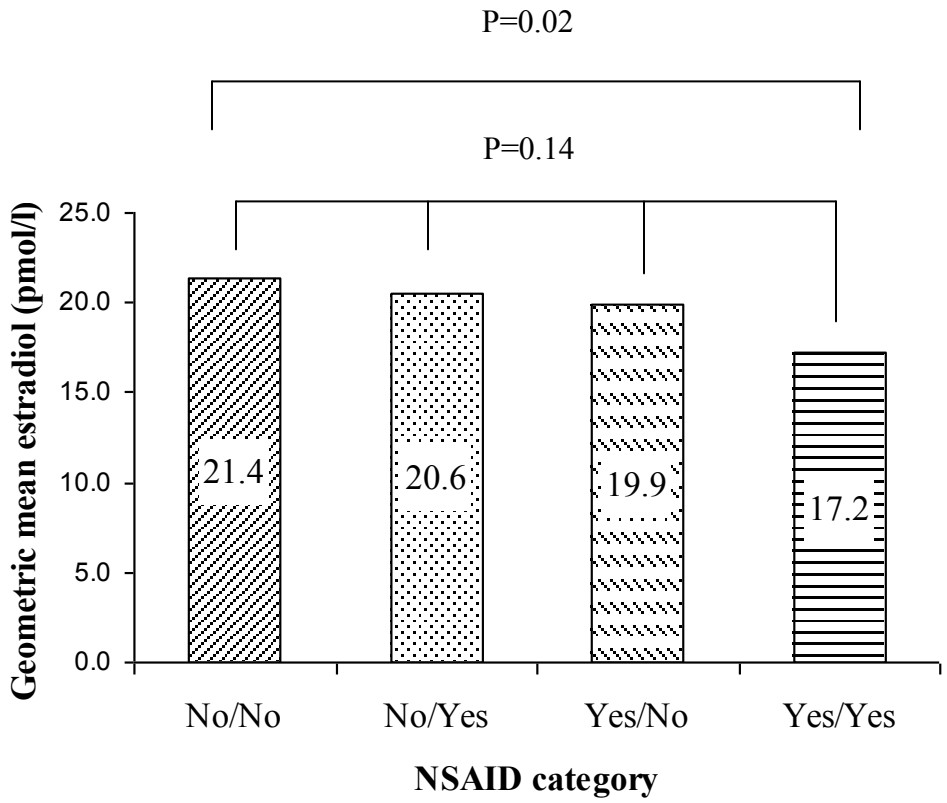


**Figure B20. Adjusted geometric mean estradiol according to self-reported NSAID use**

Serum total estradiol was adjusted for age at blood draw, BMI, race, years menopausal, and current alcohol intake in a general linear model. **No/No**=Participant's current medication list did not indicate use of a NSAID and the participant verbally responded that they did not consume an aspirin or anti-inflammatory agent within 48 hours of blood draw (N=96; mean=21.4; 95%CI=18.7, 24.5). **No/Yes**= Participant's current medication list did not indicate use of NSAID, but participant verbally responded that they consumed an aspirin or anti-inflammatory agent within 48 hours of blood draw (N= 24; mean=20.6; 95%CI=16.7, 25.4). **Yes/No**= Participant's current medication list indicated use of a NSAID, but the participant verbally responded that they did not consume an aspirin or anti-inflammatory agent within 48 hours of blood draw (N=40; mean=19.9; 95%CI=15.1, 26.1). **Yes/Yes**= Participant's current medication list indicated use of a NSAID, and participant verbally responded that they consumed an aspirin or anti-inflammatory agent within 48 hours of blood draw (N=100; mean=17.2; 95%CI=15.1, 19.6).

# APPENDIX C: ERYTHROCYTE OMEGA-6 AND OMEGA-3 FATTY ACIDS AND MAMMOGRAPHIC BREAST DENSITY ADDITIONAL ANALYSES

**Table C38. Geometric mean (95% confidence interval) breast density according to tertile of erythrocyte fatty acid composition**

