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Mammary tumor development is directly inhibited by lifelong n-3 polyunsaturated fatty acids☆,☆☆

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Abstract

Introduction: Despite the advocacy that diet may be a significant contributor to cancer prevention, there is a lack of direct evidence from epidemiological and experimental studies to substantiate such claims. Experimental studies suggest that n-3 polyunsaturated fatty acids (n-3 PUFA) from marine oils may reduce breast cancer risk, however, findings are equivocal. Thus, in this study, novel transgenic mouse models were employed to provide, for the first time, direct evidence for an anti-cancer role of n-3 PUFA in mammary tumorigenesis.
Methods: fat-1 Mice, which are capable of endogenous n-3 PUFA synthesis, were bred with mouse mammary tumor virus (MMTV)-neu(ndl)-YD5 mice, an aggressive breast cancer model. The resultant offspring, including novel hybrid progeny, were assessed for tumor onset, size and multiplicity as well as n-3 PUFA composition in mammary gland and tumor tissue. A complementary group of MMTV-neu(ndl)-YD5 mice were fed n-3 PUFA in the diet.
Results: Mice expressing MMTV-neu(ndl)-YD5 and *fat-1* displayed significant ($P<.05$) reductions in tumor volume (~30%) and multiplicity (~33%), as well as reduced n-6 PUFA and enriched n-3 PUFA in tumor phospholipids relative to MMTV-neu(ndl)-YD5 control mice. The effect observed in hybrid progeny was similarly observed in n-3 PUFA diet fed mice.
Conclusion: Using complementary genetic and conventional dietary approaches we provide, for the first time, unequivocal experimental evidence that n-3 PUFA is causally linked to tumor prevention.

Keywords: Breast cancer; Mammary tumorigenesis; n-3 PUFA; *fat-1* mice; MMTV-neu(ndl)-YD5 mice; maternal nutrition

1. Introduction

Despite the advocacy that diet may contribute largely to the prevention of cancer there remains only probable evidence for many nutrients [1]. Human epidemiological studies have provided little causal evidence linking specific dietary nutrients to cancer prevention, especially for breast cancer. In fact, only strong evidence has been found in support of a positive association between increased alcohol and body fatness with increased breast cancer risk [1]. The inherent challenges in conducting and measuring diet in epidemiological studies is well recognized, which has hindered our ability to firmly establish linkages between dietary nutrients and cancer risk [2–4].

Breast cancer (BC) is the most common cancer among women worldwide [5–7]. Though it has yet to be determined what initially causes the onset of BC, research suggests potential links to dietary habits and fat intake. Diet has been linked to having a beneficial impact on long-term health and disease outcomes [8], and in the case of BC, specific dietary fatty acids have been suggested to play an important role [9]. Epidemiological studies have found significant differences in BC incidence between populations consuming Western diets and those consuming Asian diets [10–12]. Westerners typically consume more n-6 polyunsaturated fatty acid (n-6 PUFA), such as linoleic acid (LA) which is metabolized to arachidonic acid (AA), and not enough n-3 PUFA. In contrast, Asians typically consume high

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amounts of n-3 PUFA, such as alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [13,14]. Metabolism of n-6 PUFA gives rise to pro-inflammatory eicosanoids, whereas n-3 PUFA produce less-inflammatory eicosanoids [15], and since these two classes of PUFA compete for shared metabolic enzymes, the presence of n-3 PUFA results in reduced production of n-6 PUFA-derived eicosanoids [16,17]. Though a number of human studies have associated n-3 PUFA with a reduction in BC risk [18–21], the totality of evidence is inconclusive [22–24]. In contrast, experimental studies in rodent models have provided more consistent evidence supporting an anti-cancer role for n-3 PUFA, which was recently reviewed by Signori et al. [25]. Importantly, experimental studies have shed light on potential factors which may help explain the inconsistency observed in human studies [25]. For example, the influence of early life dietary exposures on mammary gland (MG) development during puberty and other critical periods may influence BC risk later in life [26]. This is due to alterations in the structure of the MG which can have lasting effects on BC risk by affecting the number of tumor initiation sites known as terminal end buds (TEB).

Due to confounding factors inherent in diet-based studies in humans and rodents, a genetic approach would provide a direct means with which to study the impact of n-3 PUFA exposure on mammary tumor development. The novel *fat-1* transgenic mouse was created to meet this need. Mice carrying the *fat-1* transgene, which encodes an n-3 desaturase derived from the roundworm *Caenorhabditis elegans*, endogenously produce n-3 PUFA from n-6 PUFA obtained in the diet [27].

The comparative nature of MG development between humans and murine animals [28–30] has allowed for numerous mouse models of BC to be developed in order to recapitulate molecular pathways activated during human mammary tumor development. The mouse mammary tumor virus (MMTV)-neu(ndl)-YD5 model provides a means of modeling human mammary tumors that over-express HER-2 via the murine-equivalent *neu* oncogene [31]. The MMTV-neu(ndl)-YD5 model is a highly aggressive BC model that develops mammary tumors palpable by 3 months of age [31]. Additionally, *neu*-based BC models develop mammary tumors that histologically resemble human mammary adenocarcinomas [31,32]. Based on its highly aggressive phenotype, the MMTV-neu(ndl)-YD5 mouse represents a relevant model system for elucidating potential strategies aimed at prevention and/or treatment of HER-2 positive BC.

In this study, the *fat-1* and MMTV-neu(ndl)-YD5 mouse models were crossed to create a novel double-hybrid mouse that provides a purely genetic approach to investigate the effects of lifelong n-3 PUFA exposure on mammary tumor development. To our knowledge, no such approach has been used previously, and for the first time the present study demonstrates a causal and beneficial effect of lifelong n-3 PUFA exposure in preventing mammary tumor development.

2. Methods and materials

2.1. Animals and diet

Mice were housed in a temperature and humidity controlled room. Two transgenic mouse strains were used to conduct this study; *fat-1* mice and MMTV-neu(ndl)-YD5 mice. *fat-1* females on a mixed background were obtained as a gift from Dr. Kang, Harvard University. *fat-1* mice were backcrossed for 11 generations to an FVB background (Charles River). MMTV-neu(ndl)-YD5 mice on an FVB background were obtained as a generous gift from Dr. Muller (McGill University). *fat-1* females were then crossed with MMTV-neu(ndl)-YD5 males, producing four genetically distinct groups: wild-type (WT) (n=10), *fat-1* (n=12), MMTV-neu(ndl)-YD5 (MMTV) (n=10), and MMTV-neu(ndl)-YD5/*fat-1* (MMTV/*fat-1*) (n=12) mice. All offspring were weaned from their mother at 3 weeks of age. These harems and progeny were maintained on an n-6 PUFA diet. A complementary diet group was also set up, harems of wild-type females were crossed with MMTV-neu(ndl)-YD5 males and maintained on an n-3 PUFA diet. The progeny was a mix of wild-type mice and MMTV-neu(ndl)-YD5 mice. All progeny were weaned from their mothers at 3 weeks of age, genotyped and only MMTV-neu(ndl)-YD5 mice were kept (n=8). Only female offspring were kept for experiments and males were euthanized at weaning. All experimental procedures were approved by the institutional animal care committee (University of Guelph).

