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Saraswoti Khadge

Geoffrey M. Thiele

J. Graham Sharp

Timothy R. McGuire

Lynell W. Klassen

See next page for additional authors

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Authors

Saraswoti Khadge, Geoffrey M. Thiele, J. Graham Sharp, Timothy R. McGuire, Lynell W. Klassen, Paul N. Black, Concetta DiRusso, and James Talmadge

Long-Chain Omega-3 Polyunsaturated Fatty Acids Modulate Mammary Gland Composition and Inflammation

Saraswoti Khadge¹ · Geoffrey M. Thiele^{1,2,3} · John Graham Sharp⁴ · Timothy R. McGuire⁵ · Lynell W. Klassen^{2,3} · Paul N. Black⁶ · Concetta C. DiRusso⁶ · James E. Talmadge^{1,2}

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Abstract

Studies in rodents have shown that dietary modifications as mammary glands (MG) develop, regulates susceptibility to mammary tumor initiation. However, the effects of dietary PUFA composition on MGs in adult life, remains poorly understood. This study investigated morphological alterations and inflammatory microenvironments in the MGs of adult mice fed isocaloric and isolipidic liquid diets with varying compositions of omega (ω)-6 and long-chain (Lc)- ω 3FA that were pair-fed. Despite similar consumption levels of the diets, mice fed the ω -3 diet had significantly lower body-weight gains, and abdominal-fat and mammary fat pad (MFP) weights. Fatty acid analysis showed significantly higher levels of Lc- ω -3FAs in the MFPs of mice on the ω -3 diet, while in the MFPs from the ω -6 group, Lc- ω -3FAs were undetectable. Our study revealed that MGs from ω -3 group had a significantly lower ductal end-point density, branching density, an absence of ductal sprouts, a thinner ductal stroma, fewer proliferating epithelial cells and a lower transcription levels of estrogen receptor 1 and amphiregulin. An analysis of the MFP and abdominal-fat showed significantly smaller adipocytes in the ω -3 group, which was accompanied by lower transcription levels of leptin, IGF1, and IGF1R. Further, MFPs from the ω -3 group had significantly decreased numbers and sizes of crown-like-structures (CLS), F4/80+ macrophages and decreased expression of proinflammatory mediators including Ptg2, IL6, CCL2, TNF α , NF κ B, and IFN γ . Together, these results support dietary Lc- ω -3FA regulation of MG structure and density and adipose tissue inflammation with the potential for dietary Lc- ω -3FA to decrease the risk of mammary gland tumor formation.

Keywords Diet · PUFA · Omega-3 · Mammary gland · Fish oil · Mammary gland-density

Highlights

- Dietary long-chain omega-3 fatty acids (Lc- ω -3FAs) modulate mammary ductal end-point density and branching density in adult mice compared to mice receiving a high ω -6 FA containing diet.
- Dietary Lc- ω -3FAs downregulate the levels of proinflammatory cytokines, adipokines and growth factors and their receptors in association with decreased epithelial cell proliferation as compared to mice maintained on a high omega-6 (ω -6) FA containing diet
- Dietary Lc- ω -3FAs regulate the mammary fat pad (MFP) FA profile and is associated with adipocyte hypertrophy
- Dietary Lc- ω -3FAs decrease MG and abdominal adipose tissue inflammation relative to mice receiving a high ω -6 FA containing diet.

✉ James E. Talmadge
jtalmadg@unmc.edu

¹ Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, NE, USA

² Department of Internal Medicine, University of Nebraska Medical Center, Omaha, NE, USA

³ Veteran Affairs Nebraska-Western Iowa Health Care System, Omaha, NE, USA

⁴ Department of Genetics, Cell Biology and Anatomy, University of Nebraska Medical Center, Omaha, NE, USA

⁵ Department of Pharmacy Practice, University of Nebraska Medical Center, Omaha, NE, USA

⁶ Department of Biochemistry, University of Nebraska-Lincoln, Lincoln, NE, USA

Abbreviations

AA	Arachidonic acid
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
FA	Fatty acid
IGF	Insulin like growth factor
Lc	Long-chain
MFP	Mammary fat pad
MG	Mammary gland
MUFA	Monounsaturated fatty acid
PUFA	Polyunsaturated fatty acid
qRT-PCR	Quantitative real-time polymerase chain reaction
SFA	Saturated Fatty Acid
SPF	Specific pathogen free
TEB	Terminal end bud

Introduction

A growing body of evidence suggests a linkage between mammary gland (MG) development and diet [1] that can also modify breast cancer risk [2]. The majority of studies into dietary PUFA regulation of MG development have been undertaken with in-utero, fetal, post-natal and prepubertal manipulated rodents but are rarely undertaken in adult mice. These studies have shown that feeding a long-chain omega-3 fatty acid (Lc- ω -3FA; fish oil) containing diet reduces the number of terminal end buds (TEB), which are a marker of MG development [3–5]. Indeed, rodent studies have suggested that ω -6 and ω -3 PUFA have opposing effects on TEB formation, with ω -3 FA reducing the number of TEBs [1, 5, 6] and mammary gland-density [7], while high ω -6 FA diets reduce the differentiation of TEBs to alveolar buds [3]. During puberty, the majority of TEBs differentiate into terminal ducts or alveolar buds, while lateral branching of MG ducts occurs post-pubertal. As tumor initiation typically occurs post pubertal it is critical to study the effect of PUFA on MG differentiation in adult mice. Further, it is critical that studies into dietary regulation of MG development be undertaken using iso-lipidic diets differing only in PUFA composition [5]; specifically, the presence or absence of Lc- ω -3FA. It is clear that dietary PUFA composition can regulate carcinogenesis and tumor progression [8]; however, the potential for dietary epigenetic modification of molecular targets, in early life, requires additional investigation [9] as do the effects on post-pubertal MG structure and composition.

Mammary fat pads (MFP) in rodents are comprised of epithelial cells in the MG (or ductal tree), and adipose tissue, stroma and extracellular matrix which provides physical support for the MG ductal network [10]. Recently, mammary adipocytes have also been shown to have an active role in the chronic inflammation [11] that is associated with adipocyte IL-6 secretion, and likely plays a regulatory role in

mammary cancer induction and progression [12, 13]. Adipocytes within the MFP contribute to the regulation of MG development. For example, in the absence of mammary fat in A-ZIP/F-1 transgenic mice, abnormal MG development occurs [14]. MG adipocytes also regulate changes in mammary tissue during pregnancy, lactation, and involution, via local signals between mammary epithelium and adipocytes [12].

Emerging research suggests that the timing of PUFA exposure is critical to the increased risk of mammary cancer [15]. As MG development is age-dependent, specifically during puberty; most studies to date have used pre-pubertal exposure [1, 6]. These studies have demonstrated that pre-pubertal ω -3 PUFA incorporation into the MG regulates the morphological development of the MG during puberty, [6] and reduces mammary tumorigenesis [16]. However, the role of dietary Lc- ω -3FA exposure in post-pubertal life, and its regulation of the MG stromal microenvironments and morphogenesis is little studied.