| Fatty acids (wt. %) | Percent density* | p trend† | Dense area* | p trend† |
|---|---|---|---|---|
| n-6 PUFAs | | | | |
| Total n-6 ‡ | | 0.29 | | 0.71 |
| ≤37.32 | 24.1 (20.6, 28.0) | | 35.9 (30.3, 41.9) | |
| 37.33-39.49 | 23.7 (20.3, 27.4) | | 34.4 (29.1, 40.1) | |
| ≥39.50 | 27.2 (23.4, 31.2) | | 37.5 (31.9, 43.7) | |
| 18:2n-6 (LA) | | 0.32 | | 0.20 |
| ≤14.69 | 27.2 (23.4, 31.2) | | 38.5 (32.8, 44.8) | |
| 14.70-16.84 | 23.5 (20.1, 27.2) | | 36.3 (30.9, 42.2) | |
| ≥16.85 | 24.3 (20.8, 28.1) | | 33.1 (27.8, 38.8) | |
| 20:4n-6 (AA) | | 0.07 | | 0.16 |
| ≤15.24 | 22.6 (19.3, 26.3) | | 31.9 (26.8, 37.5) | |
| 15.25-16.57 | 24.7 (21.3, 28.5) | | 38.4 (32.8, 44.5) | |
| ≥16.58 | 27.6 (23.8, 31.6) | | 37.5 (31.9, 43.6) | |
| n-3 PUFAs | | | | |
| Total n-3 § | | 0.54 | | 0.64 |
| ≤6.68 | 25.2 (21.6, 29.1) | | 36.9 (31.3, 43.0) | |
| 6.69-8.41 | 26.2 (22.6, 30.0) | | 36.0 (30.6, 41.8) | |
| ≥8.42 | 23.6 (20.1, 27.3) | | 34.9 (29.5, 40.8) | |
| 18:3n-3 (ALA) | | 0.16 | | 0.20 |
| ≤0.19 | 27.3 (23.6, 31.2) | | 38.8 (33.2, 44.9) | |
| 0.20-0.25 | 24.0 (20.6, 27.8) | | 35.3 (29.9, 41.1) | |
| ≥0.26 | 23.6 (20.1, 27.3) | | 33.6 (28.3, 39.3) | |
| 20:5n-3 (EPA) | | 0.22 | | 0.21 |
| ≤0.64 | 24.8 (21.2, 28.5) | | 36.6 (31.1, 42.6) | |
| 0.65-0.90 | 28.9 (25.2, 32.9) | | 40.1 (34.4, 46.2) | |
| ≥0.91 | 21.5 (18.3, 25.1) | | 31.4 (26.3, 37.0) | |
| 22:6n-3 (DHA) | | 0.80 | | 0.78 |
| ≤3.69 | 24.7 (21.1, 28.6) | | 35.1 (29.7, 41.1) | |
| 3.70-4.86 | 26.1 (22.5, 30.0) | | 36.3 (30.9, 42.2) | |
| ≥4.87 | 24.1 (20.6, 27.8) | | 36.3 (30.8, 42.2) | |

**Table C38 (continued)**

| Fatty acids | Percent density* | p for trend† | Dense area* | p for trend† |
|---|---|---|---|---|
| 6:3 Ratios | | | | |
| Total n-6:n-3 | | 0.21 | | 0.57 |
| ≤4.48 | 22.5 (19.1, 26.1) | | 34.3 (28.9, 40.2) | |
| 4.49-5.73 | 26.7 (23.1, 30.5) | | 36.7 (31.3, 42.6) | |
| ≥5.74 | 25.8 (22.2, 29.8) | | 36.7 (31.1, 42.8) | |
| LA:ALA | | 0.40 | | 0.47 |
| ≤64.00 | 24.5 (21.1, 28.3) | | 35.8 (30.4, 41.6) | |
| 64.01-78.05 | 23.7 (20.2, 27.4) | | 33.3 (28.1, 39.0) | |
| ≥78.06 | 26.8 (23.1, 30.8) | | 38.8 (33.1, 45.0) | |
| AA:EPA | | 0.17 | | 0.28 |
| ≤16.92 | 22.5 (19.1, 26.2) | | 32.6 (27.3, 38.3) | |
| 16.93-25.89 | 26.1 (22.6, 30.0) | | 38.1 (32.6, 44.1) | |
| ≥25.90 | 26.3 (22.6, 30.3) | | 37.1 (31.5, 43.3) | |
| AA:EPA+DHA | | 0.21 | | 0.35 |
| ≤2.67 | 22.7 (19.3, 26.4) | | 34.1 (28.8, 40.0) | |
| 2.68-3.77 | 26.2 (22.6, 30.0) | | 35.6 (30.2, 41.4) | |
| ≥3.78 | 26.1 (22.4, 30.0) | | 38.1 (32.4, 44.2) | |

NOTE: N=248. Fatty acids are reported as a percentage by weight of the total fatty acids (weight percent, wt. %). PUFA, polyunsaturated fatty acid; LA, linoleic acid; AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; and DHA, docosahexaenoic acid. Tertile cutpoints were determined from entire study population (n=248). Values were adjusted for age (continuous), BMI (continuous), age at menopause (continuous), alcohol intake (none, <12g/day, ≥12g/day), current smoker (yes vs.no), previous breast biopsy (yes vs. no), nulliparous (yes vs. no), ever breastfed for > 1 month (yes vs. no), and past hormone therapy use (yes vs. no). 7 participants were excluded for missing variables