Harems breeding MMTV mice and MMTV/*fat-1* mice were fed a diet containing 10% w/w safflower oil, as were all their resulting offspring throughout life. Harems breeding for MMTV on n-3 diet were fed a diet containing 7% safflower oil and 3% menhaden oil, as were all their resulting offspring throughout life. Diets were provided by Research Diets Inc. See Table 1 for overall diet composition and Table 2 for fatty acid composition of diet. Food and water were provided *ad libitum* to all mice and were refreshed as needed.

2.2. Puberty onset and body growth

Starting at 3 weeks of age, mice were checked daily for vaginal opening, a marker of puberty onset [33]. Mice were weighed weekly on an electronic scale to track weight gain from 3–20 weeks of age.

2.3. Genotyping

Presence of the transgenes was determined by polymerase chain reaction (PCR). Briefly, tail-snips were digested overnight at 55°C with proteinase K and 1% sodium dodecyl sulphate. DNA was extracted with buffer saturated phenol, precipitated with ethanol washings, and re-suspended in Tris-EDTA buffer. Master mix was composed of 0.3 mM dNTP (Fermentas #R0192), 1.5 mM $MgCl_2$, 1.25 units of Platinum Taq and 10× PCR buffer (Invitrogen #10966-034) and the appropriate primers for transgenes *fat-1* [5′-CTGCACCACGCCTTCACCAACC-3′(F) 5′-ACACAGCAGCATTCCAGAGATT-3′(R)) and MMTV-neu(ndl)-YD5 (5′-TTCCGGAACCCACATCAGGCC-3′(F) 5′-GTTTCCTGCAGCAGCCTACGC-3′(R)]. DNA samples were amplified with a thermal cycler (Applied Biosystems Veriti 96 Well Thermal Cycler) using the required temperature program for the specified transgene [34]. Amplified DNA samples were run on a 2% agarose gel containing ethidium bromide and visualized under UV light using GeneSnap EZChrom software.

2.4. Phenotyping

To confirm the expression, or lack thereof, of the *fat-1* gene, tissue samples were analyzed by gas chromatography. Tail-snips were crushed by mortar and pestle in the presence of liquid nitrogen. Total lipids were extracted by the Folch method, transesterified by boron trifluoride methanol (14% BF_3-MeOH), and resultant FAME was analyzed by gas liquid chromatography as previously described [35]. Samples were run on Agilent Technologies 7890A GC System. Mice were identified as wild-type when chromatograms displayed high levels of LA and AA and low levels of ALA, EPA and DHA. Alternatively, mice were identified as transgenic for *fat-1* when chromatograms displayed reduced levels of LA, AA and high levels of ALA, EPA and DHA.

2.5. Tumor palpation

Mouse MG were palpated for tumors 3 times per week, starting at 8 weeks of age. Tumor sizes were measured 2 times per week by two independent researchers, using electronic callipers. Length (sagittal plane) and width (transverse plane) were measured, and volume was calculated as follows: (length×(width)^2)/2.

Table 1
Diet composition

Macronutrient	n-6 PUFA diet		n-3 PUFA diet	
	g %	kcal %	g %	kcal %
Protein	21	20	21	20
Carbohydrate	60	58	60	58
Fat	10	22	10	22
Total		100		100
kcal/g	4.1		4.1	
Ingredient	**gm/Kg Diet**	**Kcal**	**gm/Kg Diet**	**Kcal**
Casein	200	800	200	800
L-Cystine	3	12	3	12
Corn starch	336.736	1347	336.736	1347
Maltodextrin 10	132	528	132	528
Sucrose	100	400	100	400
Cellulose, BW200	50	0	50	0
Safflower oil	97	873	67.9	611
Menhaden oil	0	0	29.1	262
t-Butylhydroquinone	0.019	0	0.019	0
Mineral mix S10022G	35	0	35	0
Vitamin mix V10037	10	40	10	40
Choline bitartrate	2.5	0	2.5	0
Total	966.255	4000	966.255	4000

Composition of AIN-93G modified diet with 10% safflower oil (n-6 PUFA diet) and AIN-93G modified diet with 7% safflower oil/3% menhaden oil (n-3 PUFA diet) as provided by manufacturer, Research Diets.

Table 2
Fatty acid composition of diets

Fatty acid	n-6 PUFA Diet containing 10% safflower oil	n-3 PUFA diet containing 3% menhaden oil; 7% safflower oil
12:0	0.0	0.0
14:0	0.2	2.6
15:0	0.0	0.3
16:0	6.3	10.4
16:1c9	0.1	3.2
18:0	2.6	3.0
18:1c9	14.6	12.2
18:1c11	0.7	1.4
18:2n6	73.6	53.7
18:3n6	0.0	0.1
18:3n3	0.2	0.6
18:4n3	0.0	1.1
20:0	0.4	0.4
20:1c11	0.1	0.4
20:2n6	0.0	0.2
20:3n6	0.1	0.1
20:3n3	0.0	0.1
20:4n6	0.0	0.4
20:5n3	0.0	3.9
22:0	0.3	0.4
22:1n9	0.1	0.1
22:2n6	0.0	0.0
22:3n3	0.0	0.1
22:4n6	0.0	0.1
22:5n6	0.0	0.3
22:5n3	0.0	0.8
22:6n3	0.3	4.0
24:0	0.2	0.2
24:1	0.0	0.3
Total n-6	73.7	54.9
Total n-3	0.6	10.5
Total SFA	10.0	17.1
Total MUFA	15.6	17.5
Total PUFA	74.3	65.4

Fatty acid composition (%) of n-6 and n-3 PUFA diets. Lipids were extracted from two individuals pellets from each diet and analyzed by gas chromatography. SFA, saturated fatty acids; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

2.6. Euthanization

At 20 weeks of age, vaginal smears were taken from mice and classified into one of four estrous cycle stages; proestrus, estrus, metaestrus or diestrus. The vagina was flushed with phosphate buffer saline solution, and the solution was then viewed on a glass slide under a Nikon Eclipse TS100 microscope for classification by cell characteristics [36]. If in proestrus, estrus or metaestrus, mice were euthanized via carbon dioxide asphyxiation followed by cervical dislocation. If the mice were in diestrus, euthanization was delayed in order to control for the impact of hormone fluctuations on cell proliferation profiles [37]. In the case of animals in diestrus, estrous check was repeated the next day. This was done for a maximum of two days past the set euthanization date, at which point estrous stage was checked and recorded, and mice were taken regardless of stage, in order to maintain consistency in tumor collection measurements.

Mice were euthanized prior to the 20-week time point if they lost >20% body weight, or exceeded the maximum allowable tumor sizes of either 17 mm (in length or width) or 5000 mm^3 total volume. Mice meeting any of these characteristics were euthanized immediately and tissues harvested as described in the following section.

