Mammary Gland Density Predicts the Cancer Inhibitory Activity of the N-3 to N-6 Ratio of Dietary Fat

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Abstract

Background This study investigated the effect of a broad range of dietary ratios of n-3:n-6 fatty acids on mammary gland density (MD) and mammary cancer risk.

Methods Cancer was induced in female rats by N-methyl-N-nitrosourea. Purified diet that provided 30% of dietary kcal from fat was formulated to contain ratios of n-3:n-6 fatty acids from 25:1 to 1:25. MD was determined by digital analysis, fatty acids by gas chromatography/flame ionization detection and other plasma analytes via ELISA.

Results MD was reduced dose dependently at n-3:n-6 ratios from 1:1 to 25:1 (r=-0.477, P=0.038), with a 20.3% decrease of MD between n-3:n-6 of 1:1 versus 25:1, P<0.001. Mammary carcinogenesis was inhibited in the absence or presence of tamoxifen (1 mg/kg diet) in a manner predicted by MD. Plasma n-3 fatty acid concentrations failed to increase above an n-3:n-6 ratio of 5:1, and changes in specific plasma n-3 or n-6 fatty acids were not predictive of MD or cancer inhibitory activity. A strong reciprocal effect of the n-3:n-6 ratio on plasma leptin (decreased, P=0.005) and adiponectin (increased, P<0.001) was observed indicating adipose tissue function was modulated. However, neither cytokine was predictive of MD. Plasma IGF-1 decreased with increasing dietary n-3:n-6 ratio (P=0.004) and was predictive of the changes in MD (r=0.362, P<0.005).

Conclusions These findings indicate that 1) MD predicted the carcinogenic response, 2) the n-3:n-6 ratio exerts effects in the presence or absence of hormonal regulation of carcinogenesis and 3) signaling pathways regulated by IGF-1 are potential targets for further mechanistic investigation.
Introduction

While the general perception exists that diets enriched in n-3 relative to n-6 fatty acids have a broad range of health-related benefits, a review of the literature reveals that the epidemiological data in support of an inverse relationship between n-3 fatty acid consumption and breast cancer risk are mixed (1-6). Moreover, the preclinical data on the role of n-3 fatty acids in mammary carcinogenesis is similarly mixed (inhibition, no effect, or promotion of carcinogenesis) and the quality of the experiments varies so markedly that it is difficult to compare results across studies (7-18). These circumstances prompted the work reported herein.

One observation that emerged from the review of the preclinical evidence was that the majority of animal carcinogenesis experiments have been done using diets that were high in the percent of dietary calories provided from fat (40 to 50%) and that few experiments actually varied the ratio of n-3 to n-6, despite the fact that considerable emphasis has been placed on this ratio in hypothesized mechanisms of action (1). Both issues are problematic from a translational perspective since recent clinical trials suggest that it is unusual for women at risk for breast cancer to exceed 35% of dietary calories from fat and that most women are likely to ingest n-3 fatty acids from a dietary supplement (19-21). Thus it is important to understand the range of ratios of n-3 to n-6 fatty acids to which women are likely to be exposed. Mindful of these issues, we decided to formulate a series of purified diets modeled after the AIN-93G formulation but with the major exception that the level of dietary fat was modified to reflect that currently recommended in the US Dietary guidelines (22). Thus diets were formulated to provide 30% of dietary calories from fat and an equal amount of those calories from
saturated, monounsaturated, and polyunsaturated fatty acids. Within the polyunsaturated fatty acids, we sought to vary the ratio of n-3 to n-6 fatty acid from 25:1 to 1:25 in order to provide a robust evaluation of the role of this ratio in affecting the post initiation phase of chemically induced mammary carcinogenesis.

Our laboratories are keenly interested in conducting preclinical animal studies that have translational potential to the human population. For this reason, our initial effort was designed to test the effects of the translationally relevant diet formulations described above on mammary gland density and then to use that data to make predictions about the effects of diet on the post initiation phase of mammary carcinogenesis. This concept was based on the pioneering work of a number of clinical investigators who have reported that mammary gland density is an independent risk factor for the development of breast cancer (23-25). A goal of this work was to determine if a rodent bioassay for mammary gland density, which is distinct from but that has parallels to the determination of mammary gland density in women, would be useful in screening for interventions that ultimately impact the carcinogenic process.

Strong arguments can be made that host systemic factors contribute to risk for breast cancer (26). A rich literature exists on the effects of n-3 fatty acids on host systemic factors (1, 3, 27). When the findings from the literature related to n-3 fatty acids are juxtaposed with the host systemic factors associated with breast cancer, there is remarkable overlap, particularly with regard to signaling pathways involved in insulin sensitivity and chronic inflammation. Hence, we also report initial findings from our exploratory analyses of these relationships. The carcinogenesis experiments in which the dietary n-3:n-6 ratio was varied were done in the presence or absence of treatment
with tamoxifen to ascertain the dependence of effects of the ratio on hormonal regulation of the mammary carcinogenesis. We report that mammary gland density predicted the carcinogenic response, that the n-3:n-6 ratio exerts effects in the presence or absence of hormonal regulation of carcinogenesis and that signaling pathways regulated by IGF-1 are potential targets for further mechanistic investigation.
MATERIALS AND METHODS

Chemicals and Reagents Concentrated omega-3 oil was purchased from American International Chemical, Inc. (Framingham, MA). All experimental diets were purchased from Research Diets (New Brunswick, NJ). All fatty acid standards for GC analyses were obtained from Nu Check Prep, Inc. (Elysian, MN) with the exception of pentadecanoic acid which was obtained from Sigma-Aldrich (St. Louis, MO). Carcinogen: N-methyl-N-nitrosourea (Ash Stevens, Detroit, MI) was stored at -80°C prior to use. Glucose-hexokinase liquid stable reagent was purchased from Thermo Fisher Scientific Inc. (Waltham, MA). Enzyme-linked immunosorbent assay (ELISA) kit of C-reactive protein was from Helica Biosystems Inc. (Fullerton, CA). Multiplex and signalplex kits for insulin, leptin, IL-6 and TNFα and insulin growth factor-1 as well as ELISA kit of adiponectin was from Millipore (Billerica, MA). Carmine was purchased from Polysciences (Warrington, PA).