*Square root transformation was applied to percent density and dense breast area

† Linear trend tests were performed by treating the fatty acid tertile groups as continuous variables

‡18:2n-6+18:n-6+20:2n-6+20:3n-6+20:4n-6+22:4n-6+22:5n-6

§18:3n-3+20:4n-3+20:5n-3+22:5n-3+22:6n-3

**Table C39. Spearman correlation coefficients between erythrocyte n-6 and n-3 fatty acids and mammographic features including 9 participants with estradiol levels >150pmol/L**

| Fatty acids (wt.%) | Percent density | Dense area | Nondense area |
|---|---|---|---|
| N-6 PUFAs | | | |
| Total n-6* | -0.01 (0.88) | -0.02 (0.81) | 0.02 (0.81) |
| | 0.01 (0.83) | -0.03 (0.61) | -0.05 (0.42) |
| 18:2n-6 (LA) | 0.07 (0.24) | -0.07 (0.29) | -0.19 (0.002) |
| | -0.03 (0.68) | -0.08 (0.20) | -0.09 (0.16) |
| 20:4n-6 (AA) | <0.01 (0.99) | 0.07 (0.25) | 0.09 (0.14) |
| | 0.07 (0.24) | 0.08 (0.23) | -0.01 (0.89) |
| N-3 PUFAs | | | |
| Total n-3† | 0.11 (0.08) | 0.04 (0.57) | -0.16 (0.008) |
| | 0.03 (0.63) | 0.05 (0.46) | <0.01 (0.95) |
| 18:3n-3 (ALA) | 0.04 (0.56) | -0.06 (0.34) | -0.13 (0.04) |
| | -0.04 (0.55) | -0.06 (0.30) | -0.03 (0.59) |
| 20:5n-3 (EPA) | 0.06 (0.32) | -0.03 (0.59) | -0.16 (0.01) |
| | -0.02 (0.72) | -0.03 (0.64) | -0.03 (0.59 |
| 22:6n-3 (DHA) | 0.11 (0.07) | 0.07 (0.29) | -0.13 (0.04) |
| | 0.05 (0.44) | 0.08 (0.21) | <0.01 (0.95) |
| 6:3 Ratios | | | |
| Total n-6:n-3 | -0.10 (0.11) | -0.03 (0.61) | 0.15 (0.02) |
| | -0.03 (0.69) | -0.04 (0.48) | 0.01 (0.82) |
| LA:ALA | -0.02 (0.74) | 0.03 (0.62) | 0.07 (0.27) |
| | 0.01 (0.82) | 0.03 (0.65) | 0.02 (0.79) |
| AA:EPA | -0.06 (0.33) | 0.05 (0.46) | 0.18 (0.005) |
| | 0.04 (0.55) | 0.04 (0.50) | 0.03 (0.61) |
| AA:EPA+DHA | -0.10 (0.11) | -0.02 (0.70) | 0.16 (0.01) |
| | -0.01 (0.89) | -0.03 (0.60) | <0.01 (0.97) |

NOTE: N=257. Fatty acids are reported as a percentage by weight of the total fatty acids (weight percent, wt. %). PUFA, polyunsaturated fatty acid; LA, linoleic acid; AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; and DHA, docosahexaenoic acid. 1st line is unadjusted estimates. 2nd line is estimates adjusted for age and BMI. P between parentheses.

*18:2n-6+18:n-6+20:2n-6+20:3n-6+20:4n-6+22:4n-6+22:5n-6

†18:3n-3+20:4n-3+20:5n-3+22:5n-3+22:6n-3

**Table C40. Partial Spearman correlation coefficients between erythrocyte n-6 and n-3 fatty acids and mammographic characteristics among women (n=120) whose date of mammogram was <30 days from blood draw and film quality not rated as poor**

| Fatty acids (wt.%) | Percent density | Dense area | Nondense area |
|---|---|---|---|
| N-6 PUFAs | | | |
| Total n-6* | -0.03 (0.77) | -0.03 (0.77) | 0.10 (0.26) |
| 18:2n-6 (LA) | 0.14 (0.13) | 0.10 (0.27) | -0.08 (0.37) |
| 20:4n-6 (AA) | -0.10 (0.27) | -0.04 (0.66) | 0.14 (0.12) |
| N-3 PUFAs | | | |
| Total n-3† | -0.03 (0.77) | -0.004 (0.96) | -0.04 (0.64) |
| 18:3n-3 (ALA) | 0.15 (0.11) | 0.15 (0.11) | -0.07 (0.46) |
| 20:5n-3 (EPA) | 0.05 (0.60) | 0.05 (0.60) | -0.11 (0.22) |
| 22:6n-3 (DHA) | -0.03 (0.73) | -0.01 (0.92) | -0.02 (0.79) |
| 6:3 Ratios | | | |
| Total n-6:n-3 | 0.02 (0.85) | 0.001 (0.99) | 0.06 (0.51) |
| LA:ALA | -0.12 (0.21) | -0.12 (0.19) | 0.06 (0.49) |
| AA:EPA | -0.08 (0.40) | -0.07 (0.45) | 0.15 (0.11) |
| AA:EPA+DHA | -0.01 (0.92) | -0.01 (0.90) | 0.08 (0.41) |