2.7. Tissue collection

At time of euthanization, blood was collected by cardiac puncture, allowed to sit for 30 min before separating into cells and serum by centrifuge (10 x *g* for 5 min), and stored in a −80°C freezer. For tumor-bearing mice, MG and tumors were photographed, and final tumor volume measurements were taken using digital callipers. Tumors were cleaned of surrounding tissue, removed and weighed. Tumor tissue samples were snap-frozen in liquid nitrogen. The fifth MG, liver and uterus were removed, weighed and snap-frozen.

2.8. Fatty acid analysis

Lipid extraction. Lipids from MG and tumor tissue were extracted in the same manner. Tissue samples were thawed on ice. 0.1 g of tissue was homogenized in 0.1M KCl. Homogenates were added to 2:1 $CHCl_3$:MeOH. Samples were vortexed and chilled overnight. Samples were centrifuged and the chloroform layer was collected, dried under nitrogen and reconstituted to 10 mg/ml.

MG tissues were analyzed by lipid-class thin-layer chromatography (TLC), and tumor tissues were analyzed by phospholipid-class TLC in the following manner. The large proportion of triglycerides present in the MG prevented separation of individual phospholipid classes, therefore lipids were separated by lipid classes. Lipid class analysis is sufficient to demonstrate that n-3 PUFA were present in the target tissue of interest. Phospholipid class analysis of tumors provides more detailed information regarding potential mechanisms of action.

Lipid-class TLC. samples were run on an activated G-plate (Analtech #01011). The TLC solvent was made fresh by combining 80 ml petroleum ether, 20 ml ethyl ether and 1 ml acetic acid. The TLC plate was run and then lightly sprayed with 0.1% (w/v) ANSA (Fluka #GA12046). Lipid class bands (Triglyceride, free fatty acids and phospholipids) were visualized under UV light by comparing to standard, and collected for analysis. A C17:0 FFA standard was included using a Hamilton syringe.

Phospholipid-class TLC. Samples were run on an activated H-plate (EMD Chemicals #5721-7). The TLC solvent was made fresh by combining 30 ml chloroform, 9 ml methanol, 25 ml 2-propanol, 6 ml 0.25M KCl, and 18 ml triethylamine. The TLC plate was run and then lightly sprayed with 0.1% (w/v) ANSA (Fluka #GA12046). Phospholipid bands were visualized under UV light. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS) were collected for analysis. A C17:0 FFA standard was included using a Hamilton syringe.

Methylation for MG and tumor tissues was the same, as described below.

Methylation. Hexane and 14% BF_3-MeOH (Sigma B1252) were added to each sample and incubated for 90 min at 100°C. ddH_2O was then added to stop methylation. Samples were centrifuged and the hexane layer was collected. Samples were then dried under nitrogen, reconstituted in hexane and run on Agilent Technologies 7890A GC System.

Sample size from each group was as follows: MG tissue from MMTV (n=4); MMTV/*fat-1* (n=4) and MMTV-n-3 (n=8) and tumor tissue from MMTV (n=8), MMTV/*fat-1* (n=10) and MMTV-n-3 (n=8).

2.9. Statistical analyses

SASv9.1 was used to perform the following statistical analyses. One-way ANOVA was conducted for puberty onset between all groups, as well as for tumor latency in MMTV versus MMTV/*fat-1* mice. P<.05 by post hoc LSD was considered significant. A one-way analysis of variance (ANOVA) was conducted for tumor latency and tumor growth rate, with P<.05 considered significantly different. A repeated-measures test was conducted for total tumor volume and tumor multiplicity over the 20 week time course to determine differences between groups over time, as well as for body weight gain between groups, with P<.05 considered significant. One-way ANOVA were conducted to compare liver, uterus and mammary gland tissue weights between groups, all of which were corrected for body weight. P<.05 by post hoc LSD was considered significant. Values are reported as mean±SD. All phospholipid data was analyzed by one-way ANOVA, where P<.05 by post hoc Tukey was considered significant.

For the purposes of this study, the following definitions for tumor parameters were used. Tumor latency values were determined by time to palpation of first tumor. Total tumor volume values were calculated as the sum of all tumor volumes carried by each mouse. Tumor multiplicity values were determined by the total number of tumors palpated in each mouse.

3. Results

3.1. Tumor volume

Total tumor volume accumulated over time was compared pairwise between MMTV (n=10), MMTV/*fat-1* (n=12) and MMTV on n-3 PUFA diet (n=8) groups by repeated measures. Total tumor volume carried by MMTV/*fat-1* mice was significantly (P<.05) less than total tumor volume carried by MMTV mice, over the 20 week time course (Fig. 1). Similarly, total tumor volume carried by MMTV mice on n-3 PUFA diet was significantly (P<.05) less than that carried by MMTV control mice, with a 30% difference in final tumor volume between groups (Fig. 1). Furthermore, total tumor volume carried by MMTV mice on n-3 was also significantly (P<.05) less than MMTV/*fat-1* mice (Fig. 1). At euthanization, final total tumor volumes carried by MMTV/*fat-1* (2061±1082 mm^3) and MMTV mice on n-3 PUFA diet (1075±838 mm^3) mice were also significantly less (P=.05) than total tumor volumes carried by MMTV controls (2952±1768 mm^3). Final tumor volumes were taken at 20 weeks of age, except when tumor size required euthanization prior to 20 weeks of age. Of 22 tumor-carrying mice, four mice were taken prior to the 20 week time point