Experiment design Four experiments were conducted to determine how the n-3:n-6 dietary fatty acid ratio affects mammary gland density, circulating molecules and the carcinogenic response as illustrated in Supplementary Figure S1. The post initiation phase of chemically induced mammary carcinogenesis was investigated.

Experiment 1: Mammary gland density screening For the investigation of the effect of dietary n-3:n-6 fatty acids on mammary gland density, plasma hormones, cytokines and fatty acids, sixty-three female Sprague Dawley rats from Charles River (n=9/group) were randomized to one of 7 dietary formulations that varied in their ratio of n-3:n-6 as follows: 25:1, 10:1, 5:1, 1:1, 1:5, 1:10, 1:25 (Table 1). Rats were weighed three times...
per week. Given the screening nature of the study, the experiment was terminated after two weeks of treatment to maximize the sensitivity to detect changes in mammary gland density.

**Experiments 2-4: carcinogenesis studies** For the investigation of the effect of dietary n-3:n-6 fatty acids at 1:1, 5:1, 10:1, and 25:1 with or without 1.0 mg tamoxifen citrate/kg diet on carcinogenic response in the mammary gland, three parallel studies were conducted. In each study, female Sprague Dawley rats (20 days of age) were randomized to dietary groups (30 rats/group). The n-3:n-6 ratio = 1:1 treatment group, plus or minus tamoxifen was common to all three experiments and is designated as the referent group. In Experiment 2, n-3:n-6 ratios = 5:1 or 1:5, each plus or minus tamoxifen were investigated. In Experiment 3, the n-3:n-6 ratio = 10:1 plus or minus tamoxifen was investigated. In Experiment 4, the n-3:n-6 ratio= 25:1 plus or minus tamoxifen was investigated. Rats were weighed twice per week. Rats were palpated for detection of mammary tumors twice per week starting from 19 days post carcinogen. Rats were housed 3 per cage in solid bottomed polycarbonate cages equipped with a food cup and the experimental diets were fed for eight weeks. Animal rooms were maintained at 22 ± 1°C with 50% relative humidity and a 12-h light/12-h dark cycle. The work reported was reviewed and approved by the Institutional Animal Care and Use Committee and conducted according to the committee guidelines.

**Carcinogen administration** At 21 days of age, rats were injected with 50 mg N-methyl-N-nitrosourea/kg body weight (i.p.) as previously described (28). Seven days following carcinogen injection, all rats were randomized into treatment groups, thirty rats per group, and were fed their respective experimental diets ad libitum.
**Diet formulation**  Novel diets were formulated (Table 1). These formulations were developed with technical input from Research Diets (New Brunswick, NJ). Research Diets manufactured the diets which are now commercially available. The detailed fatty acid composition of these diet formulations was tabulated based on vendors’ product information and that data is summarized (Supplementary Table 1). The percent of dietary calories from fat was 30% across all dietary formulations. The actual dietary concentrations of fatty acids as determined by GC/MS are summarized (Supplementary Table 2).

**Necropsy**  Following an overnight fast, rats were necropsied over a 3-hour time interval. Rats were euthanized via inhalation of gaseous carbon dioxide. The sequence in which rats were euthanized was stratified across groups so as to minimize the likelihood that order effects would masquerade as treatment associated effects. After the rat lost consciousness, blood was directly obtained from the retro-orbital sinus and gravity fed through heparinized capillary tubes (Fisher Scientific, Pittsburgh, PA) into EDTA coated tubes (Becton Dickinson, Franklin Lakes, NJ) for plasma. The bleeding procedure took approximately 1 min/rat. Plasma was isolated by centrifugation at 1000 x g for 10 min at room temperature. Following blood collection and cervical dislocation, the rat was skinned and the abdominal-inguinal mammary glands chains from all animals were carefully excised with emphasis on retrieving the surrounding pleural membrane and prepared as whole mounts on 75 x 50 mm glass microscope slides and fixed in 10% neutral buffered formalin. The fixed whole mounts were processed and stained with 0.4% alum carmine as previously described (29). Digital images of whole mounts were captured for the analysis of mammary density.
For the carcinogenesis study, following blood collection and cervical dislocation, rats were then skinned and the skin was examined under translucent light for detectable mammary pathologies. All grossly detectable mammary gland pathologies were excised. In addition, whole mounts of abdominal-inguinal mammary gland chains were prepared and tissue was fixed in 10% neutral buffered formalin. The fixed whole mounts were subsequently processed, stained in 0.4% carmine and digital images captured and then evaluated under 10x magnification for detection of any abnormality that might be a mammary pathology as previously described in detail. All lesions and abnormalities were processed for histological classification as described in (29, 30). Only mammary adenocarcinomas are reported.