Based on these observations, we examined the hypothesis that dietary Lc- ω -3FA regulates mammary gland morphology. We report that in the absence of dietary Lc- ω -3FA the FA composition within the adipose tissue of MG varies, resulting in local-regional inflammation; increased ductal branching density and mammary epithelial cell proliferation, all of which may potentially contribute to MG carcinogenesis.

Materials and Methods

Animals, Diet and Tissue Collection

Female BALB/c mice (6 weeks old) purchased from Charles River Laboratories were housed in groups of five, under standard conditions of temperature and humidity, with a 12 h light-dark cycle. The mice were caged in micro-isolators under HEPA filter air supply. Routine health monitoring was undertaken to ensure specific pathogen-free (SPF) conditions. The Institutional Animal Care and Use Committee at the University of Nebraska Medical Center approved the animal protocol for this study.

Following shipment and acclimation for about 4 weeks, 10-week old mice were randomly divided into the experimental dietary groups; omega-6 (ω -6) and omega-3 (ω -3) ($n = 20$). The ω -6 diet consisted of the Lieber-DeCarli control diet (Dyets # 710027) containing 28.4 g/L olive oil; while the ω -3 diet was customized by using the base diet (Dyets # 710166) without olive oil and adding; 8.4 g/L olive oil and 20 g/L of fish oil (NutriGold Triglyceride Omega-3 Gold capsules; Lot# 0081–3180-2) (Table 1). Both liquid diets provided 1.0 Kcal energy per ml of diet and equal calories from macronutrients (35% derived from fat, 47% from carbohydrate, and 18% from protein). The ω -6 and ω -3 diets were iso-caloric and iso-lipidic diets, differing primarily by the absence or presence

Table 1 Composition of the diets: The omega-6 diet (ω -6 diet) is the Lieber-DeCarli control diet, which contains 28.4 g/L of olive oil. Omega-3 diet (ω -3 diet) was customized by replacing 20 g/L olive oil with the ω -3 oil (NutriGold omega 3 capsule). All the other components were the same in both of the diets to maintain iso-composition and iso-caloric properties

Ingredient in diet	ω -6 Diet Grams/L	ω -3-Diet Grams/L
Casein	41.4	41.4
L-Cystine	0.5	0.5
DL-Methionine	0.3	0.3
Corn oil	8.5	8.5
Olive oil	28.4	8.4
Safflower oil	2.7	2.7
Maltose dextrin	115.2	115.2
Cellulose	10	10
Mineral mix	8.75	8.75
Vitamin mix	2.5	2.5
Choline bitartrate	0.53	0.53
Xanthum gum	3	3
Encapsulated omega-3 oil	0	20
Total	221.8	221.8

of Lc- ω -3FA from fish oil and olive oil respectively. Omega-3 capsules and Lieber-DeCarli powder diets were stored at 4 °C for up to 2 weeks and at -20 °C for longer times. Diets were prepared and delivered every 24–26 h, and daily consumption monitored. The diets were given using the pair-fed model as described previously [17]. Briefly, ad libitum access to the liquid diets was provided for the first five days for acclimatization. From day six forward, the ω -6 diet group mice were pair-fed based on the mean consumption of the ω -3 diet group from the previous day, and continued throughout the study for 10 or 20 weeks. Body weights were determined and recorded every week. At the end of the experiment, the fourth MFP and abdominal fat tissue were removed, weighed and processed.

Fatty Acid Analysis of Diets and Tissues

Portions of freshly collected MFP tissues were snap frozen and stored at -80 °C until used for FA analysis. Total lipid extraction from tissues used antioxidant β -hydroxy-toluene (0.05%, wt/vol) as previously described [17–19]. Heptadecanoic and nonadecanoic acid (100 μ g each) were added to all samples to estimate the recovery of FA. Methanol (0.5 ml) containing 1% (wt/vol) sulfuric acid and 0.25 ml of toluene, was added to each sample and incubated at 60 °C overnight to hydrolyze complex lipids and methylate FA. Following incubation, 1.25 ml of water containing sodium chloride (5%, wt/vol) was added and esters extracted using hexane (2 $\times$ 1.25 ml per sample). The hexane layer was washed using one ml of water containing potassium

bicarbonate (2%, wt/vol), and dried over anhydrous sodium sulfate. The solvent was removed under nitrogen and the FA methyl esters analyzed by gas chromatography-mass spectrometry (GC-MS) using an Agilent 6890 series gas chromatograph equipped with a 5873 mass-selective detector. The FA concentration was analyzed and reported as μ g/ml or μ g/mg of the sample and compared between diets and tissues from ω -3 and ω -6 dietary treatment groups.

Histology and Immunohistochemistry (IHC)

MFP and abdominal fat tissues were fixed in zinc fixative for 24–48 h, and transferred to 70% ethanol and then embedded in paraffin, and sectioned at 4–6 μ m. Sections were stained with hematoxylin and eosin (H & E), examined under light microscopy using a Zeiss Axioplan-2 microscope with a HRc camera, and analyzed using Zeiss AxioVision Rel 4.8 software. Two independent investigators examined the sections as “blinded” group assignments and an experienced pathologist validated the results. Adipocyte size analysis was performed using *MRI Adipocyte Tools*, a plug-in tool from Image-J as per the software protocol using H & E stained sections of MFP. Images were captured from 10 randomly selected fields at 100 $\times$ magnification. The adipocyte area was analyzed, as above, such that mean adipocytes area from the 10 fields/mouse and the mean number of adipocytes/100 $\times$ field [$n = 5$] was calculated. Images were captured at 200X and used for the adipocytes size analysis from the abdominal fat tissue. Crown like structures (CLS) were identified as adipocytes with at least two infiltrating immune cells (macrophage morphology) around the cell in H & E stained MFP sections.

Rabbit polyclonal antibody to F4/80 (ab100790 Abcam Inc., Cambridge, MA) was used to detect infiltrating macrophages, and rabbit monoclonal antibody to Ki67 (ab16667, Abcam Inc.) was used as a marker of proliferating cells. For IHC staining, MFP tissue sections were deparaffinized, rehydrated and antigen retrieved by sodium citrate method as described previously [17]. The slides were washed in TRIS-buffered saline, pH 7.6 (TBS) containing Tween-20 (TBST) and endogenous peroxidase/phosphatase was blocked with BLOXALL (SP6000, Vector labs, Burlingame, CA). Non-specific reactions were blocked using 2.5% normal horse serum blocking solution (S-2012, Vector labs) for 30 min at room temperature (RT). Primary antibodies were diluted in antibody diluent (559,148; BD Bioscience), and the slides incubated with antibody overnight at 4 °C. For negative controls, serial sections were processed in the absence of primary antibody but with all other reagents. Following primary antibody incubation and washing the sections were incubated in ImmPRESS-AP, anti-rabbit Ig (MP-5401, Vector labs) for 30 min at RT in a humid chamber. Staining was detected using ImmPACT Vector Red alkaline phosphatase reagent (SK-5105) and counterstained with hematoxylin solution

(MHS32; Sigma-Aldrich, St. Louis, MO) followed by 0.1% sodium bicarbonate. Images were captured on a Zeiss Axioplan-2 microscope as described above. The number of F4/80⁺ cells in adipose tissue and Ki67⁺ cells in mammary ductal epithelium were counted using 100× fields in 10 random fields per sample ($n = 5$).