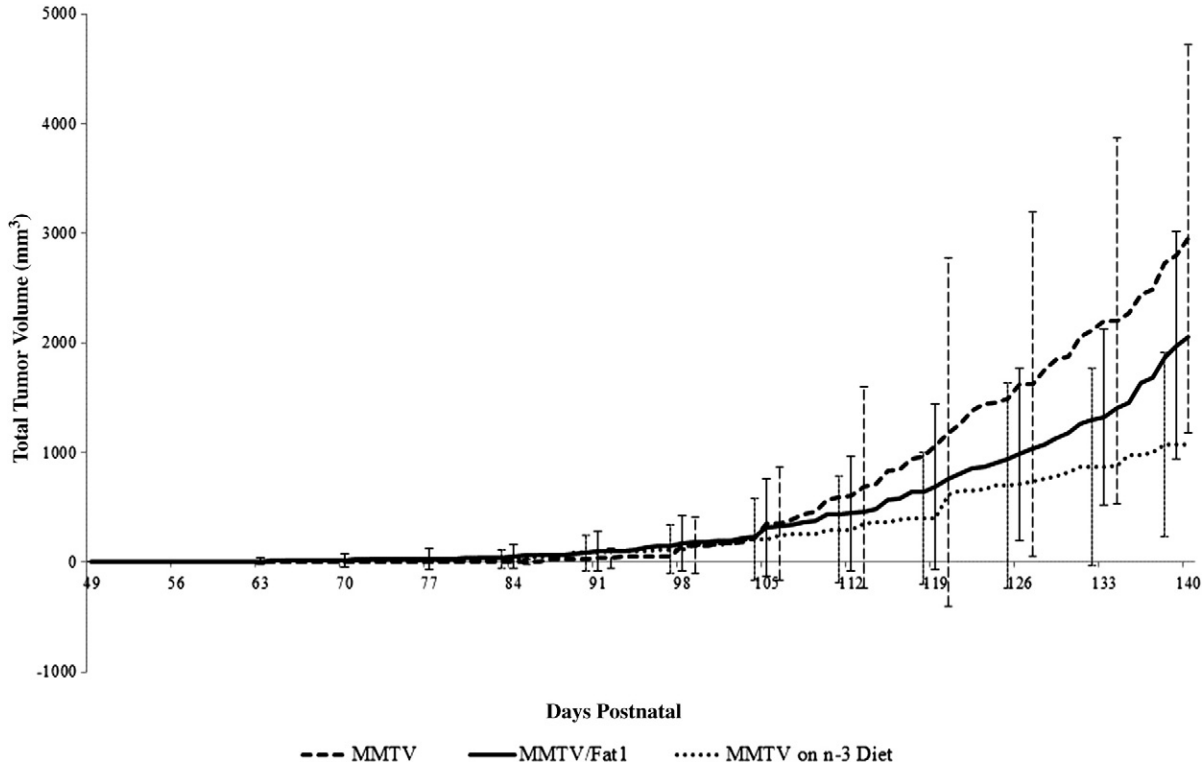

Fig. 1. Total tumor volume. Total tumor volume in MMTV (n=10), MMTV/*fat-1* (n=12) and MMTV on n-3 PUFA diet (n=8) were tracked over 20 weeks. Length and width of palpated tumors were recorded, and tumor volumes were calculated using the following equation: [(length×(width)^2]/2. Measurements were taken twice per week. All groups are significantly different (P<.05) than each other by pairwise repeated measures analysis.

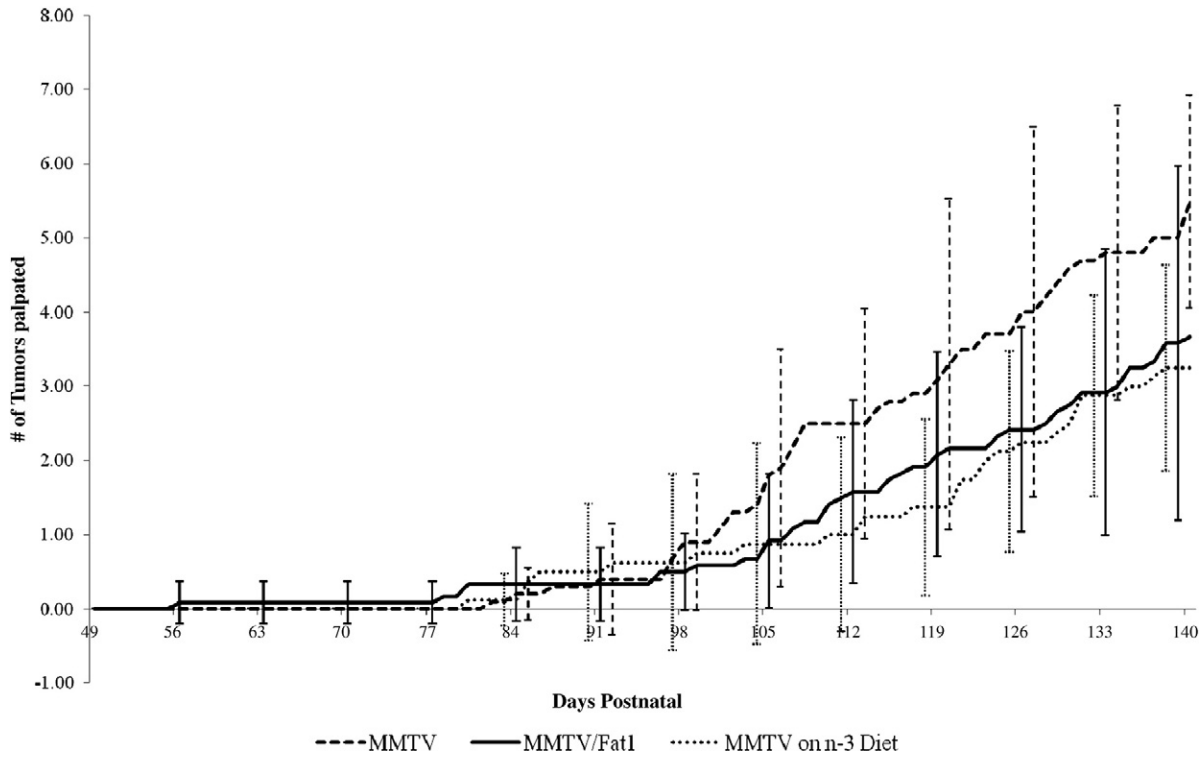

Fig. 2. Tumor multiplicity. Tumor multiplicty in MMTV (n=10), MMTV/*fat-1* (n=12) and MMTV on n-3 PUFA diet (n=8) were determined by palpation over 20 weeks. Mice were palpated for tumors three times per week. Tumor multiplicity over time in MMTV/*fat-1* and MMTV on n-3 PUFA diet were both significantly different (P<.05) compared to the MMTV control group by repeated measures analysis. There was no difference between MMTV/*fat-1* and MMTV mice on n-3 PUFA diet.

(two mice each from MMTV controls and MMTV/*fat-1* mice were taken between ~17 and 19 weeks of age).

3.2. Tumor multiplicity

Total tumor multiplicity over time was compared pairwise between groups by repeated measures. The number of tumors palpated in MMTV/*fat-1* mice was significantly less ($P<.05$) than the number palpated in MMTV control mice, over the 20 week time course (Fig. 2). Similarly, number of tumors palpated in MMTV mice on n-3 PUFA diet was significantly less ($P<.05$) than the number palpated in MMTV mice, with a 33% difference in final tumor multiplicity between groups (Fig. 2). There was no significant difference in tumor multiplicity over time between MMTV/*fat-1* mice and MMTV on n-3 PUFA diet. At euthanization, total number of tumors was significantly reduced ($P<.05$) in MMTV/*fat-1* mice (3.7±2.3 tumors) and MMTV on n-3 PUFA diet (3.3±1.4 tumors) compared to MMTV control mice (5.5±1.4 tumors). Final tumor multiplicities were taken at 20 weeks of age, except when tumor size required euthanization prior to 20 weeks of age, as described in Tumor Volume previously.

3.3. Tumor growth rate and histology

Since both tumor volume and tumor multiplicity were similarly reduced, tumor growth rate was analyzed between groups. MMTV grew 25.6±12.0 mm^3 per day and MMTV/*fat-1* exhibited 31.9±17.77 mm^3 per day and MMTV on n-3 PUFA diet exhibited 19.0 (±8.7) mm^3 per day. There was no statistical difference in the rate of tumor growth between groups. Hematoxylin and eosin (H&E)-stained tumor samples were analyzed and all were found to be adenocarcinomas with no morphological differences between groups (Fig. 3).