**Measurement of mammary gland density** All whole mounts of the abdominal-inguinal mammary gland chains were photographed and the images obtained were digitized. Digital images of the whole mounts were captured using a semi-automated image acquisition system. The components of this system include a 3.0 megapixel CMOS digital camera (Clemex Technologies, Inc. Longueuil, Canada) mounted on a Leica Z16 APO monocular zoom lens 16:1 with a magnification range of 0.57 - 9.2x. The camera and lens are mounted on a Leica Z motor attached to a transmitted light base with a 100 x 100 mm motorized stage (Clemex Technologies, Inc.). An X-Y control box and joystick (Clemex Technologies, Inc.) in conjunction with a Pentium 4 desktop PC (Dell, Round Rock, TX) and Captiva v4.0 software (Clemex Technologies, Inc.) are used for image capture. The mammary gland WMs were placed on a 6 mm thick sheet of white acrylic plastic (Gagne, Inc. Johnson City, NY) mounted on top of the motorized stage to act as a diffuser. Specimens were trans-illuminated using a 20V/150W halogen lamp light
source (Volpi, Auburn, NY) with daylight filter mounted at the rear of the base. A series of seamless tiled Z stack images (10x) were captured automatically using the motorized stage in conjunction with the Captiva 4.0 software (Clemex Technologies, Inc.) and X Y controller. The Captiva 4.0 software automatically merged the tiled Z stack images together into a single uniformly focused composite image based on a best contrast algorithm. Resulting images were saved as TIF files. Composite digital images were opened in Photoshop® v9.0.1 (Adobe Systems, Inc., San Jose, CA) and saved as PSD files. We employed and created customized actions (macros) in Photoshop® as tools, and developed a working laboratory manual for identifying areas of interest within the images. A screening method of mammary density was developed that enables large numbers of mammary whole mounts to be evaluated in a short period of time. Briefly, a 1 cm² area of mammary gland lying superior to the lymph node in gland 4 was selected from each composite whole mount image for screening. Measurements of mammary gland were performed using digitized alum carmine-stained images of 1 cm² screening areas of interest from all whole mounts and the entire abdominal-inguinal mammary gland chain from selected whole mounts based on screening results. Lymph nodes were digitally excised from whole mount composite images prior to analysis. Screening areas and abdominal-inguinal mammary gland chains were analyzed using Image-Pro® Plus v4.5 software (Media Cybernetics, Silver Spring, MD). Image-Pro® Plus macros for both screening areas and mammary gland chains were developed by our laboratory to expedite analysis. Images were evaluated for total area of the mammary gland fat pad occupied by mammary epithelium as well as total area of the fat pad encompassed by the mammary ductal tree. Area occupied by mammary epithelium divided by total area
encompassed by the mammary ductal tree was calculated. The technique is illustrated in Supplementary Figure S2 (31).

**Fatty acid analysis**

*Plasma* Total fatty acid levels in plasma and animal diets were determined by the direct one-step transesterification method of Lepage and Roy with resulting methylated fatty acids being analyzed by gas chromatography equipped with flame ionization detection (GC-FID) as described previously (32). The identification of fatty acid methyl esters was accomplished by comparison with the relative retention time of standards. Quantification was based on internal standard calculations as compared to calibration analyses with authentic standards.

*Diet* The method described for the analysis of fatty acids in plasma was modified for the quantitative assessment of fatty acids in the diet samples. Briefly, pellets from each diet were pulverized using a mortar and pestle. Portions (70-90 mg) from each diet sample were transferred into a reaction flask and 1 mg of pentadecanoic acid (internal standard) in 10 µl of methanol:toluene (4:1; v:v) were added. The diets were then suspended in 2 ml of methanol:toluene, cooled to 0°C and acetyl chloride (200 µl) was added. The suspension was then heated to 100°C for 60 min. Following this step, the mixture was cooled to 0°C and 5 ml of K₂CO₃ (6% aqueous solution) was added. The mixture was then extracted 3 x 5 ml each with hexane containing butylated hydroxytoluene (20 mg/l). The organic layers were separated, transferred into 25 ml volumetric flask, and brought to volume. One µl of each sample was analyzed by GC/FID. Quantification was based on comparisons of responses of a given fatty acid with the internal standard. The column used was CPWAX 52 CB from Varian (30 m X
0.25 mm; film thickness 0.25 µm), the carrier gas was helium (1ml/min) with nitrogen as a make-up gas. The following temperature program was used: initial temperature of a 100°C held for 0.5 min and then increased by 10°C/min to 260°C. This final temperature was held for additional 15 min. The injector and the detector temperatures were 280°C and 300°C, respectively.

**Assessment of circulating molecules** Glucose was determined using a kit obtained from Thermo Fisher Scientific Inc. (Waltham, MA). Insulin growth factor-1 was determined using a commercial signalplex kit, Insulin, leptin, IL-6 and TNFα were determined using a multiplex kit and adiponectin was determined by commercial enzyme-linked immunosorbent assay kit from Millipore (Billerica, MA). C-reactive protein was determined using a commercial rat enzyme immunoassay kit from Helica Biosystems Inc. (Fullerton, CA). All analyses were performed according to manufacturer’s instructions. For the signal-or multi-plex assay, beads conjugated to specific primary capture antibodies were added to wells of a 96-well multiscreen plate and fluid was uniformly removed from each well using a multiscreen resist vacuum manifold at constant pressure after incubation. Wells were washed with wash buffer using vacuum removal after each wash. Standards, positive controls and samples were then added to the respective wells and incubated with beads at room temperature with orbital shaking. After incubation, fluid was removed again as defined above. Wells were washed with wash buffer. Secondary antibody was then added to all wells followed by incubation at room temperature with shaking. After fluid removal and washing, Streptavidin-PE complex was added to each well and incubated with shaking. Then, the plate was washed with wash buffer. Finally, assay buffer was added to resuspend
beads. At all shaking points noted above, 96-well assay plates were covered with sealing tape and aluminum foil to retard photo-bleaching. In addition after each wash step, the underside of the plate was blotted with a paper towel. After the above procedures, standards and samples were immediately processed using the Bio-Plex Protein Array System and related Bio-Plex Manager software (Bio-Rad). Standard curves were prepared plotting absorbance versus the concentration of the targets. Values for experimental samples were determined using a five-parameter logistic regression model.

The ELISA assays that were used employed a quantitative “sandwich” technique. In each ELISA kit, a monoclonal antibody specific for adiponectin or C-reactive protein was precoated on the 96-well microtiter plate. All reagents were brought to room temperature and assay diluent (100 µl) was added to each well. The standards or unknown sample (100 µl) were then added to the wells in duplicate. After a 2-3 hour incubation period, the wells were washed 3-4 times with the provided wash buffer followed by addition of a second specific streptavidin-horseradish peroxidase-conjugated antibody. The plate was incubated at room temperature for 1-2 hours followed by washing and stop solution. Color development and intensity of the color was measured using an ELISA plate reader (Molecular Devices, Sunnyvale, CA). A standard curve was prepared plotting the absorbance versus the concentration of the targets. Values for experimental samples were determined via a four-parameter logistic regression model.