Whole-Mount Analysis of MG

The intact inguinal MGs (MGs) (#4) were excised from mice at necropsy. The MGs were spread onto a glass slide immediately after collection and fixed in Carnoy's solution (100% EtOH, chloroform, glacial acetic acid; 6:3:1) for 4 h in the fume hood at room temperature (RT). The fixed MGs were then gradually rehydrated in graded ethanol (70%, 35%, 15%) ending in water. The MGs were stained in carmine alum staining solution [0.2% Carmine (C6152, Sigma, MO) and 0.5% aluminum potassium sulfate dodecahydrate (#237086, Sigma, MO) with a few flakes of thymol (#16254, Sigma, MO) overnight. The stained MGs were washed in water and dehydrated in ascending graded ethanol solution (50%, 70%, 95%, 100%), cleared by incubating in xylene overnight and mounted with Permount (SP 15–100, Fisher scientific). Whole slide images were acquired using the Invitrogen™ EVOS™ FL Auto Imaging System. For quantitative analysis, higher magnification images were acquired using the Zeiss Discovery V8 Stereo Microscope with AxioCam 105 color camera. We used the term “ductal terminus/termini” for all the end structures of ducts and ductules present in the high power field (HPF). The number of ductal termini were counted in 10 images (40× magnification) per sample and used to calculate the average value for each sample. The extent of ductal branching was analyzed by counting the total number of lateral branches in 3–4 primary ducts and five secondary ducts (between lymph node and distal ends of ducts) at 12.5× magnification. The results were expressed as a number of lateral branches per mm of a duct (primary/secondary). The quantitative results of ductal termini and lateral branching were discussed as ductal end-point density and branching density respectively as described previously [20]. The total area of MG ductal tree, the length of the primary duct (from nipple to end of the duct) and lymph node area were quantified by using Image-J software.

Quantitative Real Time PCR Analysis

Tissues were collected in RNA stabilizing solution, RNAlater (#76106, Qiagen) and stored at -20 °C until use. RNA was extracted by using TRIzol reagent (15,596,026, Invitrogen). RNA concentration was

measured by using NanoDrop (ND1000, Thermofisher). In order to confirm the integrity of the RNA, the RNA (2 µg) was separated in a denaturing agarose gel (1%) in formaldehyde, and samples with two distinct bands of RNA (16S and 23S) in the gel were used for further analysis. Complimentary DNA (cDNA) was prepared from RNA by using high-capacity cDNA synthesis kit (4,374,966, Applied Biosystems; AB) as per company protocol. The qRT-PCR master mix was prepared using TaqMan gene expression master mix (4,370,074, AB), anti-mouse probes and 10 ng of cDNA in Micro Amp fast optical 96 wells plate (4,346,906, AB). The samples were run using StepOnePlus qPCR machine (Applied Biosystems). Pre-synthesized mouse probes from Thermofisher were used for the study. The probes used in the study were; GAPDH (Mm99999915_g1), IGF1 (Mm00439560_m1), IGF1R (Mm00802831_m1), ESR1 (Mm00433149_m1), amphiregulin (AREG; Mm01354339_m1), TNF alpha (TNFα; Mm00443258_m1), CCL2 (Mm00441242_m1), leptin (Lep; Mm00434759_m1); adiponectin (Adipoq; Mm00456425_m1), IL6 (Mm00446190_m1), Prostaglandin-endoperoxide synthase (PTGS2; Mm00478374_m1), interferon gamma (IGFγ; Mm01168134_m1), NFκB1 (Mm00476361_m1) progesterone receptor (PGR; Mm00435628_m1) and prolactin (PRL; Mm0059950_m1). Negative controls were run for every probe in the plate. Each qRT-PCR experiment was carried out in triplicate, and average cycle thresholds (Ct) were obtained for each dietary group of MFP ($n = 14$) and abdominal fat ($n = 8$). The relative quantity of target mRNA was determined using the Levak method [21]. Briefly, the Ct of GAPDH was subtracted from the Ct value of the target to obtain delta (d) Ct. Then the dCt of each of the samples, from the ω-6 group, was compared with the average dCt from the ω-3 group to get ddCt values. The fold change between the samples was calculated as 2^{-ddCt} as described previously [21].

Statistical Analysis

Results were expressed as a mean ± standard error of the mean (SEM) for animal weights, MFP/abdominal fat weight or weight/body weight, fatty acid levels, histology and IHC quantification values. Data from the two groups were compared by Student's t-test for independent samples or Mann Whitney's test. Plots for change in body weights were compared by repeated measures analysis. All graphs were plotted using Sigma Plot. All data were analyzed using SPSS for statistical significance. For qRT-PCR data, fold-change values were compared by one-sample t-test. In all instances, differences where $p \leq 0.05$ were considered to be statistically significant.

Results

Food Consumption, Body Weight, and MG and Abdominal Fat Weights

Pair-feeding was undertaken, as described previously [17], to assure iso-caloric and iso-lipidic consumption. To acclimate the mice to a liquid diet, the first five days of feeding was ad libitum. During this time, average liquid diet intake was assessed daily, with the observation of increased diet consumption of the ω -6 diet. Based on this observation, the ω -3 diet consumption was used as the baseline for pair feeding. During this five-day period of ad libitum feeding the ω -6 group of mice gained more weight than the ω -3 group in association with increased liquid diet consumption. Following the initiation of pair feeding, on day 6, no significant differences in food intake occurred between the dietary groups. (Fig. 1a). The initial body weights were comparable between the dietary groups. However, mice ($n = 20$) on the ω -3 diets had a significantly lower body weight gain throughout the study (Fig. 1b), despite the use of iso-caloric diets. The weights of the abdominal fat in the mice on the ω -3 diet were 45% lower (Fig. 1c) and 42% lower as abdominal fat weight relative to the body weight (Fig. 1d) compared to the ω -6 group ($n = 8$) (both $p < 0.05$). Similarly, the weight of the fourth (left inguinal) MGs were assessed after the mice were fed the diets for 20 weeks ($n = 10$). Significant decreases in the MG weights (−43%) (Fig. 1e) and MG weight relative to the body weight (−41%) (Fig. 1f) were observed in the mice on the ω -3 diets compared to the MGs from the mice receiving the ω -6 diets ($p < 0.05$) ($n = 10$).