NOTE: N=120. Fatty acids are reported as a percentage by weight of the total fatty acids (weight percent, wt. %). PUFA, polyunsaturated fatty acid; LA, linoleic acid; AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; and DHA, docosahexaenoic acid. Adjusted for age (continuous) and BMI (continuous). P values between parentheses

*18:2n-6+18:n-6+20:2n-6+20:3n-6+20:4n-6+22:4n-6+22:5n-6

†18:3n-3+20:4n-3+20:5n-3+22:5n-3+22:6n-3

**Table C41. Mammographic features by self-reported current NSAID use**

| Mammographic feature (n=248) | | NSAID non-user | NSAID user | p |
|---|---|---|---|---|
| Percent breast density | Model 1* | 24.2 (21.0, 27.6) | 25.2 (21.8, 28.8) | 0.69 |
| | Model 2† | 24.1 (21.3, 27.1) | 25.2 (22.2, 28.4) | 0.60 |
| | Model 3‡ | 24.6 (21.8, 27.5) | 25.4 (22.4, 28.5) | 0.72 |
| Dense area ($cm^2$) | Model 1* | 34.6 (30.3, 39.1) | 36.5 (32.0, 41.4) | 0.55 |
| | Model 2† | 34.6 (30.3, 39.1) | 36.5 (32.0, 41.4) | 0.55 |
| | Model 3‡ | 35.0 (30.8, 39.5) | 37.0 (32.4, 41.9) | 0.54 |
| Nondense area ($cm^2$) | Model 1* | 117.6 (104.9, 131.1) | 111.0 (98.1, 124.7) | 0.49 |
| | Model 2† | 118.2 (109.2, 127.6) | 110.4 (101.4, 119.9) | 0.24 |
| | Model 3‡ | 116.6 (107.8 125.7) | 110.8 (101.7, 120.2) | 0.38 |

NOTE: NSAID, nonsteroidal anti-inflammatory drug. A square root transformation was applied to percent breast density, dense area, and nondense area of the breast

*Model 1 unadjusted

†Model 2 adjusted for age (continuous) and BMI (continuous)

‡Model 3 adjusted for age (continuous), BMI (continuous), age at menopause (continuous), alcohol intake (none, <12g/day, ≥12g/day), current smoker (yes vs.no), previous breast biopsy (yes vs. no), nulliparous (yes vs. no), ever breastfed for > 1 month (yes vs. no), and past hormone therapy use (yes vs. no)

**Table C42. Partial Spearman correlations between erythrocyte fatty acids and mammographic features by current NSAID use**

| | NSAID non-user (n=129) | | | NSAID user (n=119) | | |
|---|---|---|---|---|---|---|
| | % density | dense areas | nondense areas | % density | dense areas | nondense areas |
| N-6 PUFAs | | | | | | |
| Total n-6* | 0.02 (0.84) | -0.02 (0.80) | 0.05 (0.58) | 0.02 (0.86) | 0.01 (0.89) | -0.02 (0.83) |
| 18:2n-6 (LA) | -0.01 (0.95) | -0.08 (0.38) | -0.09 (0.29) | -0.03 (0.75) | -0.02 (0.85) | -0.05 (0.59) |
| 20:4n-6 (AA) | 0.13 (0.15) | -0.03 (0.76) | -0.03 (0.76) | 0.03 (0.72) | 0.01 (0.91) | 0.002 (0.98) |
| N-3 PUFAs | | | | | | |
| Total n-3† | 0.10 (0.25) | 0.04 (0.63) | -0.12 (0.17) | -0.06 (0.53) | -0.01 (0.89) | 0.04 (0.65) |
| 18:3n-3 (ALA) | 0.003 (0.97) | -0.07 (0.45) | -0.12 (0.20) | -0.08 (0.41) | -0.03 (0.75) | 0.07 (0.44) |
| 20:5n-3 (EPA) | 0.12 (0.18) | 0.05 (0.59) | -0.14 (0.12) | -0.16 (0.09) | -0.12 (0.18) | 0.05 (0.60) |
| 22:6n-3 (DHA) | 0.10 (0.25) | 0.07 (0.43) | -0.09 (0.34) | -0.03 (0.74) | 0.02 (0.82) | 0.04 (0.64) |
| 6:3 Ratios | | | | | | |
| Total n-6:n-3 | -0.08 (0.39) | -0.04 (0.68) | 0.09 (0.30) | 0.05 (0.63) | 0.01 (0.93) | -0.03 (0.73) |
| LA:ALA | -0.04 (0.68) | 0.03 (0.76) | 0.13 (0.15) | 0.09 (0.32) | 0.05 (0.63) | -0.12 (0.21) |
| AA:EPA | -0.06 (0.50) | 0.01 (0.94) | 0.11 (0.20) | 0.15 (0.11) | 0.11 (0.26) | -0.05 (0.60) |
| AA:EPA+DHA | -0.04 (0.62) | -0.003 (0.98) | 0.08 (0.35) | 0.05 (0.58) | -0.002 (0.98) | -0.04 (0.65) |