3.4. Tumor latency

Time to palpation of first tumor was tracked. Average tumor latency was 100.0±12.1 days in MMTV mice, 95.7±18.9 days in MMTV/*fat-1* mice and 106.4±16.3 days in MMTV mice on n-3 diet. There was no significant difference were found between MMTV, MMTV/*fat-1* and MMTV on n-3 groups for time to palpation of first tumor based on a one-way ANOVA.

3.5. Body growth and tissue weights

There were no significant differences between groups in puberty onset, diet intake, body weight change from 3 to 20 weeks of age, or tissue weights measured at euthanization.

3.6. Mammary gland fatty acid composition

Fatty acid analysis of MG tissue from WT and *fat-1* control mice was conducted to demonstrate that observed effects were related to the incorporation of n-3 PUFA in the target tissue. MG tissue from WT mice ($n=2$) exhibited fatty acid profiles high in n-6 PUFA and limited, if any, n-3 PUFA. MG tissue from *fat-1* mice ($n=2$) displayed significant accumulation of EPA ($P<.05$) and DHA ($P<.01$) in phospholipids, compared to WT mice (data not shown).

Compared to MMTV mice ($n=4$), tumor-adjacent MG tissue taken from MMTV/*fat-1* mice ($n=4$) was significantly increased in EPA, DHA and overall n-3 PUFA ($P<.05$) (Fig. 4). MMTV mice on n-3 PUFA diet ($n=8$) displayed a significant increase ($P<.05$) in EPA, compared to MMTV control mice. MMTV/*fat-1* mice and MMTV mice on n-3 PUFA diet also displayed a significant decrease in n-6/n-3 PUFA ratio ($P<.05$), declining from 34:1 in MMTV MG tissue to 6:1 in MMTV/*fat-1* and MMTV on n-3 MG tissue (data not shown).

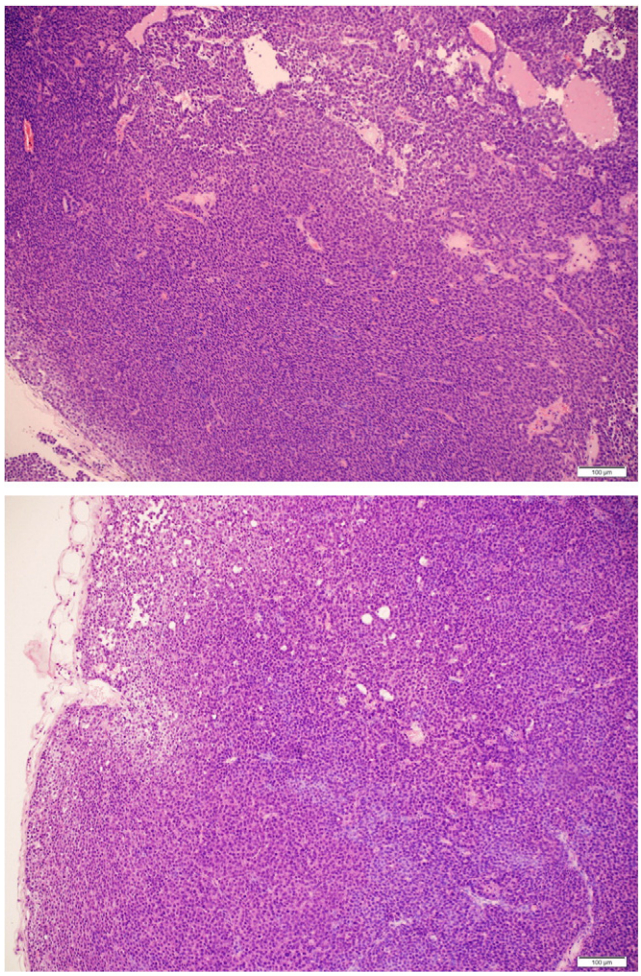

Fig. 3. Tumor morphology. Representative H&E-stained tumor samples from MMTV/*fat-1* mouse (upper) ($n=7$) and MMTV control mouse (lower) ($n=6$) are shown. All tumor samples analyzed were identified as adenocarcinomas with no morphological differences between groups.

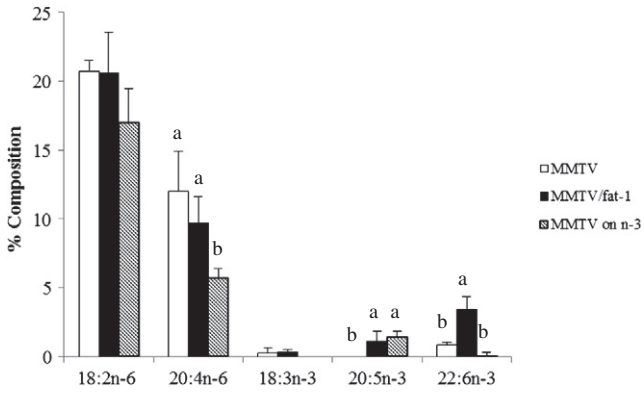

Fig. 4. Tumor-adjacent MG Fatty Acid Composition. The fatty acid composition of tumor-adjacent MG tissue differs markedly between MMTV ($n=4$), MMTV/*fat-1* mice ($n=4$) and MMTV on n-3 diet ($n=8$). Lipids from tumour-adjacent MG tissue were extracted and total phospholipids were separated by thin layer chromatography and the fatty acid composition of total phospholipids were analyzed by gas liquid chromatography. The percent composition of major n-6 and n-3 PUFA in phospholipids of tumor-adjacent MG tissue from MMTV and MMTV/*fat-1* mice are shown in the bar graph. Bars not sharing a letter are significantly different ($P<.05$) based on a one-way ANOVA test.

3.7. Tumor tissue fatty acid composition

There were significant differences in the fatty acid composition of phospholipid fractions from tumors in the three different groups, which varied between phospholipid fractions. The results from the representative PE fraction are shown in Fig. 5 and details of all phospholipid fractions are reported in Table 3. Compared to tumors from MMTV mice (n=8), tumors from MMTV/*fat-1* mice (n=10) had a significant increase in LA, a significant decrease in AA, and significant increases in ALA, EPA and DHA (Fig. 5). In the PE fraction, tumors from MMTV on n-3 PUFA diet (n=8) did not exhibit statistically different amounts of LA, ALA, EPA or DHA compared to tumors from MMTV control mice. However, tumors from MMTV on n-3 PUFA diet had a significant decrease in AA compared to tumors from MMTV mice and a significant increase in AA compared to tumors from MMTV/*fat-1* mice. The majority of n-3 PUFA in the form of EPA accumulated in the PI and PS fractions and marked reductions in AA were observed in MMTV on n-3 relative to MMTV mice (Table 3).