**Statistical Analyses** Differences among groups in the incidence of mammary adenocarcinomas were evaluated by chi-square analysis (33). Differences among
groups in the number of mammary adenocarcinomas per rat (multiplicity) were evaluated by ANOVA after square root transformation of tumor count data (33). Differences in final body weight, mammary gland density, and circulating molecules were evaluated by ANOVA. When multiple comparison across treatment groups were performed, statistical significance was adjusted by the Bonferroni method (34). The relationship between mammary gland density and circulating factors was evaluated by regression analysis (35).
Results

**Experiment 1** In this mammary gland density screening assay, the n-3:n-6 ratio of 1:1 was used as the referent group. Enriching dietary lipid with n-6 fatty acids failed to have an effect on mammary gland density, i.e. the percent of the mammary gland containing mammary epithelium (Table 2). However, with increasing levels of n-3 fatty acids (n-3:n-6 ratio= 1:1, 5:1, 10:1, and 25:1), mammary gland density was progressively reduced ($r = -0.477, P = 0.038$) and the difference reached a magnitude of 25% in the screening assay at n3/n6 ratio of 25:1 versus 1:1 (Figure 1A-C). This finding was validated in a subset analysis in which the entire abdominal-inguinal mammary gland chain (glands 4, 5, and 6) in all rats treated with either n-3:n-6 ratio of 1:1 or 25:1 were subjected to complete densitometric analysis (65.0 ± 2.7% density vs. 51.8 ± 1.8 % density, a 20.3% difference between treatment groups, $P < 0.001$). The plasma concentrations of cytokines, growth factors, and fatty acids for each treatment group are shown (Table 2). Using the 1:1 ratio as the referent group, plasma leptin, adiponectin, and IGF-1 were not significantly affected with increasing n-6 fatty acid in the diet. However, leptin ($P = 0.005$) and IGF-1 ($P = 0.004$) decreased significantly and adiponectin ($P < 0.001$) increased with increasing levels of dietary n-3 fatty acid. To determine if plasma levels of leptin, adiponectin, or IGF-1 were associated with mammary gland density, the individual data from animals in all 7 dietary groups were evaluated via regression analysis. Plasma concentrations of leptin or adiponectin were unrelated to mammary gland density. The plasma analytes on which the n-3:n-6 ratio had no significant effect are summarized (Supplementary Table 3). However, there was a significant relationship between plasma IGF-1 concentration and mammary gland
density ($r=0.362, \ P < 0.005$) (Figure 1C). Only the 25:1 n-3:n-6 dietary ratio slowed body weight gain (11%, $\ P < 0.01$); adjustment for differences among groups in body weight in the statistical evaluation did not alter the results of the mammary gland density analyses.

The plasma concentrations of select fatty acids are shown (Table 2). The results of the other fatty acid measured in the analysis of plasma are shown (Supplementary Table 4). Plasma concentrations of n-6 fatty acids, linoleic and arachidonic acid decreased with increasing dietary n-3:n-6 ratio; however, differences were not statistically significant among the ratios of n-3:n-6 of 5:1, 10:1 or 25:1. The plasma concentration of n-3 fatty acids, eicosapentaenoic acid and docosahexaenoic acid increased as the dietary n-3:n-6 dietary ratio increased; however, at 5:1, 10:1, and 25:1, the concentrations of these fatty acids actually declined as the ratio increased, although the decreases were not statistically significant.

**Experiment 2** Given the mammary gland density results of Experiment 1, the prediction was made that ratios of n-3:n-6 from 5:1 to 1:5 would not affect the process of mammary carcinogenesis since rats fed these ratios did not differ in mammary gland density. Mammary cancer incidence, cancer multiplicity and cancer burden per rat were not affected by the ratio of dietary n-3/n-6 (Table 3). However, in rats also treated with tamoxifen (1mg/kg diet), mammary cancer incidence, cancer multiplicity, and cancer burden per rat were significantly reduced. Tamoxifen is known to cause a modest
decrease in rate of body weight gain (36), and this was observed; however, the n-3:n-6 ratio had no effect on grow rate.

**Experiment 3** The hypothesis tested in this experiment was that the reduction observed in mammary gland density in rats fed the n-3:n-6 = 10:1 dietary ratio relative to the referent group (n-3:n-6 1:1 ratio) would result in a marginal cancer inhibitory effect. Relative to the referent group (n-3:n-6 1:1), mammary cancer incidence, cancer multiplicity, and cancer burden per rat were reduced by 10:1 n-3/n-6, and the effect was observed in the presence or absence of tamoxifen treatment (Table 4). Body weight gain was modestly reduced by tamoxifen or n-3:n-6 treatment, but the differences noted have been reported not to be clinically meaningful (37).

**Experiment 4** The hypothesis tested in this experiment was that the reduction observed in mammary gland density in rats fed the n-3:n-6 25:1 dietary ratio relative to the referent group (n-3:n-6 1:1) predicts cancer inhibitory activity. Mammary cancer incidence, cancer multiplicity, and cancer burden were significantly reduced by 25:1 n-3:n-6 diet and the effect was particularly robust in tamoxifen-treated rats (Table 5). Body weight gain was modestly reduced by tamoxifen or n-3:n-6 treatment, but the difference between the n-3:n-6 25:1 vs 1:1 in the tamoxifen treated groups was not statistically significant.