NOTE: Fatty acids are reported as a percentage by weight of the total fatty acids (weight percent, wt. %). PUFA, polyunsaturated fatty acid; LA, linoleic acid; AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; and DHA, docosahexaenoic acid. Adjusted for age (continuous) and BMI (continuous). P values between parentheses

*18:2n-6+18:n-6+20:2n-6+20:3n-6+20:4n-6+22:4n-6+22:5n-6

†18:3n-3+20:4n-3+20:5n-3+22:5n-3+22:6n-3

**Table C43. Geometric mean (95% confidence interval) percent breast density according to tertile of erythrocyte fatty acid composition among participants reporting no current NSAID use**

| Fatty acids (wt. %) | Percent density* | p for trend† | Dense area ($cm^2$)* | p for trend† |
|---|---|---|---|---|
| n-6 PUFAs | | | | |
| Total n-6 ‡ | | 0.35 | | 0.84 |
| ≤37.32 | 23.0 (18.7, 27.8) | | 34.3 (27.5, 41.8) | |
| 37.33-39.49 | 23.5 (18.8, 28.8) | | 34.3 (27.0, 42.5) | |
| ≥39.50 | 26.5 (21.6, 31.9) | | 35.4 (28.2, 43.5) | |
| 18:2n-6 (LA) | | 0.26 | | 0.17 |
| ≤14.69 | 28.3 (23.0, 34.2) | | 38.7 (30.7, 47.5) | |
| 14.70-16.84 | 21.8 (17.6, 26.3) | | 35.3 (28.5, 42.8) | |
| ≥16.85 | 23.8 (19.3, 28.8) | | 30.9 (24.3, 38.2) | |
| 20:4n-6 (AA) | | 0.04 | | 0.07 |
| ≤15.24 | 21.1 (17.2, 25.4) | | 29.1 (23.3, 35.6) | |
| 15.25-16.57 | 24.7 (19.7, 30.4) | | 39.1 (31.1, 48.1) | |
| ≥16.58 | 27.9 (22.9, 33.4) | | 37.7 (30.4, 45.8) | |
| n-3 PUFAs | | | | |
| Total n-3 § | | 0.65 | | 0.78 |
| ≤6.68 | 21.7 (17.2, 26.7) | | 34.1 (26.9, 42.2) | |
| 6.69-8.41 | 27.9 (23.1, 33.2) | | 37.2 (30.0, 45.1) | |
| ≥8.42 | 23.5 (18.9, 28.5) | | 32.7 (25.8, 40.5) | |
| 18:3n-3 (ALA) | | 0.29 | | 0.36 |
| ≤0.19 | 26.3 (21.4, 31.7) | | 36.8 (29.5, 45.0) | |
| 0.20-0.25 | 24.4 (19.6, 29.6) | | 35.5 (28.2, 43.6) | |
| ≥0.26 | 22.7 (18.5, 27.3) | | 32.2 (25.9, 39.2) | |
| 20:5n-3 (EPA) | | 0.34 | | 0.78 |
| ≤0.64 | 20.3 (16.3, 24.8) | | 31.2 (24.7, 38.4) | |
| 0.65-0.90 | 30.0 (25.0, 35.4) | | 40.9 (33.4, 49.2) | |
| ≥0.91 | 23.3 (18.8, 28.3) | | 32.3 (25.5, 40.0) | |
| 22:6n-3 (DHA) | | 0.41 | | 0.71 |
| ≤3.69 | 22.5 (18.1, 27.4) | | 33.6 (26.7, 41.3) | |
| 3.70-4.86 | 25.3 (20.2, 30.9) | | 34.8 (27.2, 43.2) | |
| ≥4.87 | 25.4 (20.8, 30.5) | | 35.7 (28.7, 43.4) | |