4. Discussion

This study has demonstrated that lifelong n-3 PUFA exposure inhibits multiple facets of mammary tumor development. There has been longstanding controversy in epidemiological studies regarding the potential beneficial effects of n-3 PUFA in affecting disease outcomes due to the inability to show causality. Indeed, with so many variable factors at play, conclusions could only be correlative at best. In this study, however, the unique nature of crossing a transgenic mouse capable of endogenously producing n-3 PUFA, with a relevant BC model, allowed for differences in mammary tumor growth between MMTV control mice and novel MMTV/*fat-1* mice to be solely attributed to the presence of n-3 PUFA within the tissues and tumors of the double-hybrid mice. This provides direct evidence that n-3 PUFA has anti-cancer effects in modulating mammary tumor development. Both in the genetic arm as well as, and more importantly, in the dietary arm of the study, the size and number of tumors was significantly decreased in groups exposed to n-3 PUFA.

This study provides clear evidence of reduced tumor volume and multiplicity in hybrid MMTV mice expressing the *fat-1* gene compared to MMTV control mice, which had no endogenous n-3 PUFA synthesizing capabilities. These findings demonstrate that n-3 PUFA can attenuate aspects of mammary tumorigenesis in the MMTV-neu(ndl)-YD5 model. While tumor volume and multiplicity were reduced, analysis of tumor onset and growth showed no significant differences between groups. These findings are not surprising given that 100% of all mice typically develop tumors in the highly aggressive MMTV-neu(ndl)-YD5 model [31]. Nevertheless, multiplicity was significantly reduced in MMTV/*fat-1* and MMTV on n-3 PUFA diet compared to MMTV control mice which demonstrates that n-3 PUFA can inhibit subsequent tumor development. This suggests that in a less aggressive model, both tumor latency and growth may be inhibited by n-3 PUFA.

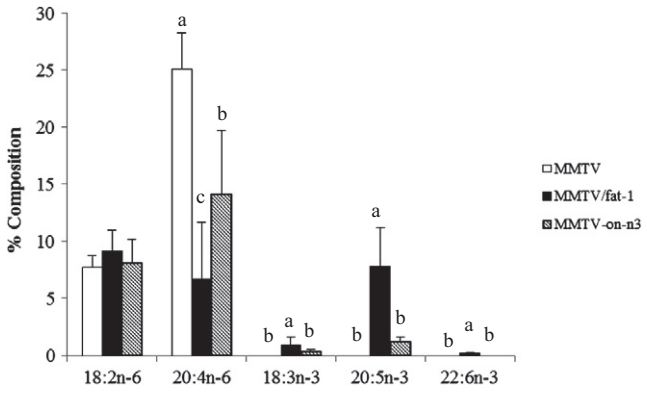

Fig. 5. Tumor fatty acid composition. The fatty acid composition of tumor phospholipid classes differs between MMTV (n=8), MMTV/*fat-1* (n=10) mice and MMTV on n-3 diet (n=8). Lipids from tumours were extracted and separated by thin layer chromatography into individual phospholipid classes and the fatty acid composition was analyzed by gas liquid chromatography. The percent composition of major n-6 and n-3 PUFA in the representative phosphatidylethanolamine fraction is shown. Bars not sharing a letter are significantly different (P<.05) based on a 1-way ANOVA test.

The findings from this study are in agreement with previous rodent feeding studies which have also shown beneficial outcomes of n-3 PUFA in mitigating mammary tumor development. One study in particular that looked at mammary tumor development in MMTV-neu mice fed menhaden oil from 7–8 weeks of age onwards also showed a significant reduction in tumor incidence (87% of corn-fed mice and 57% of fish-oil-fed mice) and tumor multiplicity by 15 months of age [38]. This study used a diet (25% energy from fat) comparable to the diet arm of the present study (22% energy from fat). Delayed tumor onset was also observed in another study when MMTVc/-neu mice were fed diets enriched in menhaden oil [39]. The evidence from several studies and the present study employing MMTV-neu related models provides concordant evidence for a protective effect of n-3 PUFA towards neu related mammary tumors. This is relevant given that a significant number of human breast cancers are HER2/neu related.

Potential mechanisms of action may be attributed to changes in membrane fatty acid composition of both the uninvolved mammary gland tissue and tumor tissue. Analysis of MG phospholipid fatty acid composition revealed significant increases in n-3 PUFA, particularly EPA (P<.05) and DHA (P<.01), as well as significant decreases (P<.05) in overall n-6/n-3 PUFA ratio, declining from 34:1 in MMTV mice to 6:1 in MMTV/*fat-1* mice. The analysis of individual phospholipid fractions of tumor tissue taken from MMTV/*fat-1* mice was found to have significant decreases in AA and significant increases in ALA, EPA and DHA (P<.01). These changes suggest potential effects on eicosanoid synthesis derived from AA, which is pro-inflammatory or cancer promoting, in contrast to EPA and DHA, which are anti-inflammatory and cancer inhibitory. Also, membrane fatty acid compositional changes may also affect signaling pathways associated with apoptosis and cell differentiation mediated through membrane rafts [40]. It was interesting to note differences observed in fatty acid profiles between the tumor tissue and the MG tissue it was extracted from, particularly in the MMTV/*fat-1* mice. Within the mammary tissue, there was a considerable amount of DHA present in phospholipids, at least double that of EPA by percent composition. However, in tumor tissue from MMTV/*fat-1* mice, we see a switch of these two long-chain n-3 PUFA, with DHA accumulation far exceeded by EPA. This may be due to a double-dosing effect experienced by these tumor cells as they may uptake fatty acids from their immediate environment as well as expressing the *fat-1* gene themselves. However, it is unclear as to why EPA is considerably more concentrated in the tumor tissue. It may indicate limited conversion of EPA to DHA, or increased retro-conversion of DHA to EPA or DPA, which was also highly enriched in the tumor tissue phospholipids. Alternatively, or perhaps in tandem, these observations may indicate a preference by tumor cells for EPA over DHA. Direct comparisons between MG and tumor tissue phospholipid data in this study, however, are not possible due the different TLC methods employed. The abundance of triglycerides present in mammary tissue from 20-week-old mice saturated the phospholipid class TLC plate preventing clear separation of individual phospholipid species, thus an alternate TLC approach for the separation of lipid classes was employed to assess total phospholipids from mammary gland tissues. Methods aside, the incorporation of these n-3 PUFA into both the mammary tissue and tumor demonstrates their direct involvement in modulating mammary tumor development, which warrants further investigation