Modulation of MG, FA Profile by the ω -3 Versus ω -6 Iso-Lipidic PUFA Diets

The FA composition of the diets and MFPs are shown in Table 2. There was a significant effect of the dietary FA composition on the lipid profile of the MFPs from mice fed the diets for 10 weeks. The ratio of ω -6: ω -3 FA of the ω -6 and ω -3 diet were 21:1 and 0.7:1 respectively. The ω -6 diet did not have detectable levels of Lc- ω -3FA such as; eicosatetraenoic acid (EPA) and docosahexaenoic acid (DHA), which were major sources of ω -3 FA in the ω -3 diet. Consistent with the dietary FA composition, significantly higher levels of ω -3 FAs such as LNA (0.7 ± 0.2), EPA (11.1 ± 1.2) and DHA (7.5 ± 0.6) $\mu\text{g}/\text{mg}$ were observed in the MFPs of mice fed the ω -3 diet. In contrast, all ω -3 FAs were below the detection levels in the MFPs of the ω -6 diet fed mice. The levels of ω -6 FAs were not significantly different between mice fed ω -6 and ω -3 diets, nor in the MFPs from mice fed the respective diets. However, a lack of Lc- ω -3FAs in the ω -6 diet significantly increased the ratio of ω -6: ω -3 FA in MFPs from the mice fed the ω -6 diet. Besides

PUFA, the levels of total saturated fatty acid (SFA) were increased (1.5 fold), and oleic acid (a major monounsaturated fatty acid from olive oil) was decreased (1.5 fold) in MFPs from mice fed an ω -3 diet compared to the levels in MFP from the ω -6 diet group. The total levels of FAs were similar between the ω -6 and ω -3 diets and not significantly different between the MFPs from the mice fed the diets.

Dietary PUFA Regulation of Mammary Ductal Branching

A histopathologic analysis of the inguinal MFPs of mice fed the diets for 20 weeks, was performed to evaluate structural/morphological alterations in MGs. ($n = 10$). A significant morphological difference was observed in the ductal tree network and the total glandular area between the two dietary groups (Fig. 2a and b). The total area of the MG tree of MFPs from ω -3 fed mice was 20% smaller ($143.3 \pm 10.4 \text{ mm}^2$) relative to the area of the MFPs from ω -6 fed mice ($177.6 \pm 11.8 \text{ mm}^2$) ($p < 0.05$) (Fig. 2c). A comparison of the quantitative differences in the ductal tree compared the number of ductal termini (end-point density) and the extent of lateral branching (branching density). Since the mice used in the study were post-pubertal adults, TEBs had differentiated into terminal ducts, alveolar buds or end buds and we use the term ductal termini for these end structures. MFPs from mice fed the ω -3 diet had 63% fewer ductal termini/HPF (25.9 ± 1.9) compared to the numbers in the MFPs from ω -6 diet fed mice (70.9 ± 5 ; $p < 0.05$) (Fig. 2d). Similarly, there were significantly fewer lateral branches per mm of primary ducts (0.7 ± 0.1) (a 60% decrease) and secondary ducts (1.3 ± 0.1) (a 65% decrease) compared to the numbers in each mm of primary duct (1.7 ± 0.1) and secondary ducts (3.8 ± 0.2) of MFPs from the ω -6 diet fed group (Fig. 2e). In the microscopic analysis, there was a significant difference in the ductal morphology, including the presence of ductal sprouts, along the ductal wall in MFPs from ω -6 diet fed mice, which were absent in the ducts of the MFPs from ω -3 diet fed mice. Despite the differences in lateral branching, there were no significant differences in the length of the primary ducts in mice fed the ω -3 diet ($25.9 \pm 1.2 \text{ mm}$) compared to the primary ductal length ($28.1 \pm 1.6 \text{ mm}$) in MFPs from ω -6 diet fed mice. Similarly, the average lymph node (LN) area ($2.7 \pm 0.1 \text{ mm}^2$) of the ω -3 diet fed mice were not significantly different from the LN area ($2.7 \pm 0.3 \text{ mm}^2$) of ω -6 diet fed mice.

Dietary PUFA Regulation of Ductal Epithelial Cell Proliferation

The ductal histology of the MFPs from mice, fed each of the diets for 20 weeks, were analyzed using Mason's trichrome stain and proliferating epithelial cells assessed by Ki67 staining. As shown in a representative Mason's

Fig. 1 Effects of Lc- ω -3FA on body weight, MG weight, and abdominal fat deposits: Mice were pair-fed omega-6 (ω -6) and omega-3 (ω -3) PUFA diets for 10–20 weeks [$n = 20$ /group]. The average amount of diet consumed/day (a). Differences in percent changes in body weights between the pair-fed groups compared by a repeated measure test (b). Differences in abdominal adipose tissues deposits (c) [$n = 10$ /group], and abdominal fat weight relative to the body weight (d). Weights of the fourth rear MG at the age of 30 weeks (e). MG weight relative to body weight (f) [$n = 10$ /group]

trichrome stained MG duct (Fig. 3a), we analyzed ductal lumen (DL) area, ductal epithelium (DE) area and ductal connective tissue (blue stained) or ductal stroma (DS) area. The ω -3 diet feeding for 20 weeks (Fig. 3b – e) resulted in a 34% decrease in the DE area ($1883.3 \pm 145.0 \mu\text{m}^2$) and a 54% decrease in the DS area ($3034.5 \pm 377.9 \mu\text{m}^2$), compared to the DE area ($2863 \pm 189.6 \mu\text{m}^2$) and the DS area ($6593.1 \pm 493.9 \mu\text{m}^2$) respectively from mice fed the ω -6 diets for the same duration ($p < 0.05$). Further, the effects on ductal size, based on a comparison of the ductal lumen (DL) area between the dietary groups, documented that mice fed an ω -3 diet had 42% smaller ducts with an average DL area of $1497.1 \pm 243.6 \mu\text{m}^2$, compared to the MFPs from ω -6 fed mice, ($2568.7 \pm 380.9 \mu\text{m}^2$) ($p < 0.05$). As there were significant differences in ductal size, we further evaluated the area of DE and DS relative to the DL. The results showed that the DS relative to DL were 33% smaller in the MFPs of ω -3 diet fed mice (3.6 ± 0.3) compared to the same in MFPs of the ω -6 group (5.3 ± 0.5) (Fig. 3f) ($p < 0.05$). However, there was no significant difference in the DE relative to

DL area between the dietary groups (Fig. 3g). These results suggest that the differences in ductal connective tissue were independent of ductal size; however, the size of ducts between the dietary groups might affect the DE area. Thus, proliferating epithelial cells were evaluated to confirm the effect of dietary PUFA on DE, which showed there were 88% fewer proliferating cells in the DE of ω -3 diet fed mice (1 ± 0.5) compared to ω -6 diet fed mice (8.3 ± 3.1) (Fig. 3h – j) ($p < 0.05$). The regulation of estrogen signaling is one mechanism of epithelial proliferation in MG ducts. To address this we assessed the relative mRNA expression of amphiregulin (AREG) and estrogen receptor-1 (ESR1) as a potential mechanism for the dietary PUFA regulation of DE cell proliferation. These studies revealed that the MFPs from mice fed the ω -6 diet had 3.1-fold and 1.4-fold higher transcription levels of AREG and ESR1, respectively, compared to MFPs from age-matched, iso-caloric ω -3 diet fed mice (Fig. 3k). However, there were no differences in the levels of progesterone receptor, and prolactin mRNA levels were below the level of detection (data not shown).