**Table C43 (continued)**

| Fatty acids (wt. %) | Percent density* | p for trend† | Dense area* | p for trend† |
|---|---|---|---|---|
| 6:3 Ratios | | | | |
| Total n-6:n-3 | | 0.76 | | 0.82 |
| ≤4.48 | 23.1 (18.5, 28.1) | | 34.5 (27.4, 42.4) | |
| 4.49-5.73 | 26.0 (21.1, 31.4) | | 33.7 (26.6, 41.6) | |
| ≥5.74 | 24.0 (19.3, 29.3) | | 35.9 (28.4, 44.2) | |
| LA:ALA | | 0.87 | | 0.87 |
| ≤64.00 | 24.4 (20.1, 29.1) | | 34.4 (28.0, 41.6) | |
| 64.01-78.05 | 24.8 (20.0, 30.1) | | 36.2 (28.8, 44.4) | |
| ≥78.06 | 23.8 (18.9, 29.2) | | 33.4 (26.0, 41.6) | |
| AA:EPA | | 0.90 | | 0.69 |
| ≤16.92 | 23.2 (18.9, 28.0) | | 32.4 (25.9, 39.7) | |
| 16.93-25.89 | 26.8 (21.7, 32.4) | | 38.0 (30.3, 46.6) | |
| ≥25.90 | 23.4 (18.8, 28.5) | | 34.2 (27.1, 42.1) | |
| AA:EPA+DHA | | 0.71 | | 0.40 |
| ≤2.67 | 23.2 (18.9, 27.9) | | 32.9 (26.4, 40.1) | |
| 2.68-3.77 | 25.8 (20.9, 31.1) | | 34.0 (26.9, 41.9) | |
| ≥3.78 | 24.4 (19.7, 29.6) | | 37.5 (30.0, 45.8) | |

NOTE: N=127. Fatty acids are reported as a percentage by weight of the total fatty acids (weight percent, wt. %). NSAID, nonsteroidal anti-inflammatory drug; PUFA, polyunsaturated fatty acid; LA, linoleic acid; AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; and DHA, docosahexaenoic acid. Tertile cutpoints were determined from entire study population (n=248). Values were adjusted for age (continuous), BMI (continuous), age at menopause (continuous), alcohol intake (none, <12g/day, ≥12g/day), current smoker (yes vs.no), previous breast biopsy (yes vs. no), nulliparous (yes vs. no), ever breastfed for > 1 month (yes vs. no), past hormone therapy use (yes vs. no).

*Square root transformation was applied to percent density and dense breast area

† Linear trend tests were performed by treating the fatty acid tertile groups as continuous variables

‡18:2n-6+18:n-6+20:2n-6+20:3n-6+20:4n-6+22:4n-6+22:5n-6

§18:3n-3+20:4n-3+20:5n-3+22:5n-3+22:6n-3

**Table C44. Geometric mean (95% confidence interval) mammographic features according to tertile of erythrocyte fatty acid composition among women reporting NSAID use at blood draw**

| Fatty acids (wt.%) | Percent density* | p for trend† | Dense area ($cm^2$)* | p for trend† |
|---|---|---|---|---|
| n-6 PUFAs | | | | |
| Total n-6 ‡ | | 0.50 | | 0.76 |
| ≤37.32 | 25.5 (19.5, 32.3) | | 38.7 (29.2, 49.7) | |
| 37.33-39.49 | 23.5 (18.6, 29.0) | | 33.6 (26.0, 42.1) | |
| ≥39.50 | 28.6 (22.5, 35.4) | | 40.8 (31.4, 51.4) | |
| 18:2n-6 (LA) | | 0.92 | | 0.81 |
| ≤14.69 | 25.8 (20.4, 31.9) | | 38.3 (29.8, 47.9) | |
| 14.70-16.84 | 25.8 (20.1, 32.2) | | 36.9 (28.1, 46.9) | |
| ≥16.85 | 25.4 (19.8, 31.8) | | 36.7 (28.0, 46.6) | |
| 20:4n-6 (AA) | 25.4 (19.2, 32.3) | 0.75 | 37.1 (27.5, 48.1) | 0.95 |
| ≤15.24 | 25.0 (20.1, 30.6) | | 38.1 (30.2, 47.0) | |
| 15.25-16.57 | 26.8 (21.0, 33.2) | | 36.7 (27.9, 46.6) | |
| ≥16.58 | | | | |
| n-3 PUFAs | | | | |
| Total n-3 § | | 0.28 | | 0.84 |
| ≤6.68 | 29.4 (23.4, 36.2) | | 40.2 (31.1, 50.6) | |
| 6.69-8.41 | 23.3 (18.1, 29.2) | | 33.1 (25.0, 42.3) | |
| ≥8.42 | 24.5 (19.0, 30.6) | | 38.8 (29.9, 48.9) | |
| 18:3n-3 (ALA) | | 0.56 | | 0.53 |
| ≤0.19 | 27.7 (22.2, 33.8) | | 40.3 (31.7, 49.9) | |
| 0.20-0.25 | 24.0 (18.9, 29.8) | | 35.4 (27.3, 44.5) | |
| ≥0.26 | 25.3 (19.3, 32.0) | | 36.1 (26.9, 46.7) | |
| 20:5n-3 (EPA) | | 0.01 | | 0.08 |
| ≤0.64 | 31.1 (24.9, 38.0) | | 43.6 (34.0, 54.5) | |
| 0.65-0.90 | 26.8 (21.3, 33.0) | | 38.1 (29.4, 47.9) | |
| ≥0.91 | 20.2 (15.4, 25.6) | | 31.4 (23.5, 40.3) | |
| 22:6n-3 (DHA) | | 0.32 | | 0.82 |
| ≤3.69 | 28.1 (21.9, 35.1) | | 37.6 (28.3, 48.2) | |
| 3.70-4.86 | 25.7 (20.4, 31.5) | | 35.7 (27.7, 44.7) | |
| ≥4.87 | 23.5 (18.0, 29.8) | | 39.2 (29.9, 49.8) | |