Table 3
Fatty acid composition of mammary tumour phospholipids

Fatty acid	PE			PI			PS			PC		
	MMTV	MMTV/*fat-1*	MMTV on n-3	MMTV	MMTV/*fat-1*	MMTV on n-3	MMTV	MMTV/*fat-1*	MMTV on n-3	MMTV	MMTV/*fat-1*	MMTV on n-3
12:0	0.0±0.0	0.0±0.0	0.1±0.3	0.0±0.1	0.1±0.1	0.0±0.0	0.1±0.2	0.1±0.1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
14:0	1.2±1.7	1.0±0.8	1.6±1.0	0.2±0.3	0.2±0.2	0.2±0.4	0.2±0.2^{b}	0.2±0.2^{b}	0.6±0.4^{a}	1.5±0.4	1.6±0.5	1.5±0.3
15:0	0.0±0.0^{b}	0.0±0.0^{b}	0.4±0.2^{a}	0.0±0.0	0.0±0.0	0.0±0.0	0.1±0.2	0.0±0.1	0.1±0.2	0.2±0.1	0.2±0.1	0.4±0.2
16:0	6.8±0.9^{ab}	6.8±0.9^{b}	8.2±2.3^{a}	8.4±1.8	8.0±1.8	8.0±1.0	5.5±2.2^{b}	4.4±1.1^{b}	6.9±1.7^{a}	30.7±1.4	30.0±2.0	30.7±1.8
16:1n-7	1.9±0.4^{b}	2.9±1.0^{ab}	3.2±1.7^{a}	0.8±0.5^{b}	1.5±0.7^{ab}	2.7±1.8^{a}	0.9±0.5^{b}	1.2±0.5^{b}	4.4±4.3^{a}	3.1±0.9^{b}	5.1±2.4^{a}	4.2±0.6^{ab}
18:0	13.6±1.8^{ab}	12.9±2.9^{b}	16.9±5.8^{a}	34.4±1.3	34.5±3.6	32.6±2.5	34.4±3.9	35.1±2.4	29.8±7.4	8.1±1.4	7.6±2.0	8.8±2.4
18:1n-9	19.1±3.2^{b}	29.3±5.7^{a}	19.5±2.2^{b}	7.3±1.3^{b}	13.2±3.0^{a}	7.3±1.0^{b}	15.4±2.9^{b}	23.4±5.7^{a}	14.4±2.7^{b}	13.2±1.4^{b}	21.0±3.1^{a}	14.6±1.0^{b}
18:1n-7	5.0±1.3^{b}	6.7±1.6^{a}	5.6±0.9^{ab}	3.9±0.9^{b}	5.5±1.4^{a}	4.1±0.5^{b}	3.3±0.9	4.2±1.9	4.4±1.1	5.7±1.1	6.6±1.2	6.5±1.1
18:2n-6	7.6±1.1^{b}	9.3±1.8^{a}	8.1±2.1^{ab}	8.5±1.2^{b}	10.5±1.7^{a}	7.8±1.8^{b}	8.4±0.6^{b}	9.3±1.9^{a}	7.4±1.4^{b}	11.9±1.2	12.9±2.7	13.1±1.5
18:3n-6	0.1±0.1	0.0±0.0	0.3±0.3	0.1±0.2	0.0±0.1	0.3±0.5	0.1±0.2	0.0±0.1	0.2±0.3	0.2±0.2^{a}	0.1±0.1^{b}	0.2±0.1^{ab}
18:3n-3	0.0±0.0^{b}	0.9±0.7^{a}	0.3±0.2^{b}	0.0±0.0	0.4±0.3	0.5±1.0	0.0±0.0^{b}	0.5±0.4^{a}	0.0±0.0^{b}	0.0±0.0^{b}	0.9±0.7^{a}	0.1±0.1^{b}
20:0	0.0±0.0^{b}	0.0±0.1^{b}	0.2±0.2^{a}	0.0±0.0	0.1±0.2	0.1±0.3	0.5±0.3	0.4±0.2	0.8±0.7	0.0±0.0^{b}	0.0±0.1^{ab}	0.1±0.1^{a}
20:1n-9	1.2±1.2^{a}	1.0±0.3^{ab}	0.5±0.2^{b}	0.6±0.3^{b}	0.5±0.2^{b}	5.1±1.0^{a}	1.0±0.7^{b}	0.9±0.3^{b}	4.8±1.1^{a}	0.9±0.7	0.7±0.2	0.5±0.1
20:2n-6	1.6±1.1^{a}	0.7±0.6^{b}	0.7±0.4^{b}	1.0±0.7^{b}	0.8±0.6^{b}	15.7±4.6^{a}	0.9±0.6^{b}	0.4±0.3^{b}	6.3±4.4^{a}	2.4±1.1^{a}	1.0±0.4^{b}	1.0±0.5^{b}
20:3n-6	4.5±1.3^{a}	1.0±0.8^{b}	3.9±1.4^{a}	5.2±1.9^{a}	2.6±1.4^{b}	0.6±1.0^{c}	7.3±1.9^{a}	1.8±1.3^{b}	0.2±0.4^{b}	3.9±1.4^{a}	0.8±0.6^{b}	3.2±0.7^{a}
20:3n-3	0.0±0.1	0.3±0.2	0.3±0.4	0.0±0.0	0.5±0.4	1.1±2.1	0.1±0.2^{b}	0.1±0.1^{b}	1.2±0.8^{a}	0.0±0.0^{b}	0.5±0.4^{a}	0.1±0.1^{b}
20:4n-6	24.8±3.3^{a}	6.7±5.0^{c}	14.1±5.6^{b}	24.4±5.2^{a}	9.3±5.6^{b}	1.0±0.6^{c}	9.6±2.0^{a}	3.1±1.7^{b}	0.2±0.5^{c}	15.3±2.9^{a}	3.5±2.6^{c}	9.5±2.1^{b}
20:5n-3	0.0±0.0^{b}	7.8±3.4^{a}	1.2±0.4^{b}	0.0±0.0^{b}	5.0±2.9^{a}	6.1±4.2^{a}	0.0±0.0^{b}	1.2±0.5^{b}	6.6±5.7^{a}	0.0±0.0^{b}	3.6±1.9^{a}	0.7±0.2^{b}
22:0	0.0±0.0	0.3±0.4	0.4±0.9	0.0±0.0	0.1±0.1	0.1±0.3	0.6±0.3^{a}	0.7±0.4^{a}	0.0±0.0^{b}	0.0±0.0^{b}	0.1±0.1^{a}	0.1±0.1^{ab}
22:1n-9	0.3±0.5^{b}	0.3±0.4^{b}	3.1±2.0^{a}	1.2±1.2	0.3±0.3	1.6±2.0	2.4±2.1	1.3±0.4	1.0±0.5	0.3±0.4^{b}	0.2±0.1^{b}	1.3±1.0^{a}
22:2n-6	0.0±0.1	0.0±0.0	0.2±0.2	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.1	0.0±0.1	0.2±0.3	0.1±0.1	0.1±0.1	0.2±0.1
22:3n-3	0.3±1.0	0.1±0.1	0.0±0.1	0.1±0.3^{b}	0.0±0.0^{b}	1.9±1.2^{a}	0.1±0.2^{b}	0.2±0.3^{b}	2.4±0.5^{a}	0.1±0.2	0.2±0.3	0.0±0.0
22:4n-6	3.8±1.1^{a}	0.7±0.7^{b}	0.7±0.3^{b}	1.7±0.8^{a}	0.5±0.3^{b}	0.0±0.1^{b}	3.4±0.9^{a}	0.9±0.6^{b}	0.1±0.2^{c}	0.9±0.5^{a}	0.3±0.2^{b}	0.3±0.0^{b}
22:5n-6	4.4±1.8^{a}	0.3±0.4^{b}	0.2±0.2^{b}	1.1±0.4^{b}	0.3±0.2^{c}	2.8±0.5^{a}	2.7±0.9^{b}	0.4±0.3^{c}	5.3±1.9^{a}	0.8±0.4^{a}	0.1±0.1^{b}	0.1±0.1^{b}
22:5n-3	0.4±0.4^{c}	4.0±0.7^{a}	2.4±0.4^{b}	0.1±0.2^{b}	3.2±0.9^{a}	0.0±0.0^{b}	0.2±0.2^{b}	3.3±0.9^{a}	0.4±0.5^{b}	0.0±0.1^{c}	1.1±0.2^{a}	0.8±0.3^{b}
22:6n-3	0.0±0.0^{b}	0.2±0.1^{a}	0.0±0.0^{b}	0.0±0.0	0.0±0.0	0.0±0.0	0.2±0.2^{b}	0.6±0.3^{a}	0.0±0.0^{b}	0.0±0.0	0.0±0.1	0.1±0.1
24:0	3.0±0.9^{b}	6.6±1.2^{a}	7.3±1.4^{a}	0.9±0.4^{b}	3.0±0.7^{a}	0.4±0.5^{b}	1.7±0.3^{b}	4.9±1.8^{a}	2.3±0.9^{b}	0.6±0.3^{c}	1.7±0.2^{b}	2.0±0.3^{a}
24:1	0.0±0.1^{b}	0.3±0.2^{ab}	0.6±0.4^{a}	0.0±0.0	0.1±0.1	0.0±0.0	0.8±0.5^{a}	1.3±0.6^{a}	0.0±0.0^{b}	0.1±0.1^{b}	0.2±0.2^{ab}	0.3±0.2^{a}
Total n-6	47.0±2.9^{a}	18.6±7.7^{c}	28.1±4.2^{b}	42.0±3.6^{a}	24.0±7.4^{b}	28.2±5.0^{b}	32.5±2.5^{a}	15.9±5.4^{b}	19.9±7.6^{b}	35.5±2.0^{a}	18.6±5.9^{c}	27.3±3.5^{b}
Total n-3	0.8±1.3^{c}	13.2±4.2^{a}	4.2±1.0^{b}	0.2±0.4^{b}	9.1±3.9^{a}	9.6±6.2^{a}	0.6±0.6^{c}	5.9±1.4^{b}	10.6±6.6^{a}	0.1±0.2^{b}	6.4±2.6^{a}	1.7±0.4^{b}
Total SFA	24.7±2.0^{b}	27.7±4.1^{b}	35.2±5.9^{a}	43.9±1.6^{a}	45.9±2.5^{a}	41.4±1.7^{b}	43.1±2.7	45.8±3.5	40.5±7.2	41.1±2.1	41.2±3.1	43.5±2.2
Total MUFA	27.5±4.6^{b}	40.5±7.5^{a}	32.5±2.5^{b}	13.9±2.5^{b}	21.0±4.9^{a}	20.8±2.0^{a}	23.8±4.6^{b}	32.4±7.8^{a}	29.0±4.5^{b}	23.4±2.5^{b}	33.7±5.2^{a}	27.4±1.7^{b}
Total PUFA	47.8±3.3^{a}	31.9±4.1^{b}	32.3±5.7^{b}	42.2±3.4^{a}	33.1±4.2^{c}	37.8±1.8^{b}	33.1±2.9^{a}	21.9±4.7^{b}	30.4±6.3^{a}	35.6±2.0^{a}	25.0±3.5^{c}	29.1±3.2^{b}