Table 2 Fatty acid profiles of the diets and mammary fat pads measured by gas chromatographic mass spectrophotometry (GC-MS) analysis

Fatty Acid (FA) Name	Carbon chain	Diet (FA $\mu\text{g/ml}$ of diet)		Mammary fat pad (FA $\mu\text{g/mg}$ of MFP $\pm$ SEM)	
		ω -3	ω -6	ω -3	ω -6
Saturated fatty acid (SFA)					
Tetradecanoate	C14:0	0	0.2	6.3 ± 0.7	2.8 ± 0.6
Hexadecenoic	C16:0	1.53	3.02	105.3 ± 8.8 *	68.5 ± 8.3
Octadecenoic	C18:0	0.28	0.76	19.8 ± 2.5	13.9 ± 2.0
Monounsaturated fatty acid (MUFA)					
9-Hexadecenoic	C16:1	0	0.2	18.2 ± 1.4	12.3 ± 2.4
9-Octadecenoic	C18:1	3.77	15.62	166.1 ± 7.0 *	252.2 ± 31.4
cis-13-Eicosenoic	C20:1	0.7	0	9.2 ± 0.5 *	3.0 ± 0.5
13-Docosenoic acid	C22:1	0.25	0	1.3 ± 0.1 *	0.0 ± 0.0
Polyunsaturated fatty acid (ω -6 FA)					
cis-8,11-Octadecadienoic (Linoleic)	C18:2	6.67	5.03	71.3 ± 3.3	55.2 ± 7.4
cis-5,8,11,14-Eicosatetraenoic (Arachedonic acid; AA)	C20:4	0.51	0	0.7 ± 0.2	0.2 ± 0.2
Polyunsaturated fatty acid (ω -3 FA)					
cis-9,12,15-Octadecatrienoic acid (Linolenic)	C18:3	0.14	0.24	0.7 ± 0.2 *	0.0 ± 0.0
7,10,13-Eicosatrienoic	C20:3	0	0	0.7 ± 0.2 *	0.0 ± 0.0
cis-5,8,11,14,17-Eicosapentaenoic (EPA)	C20:5	7.52	0	11.1 ± 1.2 *	0.0 ± 0.0
cis-4,7,10,13,16,19-Docosahexaenoic (DHA)	C22:6	2.64	0	7.5 ± 0.6 *	0.0 ± 0.0
cis-4,7,10,13,16-Docosapentaenoic acid	C22:5	0.26	0	1.7 ± 0.1 *	0.0 ± 0.0
Total omega 6 FA level		7.18	5.03	72 ± 3.5	55.5 ± 7.3
Total omega 3 FA level		10.56	0.24	21 ± 2.0 *	0.0 ± 0.0
Total MUFA level		4.72	15.82	193.5 ± 8.7	267.5 ± 32.6
Total SFA level		1.81	3.98	131.4 ± 11.9 *	85.3 ± 10.0
Total FA mass per mg/ml of MFP/diet		24.27	25.07	420.3 ± 20.1	442.2 ± 47.0
Ratio of total ω -6 FA to ω -3 FA (ω -6: ω -3)		0.7:1	21:1	3.4:1	55.5:0

Mammary fat pads were collected from the mice fed respective diets for 10 weeks. ($n = 5/\text{group}$). The ready to use diets were used for FA analysis. The results were expressed as microgram of fatty acid per milligram of MFP tissue

Dietary PUFA Regulation of MFP and Abdominal Fat Adipocytes

A comparison of adipocyte histology in the MFPs and abdominal fat deposits of mice, fed the two diets for 20 weeks, revealed (Fig. 4) that adipocytes in the MFPs of mice fed the ω -3 diet, were approximately 45% smaller compared to MFP adipocytes from ω -6 diet fed mice ($p < 0.05$) (Fig. 4a and b). The increase in MFP adipocyte size was validated by an analysis of the adipocyte number/100 $\times$ image, which documented that the adipocyte number/field was increased 67% in the MFPs of the ω -3 diet fed group, compared to the ω -6 diet fed mice (Fig. 4a and b) ($p < 0.05$). Consistent with variations in MFP adipocyte sizes, an analysis of adipocytes size and the number of adipocytes/100 $\times$ field in abdominal fat showed that mice fed the ω -3 diet had 53% smaller adipocytes and 113% more adipocytes per 100X field compared to iso-caloric ω -6 diet fed mice (Fig. 4c and d) ($p < 0.05$). We also assessed potential mechanisms for the differences in adipocyte size. This focused on alterations in fat storage/metabolism in the

MFP and abdominal fat between the dietary groups by analyzing the relative mRNA expression of genes associated with fat metabolism. The results revealed that mice on the ω -6 diet had significantly increased mRNA expression for leptin (Lep) levels (3.1-fold), insulin-like growth factor-1 (IGF1) (1.8-fold) and IGF1 receptor (IGF1R) (1.7-fold) compared to the MFPs from mice fed the ω -3 diet (Fig. 4e) ($p < 0.05$). Similarly, mRNA analysis of the abdominal fat showed that mice fed an ω -6 diet had significantly increased mRNA levels of Lep (5.1-fold), IGF1 (1.9-fold) and IGF1R (2.2-fold) compared to the mice fed diets high in ω -3 FA. (Fig. 4f).

Dietary PUFA Regulation of Adipose Tissue Inflammation

The MFPs, from groups of mice on the two FA diets for 10 and 20 weeks, were assessed for crown-like structures (CLS), macrophage infiltration and expression of inflammatory cytokines (Fig. 5). The number of CLS per 100 adipocytes and

Fig. 2 Effects of Lc- ω -3FA on MG ductal branching and morphology: MGs were analyzed for ductal termini (end-point density), lateral branching (branching density) and sprouts in mice fed the diets for 20 weeks [$n = 10/\text{group}$]. Whole mounts of carmine-alum stained fourth rear, MGs from 30-week old ω -3 diet fed mouse (**a**) and ω -6 diet fed mouse diets (**a**) [$n = 10$]. MGs were analyzed for the number (**b**). The total

ductal tree area (**c**), number of ductal terminus (**d**) and number of lateral branches/mm of primary or secondary ducts (**e**). High power analysis of ductal branches showing absence of sprouts in MFP of ω -3 diet fed mouse (**f**) and presence of sprouts (shown by arrows) in MFP of ω -6 diet fed mouse (**g**). Images of (**a** and **b**) are 12.5 $\times$ and (**f** and **g**) are 40 $\times$ magnification. [$* = p < 0.05$]