Plasma samples from the animals in this carcinogenesis study were evaluated for effects of dietary ratio and tamoxifen on host systemic factors that have been implicated in the carcinogenic process and/or to be affected by the dietary n3/n6 ratio. Three
markers of inflammation, C-reactive protein, IL-6, and TNF-α, were unaffected by the n-3:n-6 ratio and only IL-6 was reduced in tamoxifen treated rats (Table 5 and Supplementary Table 5). Plasma adipokines, leptin and adiponectin were down and up regulated, respectively, by dietary n-3:n-6 ratio but unaffected by treatment with tamoxifen. Fasting glucose, insulin, and IGF-1 were down regulated by n-3:n-6 ratio; whereas, tamoxifen affected only glucose and insulin.
Discussion

Recognizing the difficulty of evaluating the impact of dietary interventions on breast cancer risk in human populations, Experiment 1 was designed to determine if the dietary n-3:n-6 ratio would affect mammary gland density in the rat. Breast density is a recognized independent risk factor for breast cancer and has been reported to be subject to modulation by lifestyle factors such as diet (23-25). However, to our knowledge, there have not been any attempts to use mammary gland density as a screening tool in preclinical models for breast cancer nor has the effect of the n-3:n-6 ratio been evaluated. Mammary gland density was only decreased significantly as the ratio of n-3:n-6 increased from 1:1 to 25:1 (Figure 1 and Table 2).

Given these effects, a series of experiments (Experiments 2-4) were initiated to test the hypothesis that mammary gland density would predict the carcinogenic response. Experiment 2 tested the prediction that n-3:n-6 ratios from 5:1 to 1:5 would not inhibit carcinogenesis. Tamoxifen reduced cancer incidence, cancer multiplicity, and cancer burden in the absence of any effect of the n-3:n-6 ratio (Table 3). This finding, while consistent with the mammary gland density data (Table 2) and the null findings reported in (12, 36, 38), disagrees with other published reports in which high levels of dietary fish oil were reported to protect against chemically induced mammary carcinogenesis (10-18).

Experiments 3 and 4 were conducted to test the prediction that the n-3:n-6 ratios of 10:1 and 25:1 would inhibit the carcinogenic response. The 10:1 ratio resulted in numerical reductions in cancer incidence, cancer multiplicity, and cancer burden in the presence or absence of tamoxifen treatment, that were marginally significant statistically (Table 4), a finding consistent with the mammary gland density data (Table 2). However, the
cancerous response was inhibited to a significant extent when the dietary ratio of n-3:n-6 was increased to 25:1. These findings provide evidence that screening for effects on mammary gland density are predictive of cancer outcome in this model. More importantly, large effects of the n-3:n-6 ratio were noted in tamoxifen treated animals suggesting that fish oil is exerting effects on mammary carcinomas that were both sensitive and resistant to inhibition with tamoxifen, a selective estrogen receptor modifier. This finding justifies detailed studies of the molecular characteristics of the mammary carcinomas that were induced, although those experiments are beyond the scope of this paper.

In a recent review of the effects of n-3 fatty acids in rodent models for breast cancer, it was found that not only have contradictory findings been reported but also that experiments were of such variable quality, particularly as it related to documenting the fatty acid composition of the diet, that it was not possible to determine with confidence if the intended hypotheses about fish oil and breast cancer were actually evaluated (1). In addition, despite discussion of the likely importance of the ratio of n-3:n-6, only limited work has been done to test the effect of the ratio on the process of breast carcinogenesis. Hence, we set about to create a series of diets that varied in their ratio of n-3:n-6 fatty acids. The composition of these diets is shown (Table 1) and the content of the specific fatty acids contained within the diets based on formulation and as determined by GC-FID are provided (Supplementary Tables 1 and 2). The data have value in illustrating how diets with such a broad range of ratios of n-3:n-6 were formulated in a translationally relevant manner, i.e. 30% dietary kcals as fat (Table 1).
The fatty acid composition data demonstrate the point at which it is no longer possible to provide saturated, monounsaturated, and polyunsaturated fatty acids in the recommended ratio of 1:1:1 and increase dietary n-3 content (n-3:n-6 ≥10:1; Supplementary Table 1). Since the ratios that inhibited carcinogenesis were 10:1 and 25:1, these data indicate the need for using a dietary fish oil supplement to obtain benefit since such high levels of n-3 fatty acids are not easily achieved in the human diet. The dietary fatty acid analysis data illustrate the importance of actual quantification of the fatty acid profile of diets and not to depend solely on fatty acid composition data provided by a product’s vendor (Supplementary Table 2). Based on those analyses, the n-3:n-6 ratios that inhibited carcinogenesis were actually 5:1 and 15:1. This finding highlights the concern with much of the existing literature in which dietary fatty acid data is based on vendor provided information rather than analysis. This may account for apparently conflicting reports among laboratories about the effects of fish oil on the carcinogenic response variations in ratios of saturated and monounsaturated fatty acids may also exert effects on the carcinogenic process distinct from those attributed to the n3:n6 ratio.

In Experiment 2 it was expected that the observed changes in mammary gland density would be accompanied by changes in plasma concentrations of specific fatty acids, namely arachidonic acid, eicosapentaenoic acid and/or docosahexaenoic acid. However, changes in plasma concentrations of these fatty acids at the dietary ratios of n-3:n-6 that affected mammary gland density were small, and concentrations of eicosapentaenoic acid and docosahexaenoic acid failed to increase significantly above
the levels observed at the n-3:n-6 dietary ratio of 5:1, a dietary ratio that did not significantly influence mammary gland density (Table 2). While this observation could imply that eicosanoid metabolism is not involved in accounting for the reduction in mammary gland density, the expectation is that such large changes in dietary n-3:n-6 ratio would exert effects via influencing substrate availability, particularly for eicosanoid synthesis. Clearly, more work is needed to fully understand these observations. However, the effects on plasma fatty acid concentrations require not only additional studies of eicosanoid synthesis but also eicosanoid degradation since the levels of eicosapentaenoic acid and docosahexaenoic acid actually decreased numerically with increasing n-3:n-6 ratio above 5:1. The possibility that fatty acid degradation is induced is noteworthy given recent reports that eicosanoid elimination is associated with tumor suppressor activity (39-43). Also warranted by the plasma fatty acid data are studies of hepatic, adipose, and muscle lipid metabolism in response to the ratio with a particular focus on lipid oxidation and detoxification mechanisms. Alternatively, it was not possible to maintain the 1:1:1 dietary ratio of saturated:monounsaturated:polyunsaturated fatty acids when the n-3:n-6 ratios were increased to either 10:1 or 25:1 (Supplementary Table 1). Whether these differences contributed to the differences in biological activity observed also merits consideration.