Table C44 (continued)

| Fatty acids (wt.%) | Percent density† | p for trend‡ | Dense area† | p for trend‡ |
|---|---|---|---|---|
| 6:3 Ratios | | | | |
| Total n-6:n-3 | | 0.13 | | 0.55 |
| ≤4.48 | 22.0 (16.7, 28.0) | | 34.9 (26.3, 44.9) | |
| 4.49-5.73 | 26.4 (21.0, 32.5) | | 37.9 (29.4, 47.5) | |
| ≥5.74 | 28.8 (22.8, 35.4) | | 39.2 (30.2, 49.4) | |
| LA:ALA | | 0.21 | | 0.24 |
| ≤64.00 | 24.1 (18.4, 30.6) | | 36.4 (27.4, 46.7) | |
| 64.01-78.05 | 23.4 (18.3, 29.1) | | 31.9 (24.2, 40.6) | |
| ≥78.06 | 29.3. (23.7, 35.4) | | 43.8 (35.0, 53.5) | |
| AA:EPA | | 0.09 | | 0.34 |
| ≤16.92 | 22.1 (16.5, 28.6) | | 33.9 (24.8, 44.4) | |
| 16.93-25.89 | 24.9 (19.8, 30.6) | | 37.0 (28.9, 46.0) | |
| ≥25.90 | 30.0 (18.9, 28.6) | | 41.0 (31.7, 51.6) | |
| AA:EPA+DHA | | 0.24 | | 0.76 |
| ≤2.67 | 22.9 (17.0, 29.7) | | 37.4 (27.6, 48.7) | |
| 2.68-3.77 | 25.3 (19.9, 31.3) | | 35.4 (27.2, 44.6) | |
| ≥3.78 | 28.4 (22.5, 35.1) | | 39.5 (30.4, 49.7) | |

NOTE: N=114. Fatty acids are reported as a percentage by weight of the total fatty acids (weight percent, wt.%). NSAID, nonsteroidal anti-inflammatory drug; PUFA, polyunsaturated fatty acid; LA, linoleic acid; AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; and DHA, docosahexaenoic acid. Tertile cutpoints were determined from entire study population (n=248). Values were adjusted for age (continuous), BMI (continuous), age at menopause (continuous), alcohol intake (none, <12g/day, ≥12g/day), current smoker (yes vs.no), previous breast biopsy (yes vs. no), nulliparous (yes vs. no), ever breastfed for > 1 month (yes vs. no), past hormone therapy use (yes vs. no).

*Square root transformation was applied to percent density and dense breast area

† Linear trend tests were performed by treating the fatty acid tertile groups as continuous variables

‡18:2n-6+18:n-6+20:2n-6+20:3n-6+20:4n-6+22:4n-6+22:5n-6

§18:3n-3+20:4n-3+20:5n-3+22:5n-3+22:6n-3

**Table C45. Spearman correlation coefficients between erythrocyte fatty acids not of the n-6 or n-3 families and mammographic features**