infiltrating immune cells/CLS were analyzed in H & E stained MFP sections (Fig. 5a). Mice on the ω -3 diet had 64% fewer MFP-CLS (11.0 ± 0.8) compared to MFP-CLS (30.7 ± 3.2) in the ω -6 diet fed mice (Fig. 5a and b) ($p < 0.05$). Similarly, there was a 31% decrease in the number of infiltrating macrophages per CLS i.e. (2.8 ± 0.4) compared to the number of infiltrating macrophages (4.0 ± 0.6) cells/CLS in the MFPs from the ω -6 diet fed mice (Fig. 5a and c). Macrophage infiltration of MFPs was confirmed by F4/80⁺ staining (Fig. 5d). This analysis revealed that mice on the ω -3 diet had 60%

fewer adipocytes with at least one adjacent F4/80⁺ cell/s (1.5 ± 0.1) compared to adipocytes (5.2 ± 0.2) in the MFPs from ω -6 diet fed mice (Fig. 5d and e) ($p < 0.05$). As high levels of inflammatory mediators have been associated with the increased numbers of CLS in adipose tissue [11], we analyzed the mRNA levels of inflammatory cytokines by qRT-PCR. These studies revealed that the MFPs of mice fed an ω -6 diet, had higher levels of mRNA for Prostaglandin-Endoperoxide Synthase 2 (Pgs2) (2.3-fold), interleukin-6 (IL6) (2.0-fold), C-C motif chemokine ligand 2 chemokine

Fig. 3 Effects of Lc- ω -3FA on MG ductal thickness and epithelial cell proliferation: Mammary ductal histology analyzed in trichrome stained MFP sections, using 200 $\times$ images ($n = 10$ fields/mouse from five mice/group). In each duct, the area of lumen marked as (DL), epithelium (DE) and stroma or connective tissue (DS) were analyzed as illustrated (a). Masson's trichrome stained MFP from ω -3 diet fed mice (b) and ω -6 diet fed mice (c). The difference in the area of ductal stroma (d), ductal epithelium (e), stroma relative to luminal area (f) and epithelial

area relative to luminal area (g) were analyzed. MFP sections stained with the anti-Ki67 antibody were analyzed for Ki67⁺ proliferating ductal epithelial cells (h). Images showing Ki67⁺ cells in ducts of MFP from ω -3 diet fed mouse (i) and ω -6 mouse (j). Relative mRNA expression (fold change compared to the ω -3 group) of ESR1 and AREG in MFP of mice fed the ω -6 diet for 20 weeks ($n = 15$ /group) [$\# = p < 0.05$ compared to the ω -3 group]. Images were taken at a magnification of 200 $\times$ (a and f) [$* = p < 0.05$]

(CCL2) (2.3-fold), interferon gamma (IFN γ) (2.8-fold), NF κ B (1.5-fold) and TNF α (1.7-fold) in the MFPs from ω -6 diet fed mice compared to ω -3 diet fed mice (Fig. 5f).

Discussion

In this study, we provided moderate fat, iso-caloric diets to pair-fed groups of mice for 10 or 20 weeks of adult life. Our goal was to assess the effects of dietary Lc- ω -3FAs on the structural, inflammation and morphological alterations and differentiation of post-pubertal murine MGs. These studies demonstrated that a post-pubertal exposure to Lc- ω -3FAs modulates mammary gland FA composition; lateral branching, ductal epithelial proliferation and

adipose tissue inflammation relative to the mice fed ω -6 diet, which contained high levels of ω -6 FA and oleic acid but lacked Lc- ω -3FAs.

A difference in dietary consumption (likely due to palatability) may be a factor when analyzing effects specific to the dietary composition, independent of total caloric intake or obesity [17]. We report that mice fed an ω -6 diet consumed significantly more liquid diet compared to the ω -3 diet during a 5-day initial ad libitum feeding period. This difference was subsequently controlled by pair feeding based on consumption of the ω -3 diet. Despite pair feeding and isocaloric and isolipidic diet consumption, the inclusion of Lc- ω -3FA in the diet significantly decreased body weight gains and abdominal fat deposits (~45%) in mice. These results are consistent with prior studies comparing diets high in ω -3 or ω -6 FA [6, 17].

Fig. 4 Effects of Lc- ω -3FA on adipocytes histology and metabolic gene expression: Analysis of adipocytes in H & E stained mammary fat pad (MFP) (a) by measuring an average adipocyte size (area) and the number of adipocytes per 100 $\times$ field (b) [10 fields/mouse in five mice]. H and E stained sections of abdominal fat (c), adipocyte size and the number of adipocytes per 200 $\times$ field (d) [n = 10 fields/mouse in five mice]. Relative

mRNA expression (fold change compared to ω -3) of leptin, IGF1, and IGF1R in MFP (e) [n = 15/group] and an abdominal fat of mice fed an ω -6 diet for 20 weeks (f) [n = 8/group] [$\#$ = p < 0.05 compared to ω -3 group]. Images were taken at a magnification of 100 $\times$ (a and c) [$\ast$ = p < 0.05]

In addition, studies have reported a 50% reduction in retroperitoneal fat mass [22], and a 25% reduction in epididymal fat deposits in mice fed a high ω -3 FA diet [23]. This suggests that dietary Lc- ω -3FA are associated with the regulation of fat mass via stimulatory or inhibitory roles of ω -6 FA and ω -3 FA metabolites respectively in the differentiation of adipocytes [24, 25]. Further, increasing β -oxidation and suppression of lipogenic enzymes, such as leptin in adipose tissue, may provide potential mechanisms for reducing body fat by ω -3 FA exposures [26, 27].

To analyze the effects of dietary PUFA in MGs, we compared MFP weights of the fourth MG, after feeding the diets for 20 weeks, and observed 43% lighter MFPs in mice on a ω -3 diet as compared to the ω -6 diet group. This was associated with the novel observation of a significant higher incorporation of Lc- ω -3FAs in the MFPs from ω -3 diet fed mice as

compared to the MFPs from ω -6 mice. The ω -6 and ω -3 diets used in the present study contained a ω -6: ω -3 ratio of 21:1 and 0.7:1 respectively. The only ω -3 FA in the ω -6 diet was Linolenic acid (ALA), whereas Lc- ω -3FAs such as EPA and DHA were prominent ω -3 FAs in the MGs of the ω -3 diet fed mice (Table 2). Thus, our results document that the incorporation of Lc- ω -3FAs in MFP depends directly on the dietary intake rather than in vivo conversion to ω -3 FAs from dietary ALA. We note that dietary ALA can be endogenously converted to Lc- ω -3FAs in mammals; however, the process is not efficient and < 5% of ALA consumed is converted to Lc- ω -3FAs in humans [28]. The observation might explain the lack of Lc- ω -3FA in the MFPs of mice fed the ω -6 diet. The Lc- ω -3FA levels of mammary adipose tissue might be of clinical relevance as the breast adipose tissue from breast cancer patients has significantly higher and lower levels of ω -6 FA