Fatty acid composition (%) of tumours from MMTV (n=8), MMTV/*fat-1* (n=10) and MMTV on n-3 diet (n=8). Within each phospholipid class, values with different letters^{a,b} indicate significant difference (P<.05) between groups.

into mechanisms of action. In particular, given the effects on neu dependent tumour development, future work is needed to examine the effects of n-3 PUFA on neu signaling pathways.

While this study has shown that n-3 PUFA can reduce tumor outcomes, the mechanism of action remains to be determined. The model employed in this study is a simplistic yet elegant platform to study the impact of lifelong exposure of n-3 PUFA in mice expressing the *fat-1* gene. Thus, the impact of n-3 PUFA on developmental stages prior to tumor onset are potential areas for further study. Research on MG development has identified critical periods of development, including early windows such as in utero and pubescence, that are influenced by exposure to different dietary fatty acids which can modulate the risk of mammary tumorigenesis later in life [26,41,42]. This suggests that fatty acid composition of the MG during periods of rapid growth and development can influence the long-term health of mammary tissue. Rodents fed diets high in n-6 PUFA early in development have been found to develop more TEB compared to mice fed n-3 PUFA enriched diets, which have been identified as the sites of mammary tumorigenesis [33,42]. Alternatively, if the level of n-3 PUFA is increased in utero and during puberty, an anti-proliferative environment may be encouraged by reducing n-6 PUFA content and overall proliferation, resulting in a decrease in BC risk [42,43]. Thus, changes in the fatty acid composition during early periods of extensive growth and remodeling may modify risk for mammary tumorigenesis. The *fat-1* model used in this study allowed for n-3 PUFA exposure to occur throughout all early developmental windows, since expression of this transgene, and subsequent function of the n-3 desaturase, is active from conception onwards (communication with Dr. Kang).

The paradigm utilized in this study attempts to mimick the considerably low intake of EPA/DHA and high intake of n-6 PUFA in North American populations. Experimental groups were exposed to either adequate or deficient amounts of n-3 PUFA. There are two potential interpretations of the study results based on this paradigm. Firstly, the results suggest that it is the presence of n-3 PUFA which inhibit tumour outcomes. However, it is also possible that the absence of n-3 PUFA in the n-6 PUFA groups resulted in an increase in tumour development. Thus, it will be important to determine in future studies using dose dependent approaches to determine if it is the former or the latter. Nevertheless, the present study demonstrates that n-3 PUFA play an important role in tumour outcomes.

In conclusion, this study has shown that lifelong n-3 PUFA exposure can mitigate tumor development in an aggressive HER-2-positive BC model. Thus, this study has, for the first time, provided direct evidence that n-3 PUFA can inhibit mammary tumorigenesis. The fact that a food nutrient can have a significant effect on mammary tumor development is remarkable and as such has considerable implications in the field of breast cancer prevention. These findings provide the foundation for advancing nutritional literacy regarding the potential importance of n-3 PUFA in maternal and childhood diets throughout the lifecycle as a simple and effective means of mitigating lifelong BC risk.

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