| | Percent breast density | Dense breast area | Nondense breast area |
|---|---|---|---|
| Fatty acid (wt. %) | r (p) | r (p) | r (p) |
| 14.0 | -0.04 (0.50) | -0.07 (0.24) | 0.02 (0.81) |
| 15.0 | 0.07 (0.27) | -0.07 (0.28) | -0.02 (0.71) |
| 16.0 | 0.003 (0.96) | -0.02 (0.76) | 0.005 (0.94) |
| 17.0 | 0.10 (0.12) | 0.13 (0.05) | -0.02 (0.77) |
| 18.0 | -0.02 (0.78) | 0.001 (0.99) | 0.004 (0.96) |
| 16:1t | -0.05 (0.40) | -0.07 (0.30) | 0.03 (0.61) |
| 18:1 t-1 | -0.07 (0.25) | -0.07 (0.29) | 0.05 (0.46) |
| 18:1 t-2 | -0.09 (0.17) | -0.05 (0.41) | 0.12 (0.06) |
| 18:1 t-3 | -0.13 (0.05) | -0.10 (0.13) | 0.12 (0.06) |
| 18:1 t-4 | -0.13 (0.05) | -0.09 (0.14) | 0.10 (0.10) |
| 18:1 t-5 | -0.12 (0.07) | -0.09 (0.14) | 0.11 (0.10) |
| 18:2 tt | -0.08 (0.21) | -0.14 (0.02) | -0.01 (0.87) |
| 16:1n7c | -0.001 (0.98) | -0.04 (0.55) | 0.009 (0.89) |
| 18:1n9c | 0.02 (0.80) | 0.01 (0.84) | 0.007 (0.92) |
| 18:1n7c | 0.05 (0.47) | 0.04 (0.55) | 0.02 (0.81) |
| 20:1n9 | -0.02 (0.71) | -0.01 (0.84) | 0.06 (0.37) |
| 24:1n9 | 0.04 (0.55) | 0.08 (0.21) | 0.02 (0.79) |

NOTE: N=248. Fatty acids are reported as a percentage by weight of the total fatty acids (weight percent, wt. %). Adjusted for age (continuous) and BMI (continuous)

**Table C46. Spearman correlation coefficients between erythrocyte fatty acids not of the n-6 or n-3 families and mammographic features among women whose date of mammogram was <30 days from blood draw and film quality not rated as poor**

| | Percent breast density | Dense breast area | Nondense breast area |
|---|---|---|---|
| Fatty acid (wt.%) | r (p) | r (p) | r (p) |
| 14.0 | 0.03 (0.73) | -0.02 (0.87) | -0.04 (0.68) |
| 15.0 | 0.15 (0.11) | 0.15 (0.10) | -0.05 (0.62) |
| 16.0 | 0.03 (0.77) | -0.01 (0.93) | -0.02 (0.80) |
| 17.0 | 0.13 (0.17) | 0.17 (0.07) | -0.06 (0.49) |
| 18.0 | -0.10 (0.28) | -0.06 (0.53) | 0.05 (0.56) |
| 16:1t | 0.03 (0.76) | -0.01 (0.93) | -0.07 (0.47) |
| 18:1 t-1 | 0.02 (0.87) | -0.02 (0.81) | -0.08 (0.41) |
| 18:1 t-2 | -0.12 (0.20) | -0.14 (0.13) | 0.06 (0.55) |
| 18:1 t-3 | -0.14 (0.14) | -0.14 (0.13) | 0.08 (0.37) |
| 18:1 t-4 | -0.16 (0.07) | -0.18 (0.05) | 0.06 (0.52) |
| 18:1 t-5 | -0.11 (0.22) | -0.10 (0.29) | 0.10 (0.30) |
| 18:2 tt | -0.02 (0.79) | -0.16 (0.08) | -0.12 (0.20) |
| 16:1n7c | 0.10 (0.29) | 0.02 (0.79) | -0.06 (0.51) |
| 18:1n9c | 0.23 (0.01) | 0.12 (0.20) | -0.25 (0.01) |
| 18:1n7c | 0.10 (0.29) | 0.04 (0.67) | -0.06 (0.53) |
| 20:1n9 | 0.09 (0.32) | 0.03 (0.75) | -0.01 (0.89) |
| 24:1n9 | -0.14 (0.14) | -0.05 (0.61) | 0.22 (0.20) |

NOTE: N=120. Fatty acids are reported as a percentage by weight of the total fatty acids (weight percent, wt. %). Adjusted for age (continuous) and BMI (continuous)

**Table C47. Spearman's correlation coefficients between serum estradiol concentration and mammographic features**

| | Serum estradiol | | | |
|---|---|---|---|---|
| Mammographic characteristic | r* | p | r† | p |
| Percent breast density | -0.29 | <0.0001 | -0.08 | 0.24 |
| Dense breast area ($cm^2$) | -0.06 | 0.37 | -0.07 | 0.29 |
| Nondense breast area ($cm^2$) | 0.42 | <0.0001 | 0.08 | 0.19 |

*Unadjusted Spearman's correlation coefficients

†Age- and BMI-adjusted Spearman's correlation coefficients

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