The plasma from the animals on which mammary gland density was determined was also analyzed for effects on host systemic factors that have been implicated in breast cancer (26, 44). While no evidence of an effect on biomarkers of chronic inflammation was observed (i.e. C-reactive protein, IL-6, or TNF-α), the concentration of leptin was
reduced \((P = 0.005)\) and adiponectin increased \((P < 0.001)\) as the n-3:n-6 ratio increased. These data clearly signal that the dietary ratio of n-3:n-6 had an effect on adipose tissue function, a finding consistent with a number of reports. However, despite the fact that the mammary epithelium is imbedded in a subcutaneous fat pad and that leptin and adiponectin have been implicated in mammary carcinogenesis \(45-49\), no relationship was observed between the plasma concentration of these cytokines and mammary gland density. The effects of the 25:1 n-3:n-6 dietary ratio on leptin and adiponectin was confirmed in Experiment 4 and was observed in the presence or absence of tamoxifen treatment \(\text{Table 5}\). Given that the effects of the dietary ratio of n-3:n-6 fatty acids on these cytokines are pronounced, the significant effects of the enhanced n-3:n-6 ratio in tamoxifen treated animals \(\text{Tables 4 and 5}\), and recent evidence that leptin/adiponectin regulate the function of mammary epithelial cells via the LKB1/AMPK signaling network, in part by regulating aromatase activity \(50, 51\), a detailed investigation of these adipokines relative to mammary cancer risk is warranted.

Given reports that n-3 fatty acids can influence insulin sensitivity, plasma was also evaluated for evidence of an effect on glucose homeostasis \(52-55\). While fasting glucose and insulin were reduced with increasing n-3:n-6 ratio, the differences did not reach the level of statistical significance \(\text{Supplementary Table 5}\). However, plasma IGF-1 was reduced as the ratio increased and when mammary gland density for all 63 animals used in Experiment 1 were regressed on plasma IGF-1 \(\text{Figure 1C}\), a significant positive relationship was observed. This finding also was assessed in Experiment 4 in which IGF-1 levels were observed to be lower in n-3:n-6 ratio 25:1 in
both the presence or absence of tamoxifen treatment. Since the signaling pathway that is activated when plasma IGF-1 binds to its cognate cell surface receptor is misregulated in the majority of human breast cancers (56-60), this observation indicates that priority should be given to understanding how the dietary n-3:n-6 ratio modulates the expression of the various proteins and Si-RNA that regulate the signaling network of which PI-3 kinase, PTEN, and mTOR are components.

Concluding Comments  Findings from epidemiological investigations as well as those from animal carcinogenesis studies reflect a contradictory literature about the potential for n-3 fatty acid dietary enrichment to reduce the risk for breast cancer. There are an unusually large number of factors that may obscure the real protective effects of n-3 fatty acids relative to breast cancer risk. The work reported herein represents one arm of a systematic effort to resolve the fish oil-breast cancer conundrum and points to an important signaling network for detailed studies of cancer inhibitory activity. While there are many issues that must be considered including population and clinical studies, in vitro experiments using human breast cancer cells, carcinogenesis studies in animal models for breast cancer, and trans-species and trans disease mechanistic studies, this paper provides a rational basis for a new generation of in vivo mechanistic studies of fish oil mediated effects using a widely investigated animal model for breast cancer with demonstrated translational potential as evidenced by the fact that this model was used in the early phases of development of drugs such as tamoxifen and raloxifene.
Funding

This work was supported by the Susan G. Komen Foundation (KG081632).

Acknowledgements

The excellent technical assistance of Amanda Blasingame, Ana Calcagnotto, Angie Neil, Elizabeth Neil, Denise Rush, Dana Steiner and Neil Trushin are greatly appreciated.
References


Table 1. Composition of experimental diets with different ratio of omega-3 to omega-6 fatty acids

<table>
<thead>
<tr>
<th>Ingredient (g/kg diet)</th>
<th>n-3:n-6 25:1</th>
<th>n-3:n-6 10:1</th>
<th>n-3:n-6 5:1</th>
<th>n-3:n-6 1:1</th>
<th>n-3:n-6 1:5</th>
<th>n-3:n-6 1:10</th>
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<td>217.38</td>
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<td>275.52</td>
<td>275.52</td>
<td>275.52</td>
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Table 2. Effect of n-3:n-6 dietary fatty acid ratio on mammary gland density and plasma analytes*

