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ORIGINAL CONTRIBUTION

Increased dietary levels of α-linoleic acid inhibit mammary tumor growth and metastasis

Marianela Vara-Messler[1,2,3] · Maria E. Pasqualini[1,2] · Andrea Comba[1,2] · Renata Silva[1,2] · Carola Buccellati[4] · Annalisa Trenti[3] · Lucia Trevisi[3] · Aldo R. Eynard[1,2] · Angelo Sala[4,5] · Chiara Bolego[3] · Mirta A. Valentich[1,2]

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Abstract
Objective The aim of this study was to determine whether α-linolenic acid (ALA ω-3 fatty acid) enriched diet affects growth parameters when applied to a syngeneic model of mammary carcinoma.
Materials and methods BALB/c mice were divided and fed with: 1) a chia oil diet, rich in ALA or 2) a corn oil diet, rich in linoleic acid (LA ω-6 fatty acid). Mice were subcutaneously inoculated with a tumor cell line LM3, derived from a murine mammary adenocarcinoma.
Results After 35 days, tumor incidence, weight, volume and metastasis number were lower in the ALA-fed mice, while tumor latency time was higher, and the release of pro-tumor metabolites derived from ω-6 fatty acids decreased in the tumor. Compared to the control group, a lower number of mitosis, a higher number of apoptotic bodies and higher T-lymphocyte infiltration were consistently observed in the ALA group. An ALA-rich diet decreased the estrogen receptor (ER) α expression, a recognized breast cancer promotor while showing an opposite effect on ERβ in tumor lysates.
Conclusion These data support the anticancer effect of an ALA-enriched diet, which might be used as a dietary strategy in breast cancer prevention.

Keywords α-linolenic acid (ALA) · Chia oil · Mammary carcinoma · LOXs metabolites · Estrogen receptor

Introduction

Cancer is a polygenic and multifactorial disease that affects fundamental biological events such as cell proliferation and death. Breast cancer (BC) is the most common cancer: It is the primary cause of cancer deaths and the primary cause of female cancer deaths worldwide [1]. Many risk factors, such as hormonal and lifestyle, are known to play a role in the development of BC. Several studies have shown that between 80 and 90 % of cancers are related to environmental factors; it is estimated that 35 % are related to dietary factors. Consumption of red meat, along with high levels of total cholesterol and triglycerides, appears to be associated with an increased risk of BC, whereas ω-3 polyunsaturated fatty acids (PUFAs) might exert a protective role [2].

It has been shown that dietary PUFAs modify the PUFA ratios in the cell membrane by increasing ω-3 PUFA concentrations. ω-3 PUFAs serve several functions, but their primary role is as a part of all cell membranes, where they maintain membrane fluidity. Membrane-esterified PUFAs

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✉ Mirta A. Valentich
mirtavalentich@gmail.com

1 Instituto de Investigaciones en Ciencias de la Salud de Córdoba (INICSA-CONICET), Universidad Nacional de Córdoba, Enrique Barros s/n. Ciudad Universitaria, 5000 Córdoba, Argentina

2 Instituto de Biología Celular, Cátedra de Biología Celular, Histología y Embriología, Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Enrique Barros s/n. Ciudad Universitaria, 5000 Córdoba, Argentina

3 Department of Pharmaceutical and Pharmacological Sciences, University of Padua, Largo E. Meneghetti 2, 35131 Padua, Italy

4 Department of Pharmacological and Biomolecular Sciences, University of Milan, Via Balzaretti 9, 20133 Milan, Italy

5 Institute of Biomedicine and Molecular Immunology, Italian National Research Council, Via Ugo la Malfa 153, 90146 Palermo, Italy

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are released from phospholipids by the action of phospholipase A_2 (PLA_2), and these unesterified PUFAs are substrate for cyclooxygenases (COXs), lipoxygenases (LOXs), and cytochrome P450 (CYP), three families of enzymes that generate bioactive lipids [3]. Several studies have revealed that the anti-tumor effects of ω-3 PUFAs are due to a switch from classical arachidonic acid (AA 20:4 ω-6)-derived mediators. Moreover, the two ω-3 PUFAs downstream metabolite from ALA (18:3 ω-3), as eicosapentaenoic acid (EPA 20:5 ω-3) and docosahexaenoic acid (DHA 22:6 ω-3), are the precursors of novel, specialized lipid mediators with pro-resolving activity [4]. In animal models, ALA-rich diets, including flaxseed [5], canola [6], mystol [7], perilla [8], and chia [9] oil, have shown to reduce mammary tumor growth.