Fig. 5 Effects of Lc- ω -3FA on adipose tissue inflammation: Analysis of crown-like-structures (CLS) in H & E stained mammary fat pad (MFP) (a) [n = 10 fields/mouse from five mice/group]. Number of CLS per 100 adipocytes (b) and the number of inflammatory cells per CLS (c) were compared. Macrophages infiltration analyzed in F4/80 stained MFP sections (d) [n = 10 fields/mouse in five mice]. The number of adipocytes

surrounded by at least one F4/80⁺ cell/s were counted in 200 $\times$ field (e) Relative mRNA expression (fold change compared to ω -3) of inflammatory mediators, such as ptgs2, IL6, CCL2, IFN γ , NF κ B and TNF α in MFP of mice fed the ω -6 diet for 20 weeks (f) (n = 15/group) [[#] = p < 0.05 compared to ω -3 group]. Images were taken at a magnification of 400 $\times$ (a) and 200 $\times$ (d) respectively. [* = p < 0.05]

and Lc- ω -3FA respectively [29, 30]. Besides the differences in ω -3 and ω -6 FA composition between the diets, the level of oleic acid was significantly higher in ω -6 diet and in MFPs of mice fed ω -6 diet compared to the levels in the ω -3 group. However, there is no significant data on oleic acid regulation of inflammation and mammary gland density published. Rather, oleic acid is known to prevent inflammation in adipose tissue [31]. Thus, we believe the alterations in outcomes between the dietary groups, are potentially due to the differences in Lc- ω -3FA levels between the diets.

Although MFP weights may be a gross indicator of MG density in animal models, significant differences in MG adiposity might influence the MG weights. Pubertal exposure to HF diets has been shown to result in heavier MGs and stunted development in C57BL/6 mice due to higher adiposity [1], while in-utero exposure to an HF- ω -6 diet increased the TEB number and enhanced susceptibility to carcinogenesis in BALB/c mice [32]. Our data suggest that the observed difference in MFP weight might be the result of combined effects by the difference in MFP adipocyte size and alterations in MG glandular structure and composition including ductal

epithelial cells proliferation and leucocytes infiltration between the dietary groups. MG development is age-dependent such that MGs undergo extensive modeling and remodeling during fetal development, puberty and pregnancy. The majority of dietary intervention studies use pre-pubertal mice that have a correlation between a higher number of TEBs and an increased risk of cancer [1, 16, 32]. However, in this study we used post-pubertal mice whose TEBs had differentiated into terminal ducts. Despite the post pubertal status, we observed that feeding an ω -3 diet in early adulthood (10–30 weeks of age) was associated with a decreased number of ductal termini, lateral branching of primary and secondary ducts, and a different total ductal area compared to isocaloric, isolipidic ω -6 diet fed mice. Previous studies, based on ω -3 diets using a LF- ω -3 (16% energy) diet in pre-pubertal mice [16] and offspring of HF- ω -3 (39% energy) diet fed mice, had a reduced number of TEB associating with the lower risk of tumorigenesis compared to same fat level ω -6 dietary group [33]. However, when HF- ω -3 (39% energy) were compared to LF- ω -6 (16% energy), the former developed more TEB and an increase in tumorigenesis compared to the latter,

emphasizing the differential effects of fat-calories vs fat composition of the diets in MG differentiation and tumorigenesis [16]. Since we used an iso-caloric diet and pair-feeding, our observation of differences are indicative of the regulation of MG structure and morphology by dietary Lc- ω -3FA, independent of caloric intake and obesity. Total ductal tree area and ductal end-point and branching density represents epithelial and stromal mass of MG in animals, which can be considered equivalent to breast density as shown by mammographic scans in humans [20, 34]. Animal and clinical studies showed MG or breast density as a predictor of carcinogenic response or breast cancer risk [7, 35], while an increase in plasma DHA was associated with a decreased breast density in obese, postmenopausal women [36]. We did not observe a significant difference in ductal lengths between the dietary groups, which might be due to the age of the mice (post-pubertal) at the start of the diet, since mice at this age have already gained full primary duct length, as a result of the extension which occurs mostly during pubertal age [6]. Rather, the effects of the diets alter the frequency of lateral branches, ductal terminus and formation of ducts (shown by a presence of sprouts in ducts in ω -6 diet fed mice) and likely the total epithelial mass of MG. Further, future studies will need to evaluate the effects of dietary PUFA composition in mammary tumor growth and metastasis.

The observation of structural and morphological alterations in MG ductal trees was confirmed by immune-histological analysis of MG ducts. Mice fed the ω -3 diet had significantly smaller ducts, and a thinner layer of epithelium and connective tissue (stroma) in the ductal wall, as compared to the ducts from the ω -6 group. Since there was a difference in ductal size, we further evaluated those parameters relative to the ductal luminal area and found that the difference in the stroma was independent of ductal size, but the differences in epithelium thickness might be secondary to the ductal size. However, a significant decrease in proliferating ductal epithelial cells in the ω -3 group suggests a regulatory role of Lc- ω -3FA in mammary epithelial cells proliferation. Our observations of an increased ductal stroma and proliferating ductal epithelial cells in MFPs from mice receiving the ω -6 diet were in agreement with previous reports that showed higher stromal density [37] and proliferating epithelial cells [38] in mammary ducts of mice fed high fat-diet. However, effects of isocaloric PUFA diets and/or dietary Lc- ω -3FA in ductal stromal tissue have not been reported before.

Evidence from clinical studies showed that women with cytological atypia had a higher ratio of ω -6: ω -3 FA in breast tissue [39], which was associated with a reduced number of Ki-67⁺ cells with increased levels of Lc- ω -3FA in erythrocyte phospholipids [40]. Further, animal studies have shown that pre-pubertal exposure to low-fat (LF)- ω -3 was able to reduce chemical induced tumorigenesis [16] and inhibit atypical ductal hyperplasia at early stages of mammary carcinogenesis [41]. In addition, a recent finding of the regression of

established pre-neoplastic lesions, by combined treatment of fish oil and tamoxifen and its correlation with tissue levels of Lc- ω -3FA, demonstrated preventive and therapeutic roles of dietary Lc- ω -3FA in MG malignancies [42].

In our studies we detected estrogen receptors in the MFPs, consistent with prior reports of their presence on adipocytes and fibroblasts, within MFPs. This supports the critical role of estrogen receptors in epithelial proliferation, mediated by both endocrine-derived and locally produced estrogen [12, 43, 44]. We also studied message levels of AREG, a ligand for epidermal growth factor receptor (EGFR), which is induced by estrogen binding to estrogen receptor alpha (ESR1) stimulating the proliferation of mammary epithelium. Further, the overexpression of AREG has been associated with mammary tumorigenesis [45, 46]. Recent studies have shown estrogen stimulated ductal side branching by enhancing IGF-I action, while an overexpression of IGF-I was associated with ductal hyperplasia [47, 48] as observed in our studies. Further, both estrogen and IGF-I are required for progesterone-mediated formation of mature mammary gland alveoli. Thus our observation of increased lateral branching, stromal thickness and epithelial proliferation in mice fed the ω -6 diet might be due to additive effects of IGF-I to estrogen signaling, as shown by significant higher expression of AREG and ESR1 respectively in MFPs of ω -6 diet fed mice. However, as there were no differences in progesterone receptor mRNA expression in the MFPs. These observations indicate progesterone message levels might have little effect on lateral branching of the ducts in virgin, nulliparous adult mice.