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<tr>
<th>Dietary treatment</th>
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<th>n-3:n-6</th>
<th>n-3:n-6</th>
<th>n-3:n-6</th>
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<td>5:1</td>
<td>1:1</td>
<td>1:5</td>
<td>1:10</td>
<td>1:25</td>
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<tr>
<td>Breast density (%)</td>
<td>50.1 ± 1.2†</td>
<td>58.9 ± 3.5†‡</td>
<td>63.7 ± 5.3†‡</td>
<td>66.8 ± 5.0‡</td>
<td>60.4 ± 4.2‡</td>
<td>57.2 ± 4.2‡</td>
<td>62.6 ± 4.9‡</td>
</tr>
<tr>
<td>Leptin (pg/ml)</td>
<td>96.7 ± 27.9†</td>
<td>118.5 ± 18.8†</td>
<td>134.3 ± 16.8‡</td>
<td>163.3 ± 21.5‡</td>
<td>183.2 ± 31.3‡</td>
<td>264.6 ± 51.7‡</td>
<td>152.3 ± 20.5†‡</td>
</tr>
<tr>
<td>Adiponectin (µg/ml)</td>
<td>18.4 ± 0.6†</td>
<td>17.5 ± 1.6†</td>
<td>16.2 ± 1.1†</td>
<td>15.9 ± 1.2‡</td>
<td>12.0 ± 0.8‡</td>
<td>12.7 ± 1.2‡</td>
<td>10.9 ± 0.7§</td>
</tr>
<tr>
<td>IGF-1 (ng/ml)</td>
<td>331.0 ± 34.7†</td>
<td>385.6 ± 36.3†‡</td>
<td>481.0 ± 40.1‡</td>
<td>503.9 ± 31.9‡</td>
<td>481.9 ± 10.6‡</td>
<td>432.9 ± 33.5‡</td>
<td>443.5 ± 30‡‡</td>
</tr>
<tr>
<td>Linoleic acid (µmol/ml)</td>
<td>0.23 ± 0.07†</td>
<td>0.30 ± 0.08‡</td>
<td>0.38 ± 0.08‡</td>
<td>0.69 ± 0.10‡</td>
<td>0.91 ± 0.13‡</td>
<td>0.93 ± 0.13‡</td>
<td>0.87 ± 0.21‡</td>
</tr>
<tr>
<td>Arachidonic acid (µmol/ml)</td>
<td>0.52 ± 0.10†</td>
<td>0.50 ± 0.08‡</td>
<td>0.61 ± 0.16†</td>
<td>0.66 ± 0.11†</td>
<td>1.11 ± 0.38‡</td>
<td>1.34 ± 0.50‡</td>
<td>1.25 ± 0.45†</td>
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<tr>
<td>Eicosapentenoic acid (EPA) (µmol/ml)</td>
<td>0.73 ± 0.17†</td>
<td>0.87 ± 0.24†‡</td>
<td>0.99 ± 0.23‡</td>
<td>0.56 ± 0.13§</td>
<td>0.34 ± 0.15§</td>
<td>0.18 ± 0.05§</td>
<td>ND</td>
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<tr>
<td>Docosohexaenoic acid (DHA) (µmol/ml)</td>
<td>0.33 ± 0.09§</td>
<td>0.44 ± 0.10§</td>
<td>0.53 ± 0.13§</td>
<td>0.33 ± 0.07§</td>
<td>0.23 ± 0.04§</td>
<td>0.23 ± 0.05§</td>
<td>0.19 ± 0.06§</td>
</tr>
<tr>
<td>Total n-3 fatty acids (µmol/ml)</td>
<td>1.14 ± 0.25‡</td>
<td>1.39 ± 0.33‡</td>
<td>1.58 ± 0.35‡</td>
<td>0.95 ± 0.19‡</td>
<td>0.62 ± 0.16§</td>
<td>0.47 ± 0.08§</td>
<td>0.24 ± 0.07†</td>
</tr>
<tr>
<td>Total n-6 fatty acids (µmol/ml)</td>
<td>0.80 ± 0.14†</td>
<td>0.80 ± 0.14†</td>
<td>0.99 ± 0.20‡‡</td>
<td>1.35 ± 0.18‡</td>
<td>2.02 ± 0.42§</td>
<td>2.27 ± 0.58§</td>
<td>2.12 ± 0.64§</td>
</tr>
<tr>
<td>n-3:n-6 ratio</td>
<td>1.44 ± 0.27†</td>
<td>1.74 ± 0.29†</td>
<td>1.61 ± 0.28‡</td>
<td>0.70 ± 0.10‡</td>
<td>0.31 ± 0.09§</td>
<td>0.21 ± 0.03§</td>
<td>0.11 ± 0.03</td>
</tr>
</tbody>
</table>

*Values are means ± SEM. Data were analyzed by ANOVA with post hoc comparisons by the method of Bonferroni. Values within a row with different superscripts are statistically different from each other (P < 0.05). ND, not detected.
Table 3. Effect of n-3:n-6 fatty acid ratio (5:1, 1:1, 1:5) and tamoxifen on the carcinogenic response in mammary gland*

<table>
<thead>
<tr>
<th>Dietary treatment</th>
<th>n-3:n-6 5:1</th>
<th>n-3:n-6 1:1</th>
<th>n-3:n-6 1:5</th>
<th>Tmx/ n-3:n-6 5:1</th>
<th>Tmx/ n-3:n-6 1:1</th>
<th>Tmx/ n-3:n-6 1:5</th>
<th>P</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer Incidence (%)</td>
<td>90.0 †</td>
<td>96.7 †</td>
<td>96.7 †</td>
<td>70.0 ‡</td>
<td>63.3 ‡</td>
<td>73.3 ‡</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>Cancer Multiplicity (No. carcinomas/rat)</td>
<td>4.5 ± 0.6 †</td>
<td>4.3 ± 0.4 †</td>
<td>4.3 ± 0.5 †</td>
<td>2.1 ± 0.4 ‡</td>
<td>1.6 ± 0.4 ‡</td>
<td>4.5 ± 0.3 †</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>Cancer Burden (Ave. cancer mass/rat (g))</td>
<td>6.52 ± 1.10 †</td>
<td>6.75 ± 0.90 †</td>
<td>6.02 ± 1.02 †</td>
<td>0.87 ± 0.28 ‡</td>
<td>0.67 ± 0.28 ‡</td>
<td>0.86 ± 0.34 ‡</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>Final Body Weight (g)</td>
<td>248 ± 4 †</td>
<td>253 ± 5 †</td>
<td>254 ± 5 †</td>
<td>209 ± 3 †</td>
<td>211 ± 3 ‡</td>
<td>211 ± 2 †</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
</tbody>
</table>