We also assessed the effects of PUFA on adipocytes, which may regulate MG ductal morphogenesis. Adipocyte hypertrophy is one mechanism for storing excess energy, and has been observed in both omental and subcutaneous fat of obese humans [49], and in HF, high calorie-fed animals, [50] and genetically susceptible animals [51]. In our studies, we used moderate fat (MF), iso-caloric diets and a pair-fed model in BALB/c mice, which are known to be less susceptible to obesity [52]. Thus, our observation of significantly smaller adipocytes in abdominal and mammary adipose tissue from ω -3 mice suggests differential regulation of lipid metabolism, storage and/or beta-oxidation in adipose tissue by dietary ω -3 and ω -6 FAs [53–55]. Studies showed that ω -6 FAs consumption triggered adiposity in human [56] and rodents [57] potentially by promoting pre-adipocyte differentiation or adipogenesis [58], while DHA was able to inhibit differentiation to adipocytes and promote lipolysis [59]. In this study, the ω -3 diet fed group had significantly higher number of adipocyte per microscopic field, which further validates our observation of a smaller adipocytes in ω -3 group. However, since we did not analyze a total adipocytes number per MFP or abdominal fat, further studies are required to analyze PUFA regulation of adipogenesis in this pair-fed model. Further, prior reports have shown that EPA can reduce hypertrophy of abdominal

adipocytes [50, 60]. However, the role of Lc- ω -3FAs in decreasing MFP adipocyte hypertrophy, have not been done previously, even though adipocytes have been actively researched for their crucial role in MG growth, development and carcinogenesis [12].

Adipocytes' size and fat mass are important determinants in adipokine regulation of energy homeostasis. Leptin (Lep) expression increases with adipocyte size [61] and fat mass [62], while adiponectin (Adipoq) expression is inversely correlated with obesity [63]. However, the fat deposit distribution depends on the levels of leptin expression [64]. Leptin is a hormone of satiety; however, elevated plasma leptin levels do not induce a reduction of food intake nor increased energy expenditure in obese people, due to development of leptin resistance [65]. Although the mouse strain we used is less sensitive to obesity [66], the absence of Lc- ω -3FA in the ω -6 diet led mice to gain adipose tissue resulting in a significant increase in Lep and no effects on Adipoq expression in abdominal fat tissue and MFP. Our results in Lep expression are in agreement with prior studies using HF diets with and without EPA [62, 67], while no effect in Adipoq might be due to dietary FA levels, mouse strain, or type of adipose tissue analyzed. Fish oil was able to induce epididymal adipose tissue but not subcutaneous adipose tissue [68]. Besides their role in lipid homeostasis, leptin levels have been associated with the induction of pro-inflammatory cytokines [69], and enhanced proliferation of normal and malignant breast epithelial cells [70].

In addition, we observed that a lack of dietary Lc- ω -3FAs significantly enhanced expression of IGF1 and IGF1R in MFPs and abdominal fat. IGF1 was originally believed to be exclusively produced by the liver [71], but a recent study [72] showed IGF1 secretion by adipocytes and M2-macrophages. Further, ablation of IGF1R from myeloid cells results in elevated adiposity, lowered energy expenditure and increased macrophage infiltration into adipose tissue, and reduced phagocytosis [72, 73]. While IGF1 is synthesized by MG adipocytes and promotes the growth of ductal epithelium [74], overexpression of IGF1R can induce mammary epithelial hyperplasia in transgenic mice (containing human IGF-IR under a doxycycline-inducible MMTV promoter) [75] and is associated with increased risk of mammary cancer [76]. Our results are consistent with a prior study, which showed a negative correlation of dietary ω -3 FA with plasma IGF1 expression and ductal density [7]. However, the effects of ω -3 in local IGF1 expression in MG and other adipose tissue have not been previously reported; thus, our results indicate a need for additional investigation. Further, the similarities in IGF1 expression, in both MFP and abdominal fat, but differential expression pattern of IGF1R, indicates morphological similarities between adipocytes, but potentially different mechanisms of signaling.

Recent studies suggest that white blood cells, notably macrophages and eosinophils, are attracted to the end bud [77] and are essential for their development [77]. This finding raises a question regarding the regulatory role of chronic local/adipose tissue inflammation in MG pathology. Further, macrophages may also have a role in other attributes of MG morphogenesis such as lateral branching, terminal ducts, and ductal sprout formation, potential mechanisms requiring additional study. In the studies reported herein, we observed that the number/size of CLS and the number of macrophages infiltrating MGs were significantly lower in MFPs of ω -3 diet fed mice. CLS is a hallmark of chronic adipose tissue inflammation, and increased numbers of CLS in the breast (CLS-B) have been associated with increased risk and poor prognosis of breast cancer patients in both obese individuals and those with normal BMI [78, 79]. CLS formation has been shown to enhance the expression of inflammatory cytokines/chemokines potentially, further activating macrophages for clearing of apoptotic adipocytes. We observed significantly increased expression of Ptg2, NFkB, IL6, IFN γ , and the macrophage chemoattractant; CCL2 in the MFP of ω -6 diet fed mice. These findings support an increase in pro-inflammatory signaling by ω -6 FAs and its metabolite, AA in the MFP microenvironment. In these studies, the AA levels in the MGs did not differ between the ω -6 and ω -3 diet groups; however, the MFP from ω -6 diet mice did not have detectable levels of Lc- ω -3FA. Thus, our results suggested that the lack of detectable Lc- ω -3FA is more crucial relative to the levels of AA for chronic inflammation. Local Lc- ω -3FA has been shown to exert anti-inflammatory and anti-chemotactic activity regulating adipose tissue inflammation [80]. Further, constitutive expression of CCL2 by mouse mammary epithelium has been reported to increase stromal density and increase mammary tumor susceptibility [81], emphasizing a potential role of chronic inflammation in ductal epithelial proliferation and carcinogenesis that could be modified by dietary ω -3 FAs.

Findings from epidemiological, and animal studies using HF-fed or genetically obese models have provided contradictory results regarding the role of dietary Lc- ω -3FA in the MG microenvironment and morphogenesis, depending on the age of mice and mixed effects of dietary calories versus composition. In summary, this research shows that dietary Lc- ω -3FA reduces MG density by decreasing ductal end-point density, branching density, ductal stroma and epithelial proliferation in adult mice. This occurred in an amphiregulin-estrogen and IGF-I dependent manner, potentially by modulating adipocyte metabolism and adipose tissue inflammation. Further, an absence of detectable levels of Lc- ω -3FA, such as EPA and DHA in MG, might be an indicator of inflammation-associated pathologies. Future, mechanistic studies using tumor models are justified to assess a role for dietary Lc- ω -3FA inhibition of mammary tumor development in adults.

Compliance with Ethical Standards

Conflict of Interest The authors declare that there are no conflicts of interest.

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