*Values are means ± SEM except incidence. Data were analyzed by Fisher’s exact test (cancer incidence), and ANOVA (square root cancers counts per rat). Values within a row with different superscripts are statistically different from each other (P < 0.05) based on post hoc analyses adjusted for multiple comparisons. Tmx, tamoxifen.
Table 4. Effect of n-3:n-6 fatty acid ratio at 10:1 and tamoxifen on carcinogenic response in mammary gland
d

<table>
<thead>
<tr>
<th>Dietary treatment</th>
<th>n-3:n-6</th>
<th>n-3:n-6</th>
<th>Tmx/</th>
<th>Tmx/</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:1</td>
<td>10:1</td>
<td>n-3:n-6 1:1</td>
<td>n-3:n-6 10:1</td>
<td>Overall</td>
</tr>
<tr>
<td>Cancer Incidence (%)</td>
<td>86.7 †</td>
<td>76.7 †</td>
<td>50 ‡</td>
<td>36.7 §</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Cancer Multiplicity (No. carcinomas/rat)</td>
<td>3.1 ± 0.3 †</td>
<td>2.3 ± 0.3 †</td>
<td>1.0 ± 0.3 ‡</td>
<td>0.6 ± 0.2 ‡</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Cancer Burden (Ave. cancer mass/rat (g))</td>
<td>1.44 ± 0.20 †</td>
<td>1.03 ± 0.77 †,‡</td>
<td>0.52 ± 0.18 †,§</td>
<td>0.04 ± 0.01 §</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Final Body Weight (g)</td>
<td>244 ± 4 †</td>
<td>224 ± 4 †</td>
<td>197 ± 3 §</td>
<td>189 ± 3 §</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

*Values are means ± SEM except incidence. Data were analyzed by Fisher’s exact test (cancer incidence), and ANOVA (square root cancers counts per rat). Values within a row with different superscripts are statistically different from each other (P < 0.05) based on post hoc analyses adjusted for multiple comparisons. Cancer latency data were not adjusted for multiple comparisons. The results of the factorial ANOVA that were performed on cancer multiplicity and cancer burden did not provide support for the existence of an interaction between tamoxifen and dietary n3:n6 ratio. Tmx, tamoxifen.
Table 5. Effect of n-3:n-6 fatty acid ratio at 25:1 and tamoxifen on carcinogenic response in mammary gland*

<table>
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<tr>
<th>Dietary treatment</th>
<th>n-3:n-6 1:1</th>
<th>n-3:n-6 25:1</th>
<th>Tmx/n-3:n-6 1:1</th>
<th>Tmx/n-3:n-6 25:1</th>
<th>P Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer Incidence (%)</td>
<td>96.7 †</td>
<td>76.7 ‡</td>
<td>62.1 ‡</td>
<td>26.7 §</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Cancer Latency (days)</td>
<td>37.9 ± 1.5 †</td>
<td>42.1 ± 1.6 †,‡</td>
<td>47.0 ± 0.9 †</td>
<td>46.8 ± 1.3 ‡</td>
<td>&lt; 0.0001</td>
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<tr>
<td>Cancer Multiplicity (No. carcinomas/rat)</td>
<td>3.0 ± 0.4 †</td>
<td>2.1 ± 0.3 †,‡</td>
<td>1.3 ± 0.3 †,§</td>
<td>0.6 ± 0.2 §</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Cancer Burden (Ave. cancer mass/rat (g))</td>
<td>1.44 ± 0.39 †,‡</td>
<td>0.29 ± 0.09 †</td>
<td>0.35 ± 0.26 †</td>
<td>0.23 ± 0.20 ‡</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Final Body Weight (g)</td>
<td>240 ± 3 †</td>
<td>206 ± 3 ‡</td>
<td>199 ± 2 †,§</td>
<td>190 ± 3 §</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Leptin (pg/mL)</td>
<td>1052.6 ± 254.7 †,‡</td>
<td>564.6 ± 108.2 †</td>
<td>1054.6 ± 95.1 †</td>
<td>548.2 ± 58.6 §</td>
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<tr>
<td>Adiponectin (µg/mL)</td>
<td>14.68 ± 1.4 †</td>
<td>29.86 ± 3.7 †</td>
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<tr>
<td>IGF-1 (ng/mL)</td>
<td>413.4 ± 36.9 †,‡</td>
<td>340.8 ± 28.8 †,§</td>
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<td>310.2 ± 15.1 §</td>
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</table>

*Values are means ± SEM except incidence. Data were analyzed by Fisher’s exact test (cancer incidence), and ANOVA (square root cancers counts per rat). Values within a row with different superscripts are statistically different from each other (P < 0.05) based on post hoc analyses adjusted for multiple comparisons. Cancer latency data were not adjusted for multiple comparisons. The results of the factorial ANOVA that were performed on cancer multiplicity and cancer burden did not provide support for the existence of an interaction between tamoxifen and dietary n3:n6 ratio. Tmx, tamoxifen.
Figure Legends

Figure 1

(A) Carmine stained abdominal inguinal mammary gland whole mounts depicting effect of n-3:n-6 on phenotype, bars = 0.5 cm. (B) Mammary gland density analysis shows a decreasing trend in density as n-3:n-6 increases. (C) Linear regression of mammary gland density and IGF-1, upper prediction limit (UPL); upper confidence limit (UCL); estimate (EST); lower confidence limit (LCL); lower prediction limit (LPL).

Supplementary Figure S1

Study design and key differences between experiments.

Supplementary Figure S2

(A) Grayscale image of a carmine stained abdominal inguinal mammary gland whole mount. (B) Extracted epithelium used in image analysis (C) Extracted interductal fat pad fused in image analysis. (D) High resolution view of area depicted in panel A (black square). (E) High resolution view of area depicted in panel B (black square). (F) High resolution view of area depicted in panel C (black square). Bars displayed in panels A-C = 0.5 cm.
# Cancer Prevention Research

## Mammary Gland Density Predicts the Cancer Inhibitory Activity of the N-3 to N-6 Ratio of Dietary Fat


*Cancer Prev Res* Published OnlineFirst August 2, 2011.

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