Numerous studies have linked dietary PUFAs to apoptosis and a decrease in the proliferation of various cancer cell lines [10]. In addition, it has been shown that BC growth may be inhibited by DHA through changes in ERα distribution. Two-thirds of breast cancer cases are hormone-dependent cancers that express estrogen receptors (ERs) and require estrogen for tumor growth [11]. Estrogens, such as 17β-estradiol, play an important role in the modulation of mammary cancer, exerting their biological responses by binding onto two ER isoforms, ERα and ERβ [12, 13]. To our knowledge, while the effect of ω-3 PUFAs in BC development has been studied extensively, their role in ER expression is largely unknown, and in vivo data on a possible role of ALA—the most abundant ω-3 PUFAs found in Western diets—on ER expression are still lacking. This study determines the effect of an ALA-rich diet on the growth of murine mammary adenocarcinoma through the release of PUFA oxidation products and the ER expression.

Methods and materials

Materials

ω-3 PUFA source, chia seed oil, was purchased from Nutraceutica Sturla S.R.L. (Argentina), while ω-6 PUFA source, corn seed oil, was purchased from Arcor S.A. (Argentina). LM3 cells, a line of murine mammary cancer cells, were kindly provided by Dr. Bal de Kier-Joffe (Instituto Oncologico Angel Roffo, Argentina). Fatty acids were obtained from Cayman Chemical (Ann Arbor, MI, USA), and fatty acids methyl esters standard came from Nu-Chek Prep, Inc. (Minneapolis, MN, USA). 15 (S)-HETE, 5 (S)-HETE, and 13 (S)-HODE for standard curves were obtained from Biomol International LP (Philadelphia, PA, USA), and a C18 Phenosphere-Next column (5 μm; 4.6 × 250 mm) (Phenomenex, Inc. USA) for high-performance liquid chromatography (HPLC) analysis was used. A complete protease inhibitor mixture was obtained from Roche (Mannheim, Germany). A CD3 anti-mouse monoclonal antibody was purchased from BD Biosciencies Pharmingen (San Diego, CA, USA). For Ki-67 detection, MIB-5 monoclonal antibody was purchased from DAKO Corp. (Carpinteria, CA, USA) and the kit Fluorometric TUNEL System from Roche Applied Sciences (Mannhein, Germany). A complete protease inhibitor mixture was obtained from Roche (Mannheim, Germany). Anti-ERα was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and anti-ERβ from Calbiochem-Merck Millipore (Darmstadt, Germany). Anti-β-actin antibody was obtained from Sigma-Aldrich (St. Louis, MO, USA) and secondary antibodies such as anti-mouse IgG and anti-rabbit IgG came from Vector (Peterborough, UK). ECL substrate was purchased from GE Healthcare (Little Chalfont, UK). Skim milk, as well as hematoxylin and eosin, trypsin 0.25 %-EDTA, minimal essential medium (MEM), resazurin, fetal bovine serum, antibiotics solution (10.000 U/mL penicillin/10 mg/mL streptomycin), pronase and Type1-deoxyribonuclease, as well as other analytic grade chemical agents, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Experimental protocol

The experimental protocol is outlined in Fig. 1.

In vivo experiments:

Mice and diets

Our previously published results demonstrated that a murine transplantable mammary adenocarcinoma (M3) had moderate metastatic capability on BALB/c mice that had previously been fed ALA-rich diet (6 % chia seed oil) [9]. In order to enhance the model, this study utilized a murine mammary cancer line from the M3 tumor to induce tumor in mice. We also fed the mice with a 10 % chia seed oil diet in order to augment the ALA bioavailability in the tumor cell membrane.

After weaning, 40 BALB/c mice (male and female) were randomized and housed in polycarbonate cages in groups of four. Animals were kept in a 12-h light and 12-h darkness cycle at a constant temperature of 23 °C. Animal studies were conducted in accordance with the guidelines set forth by the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. All the procedures were approved by the Institutional Committee for the Care and Use of Laboratory Animals at the Facultad de Ciencias Médicas (Universidad Nacional de Cordoba, Argentina). Animals were fed with two isocaloric diets (3.86 kcal/g) differing in their fatty acid composition. The final composition of both diets consisted of (%) casein (16.0), sucrose (34.0), corn starch (39.0), fiber (2.0), salt mixture (3.5), and vitamin mixture (0.5). The chia oil diet (ChO) contained chia oil (10.0), and the control diet contained corn oil (CO) (10.0). Food and water were provided ad libitum. Animals

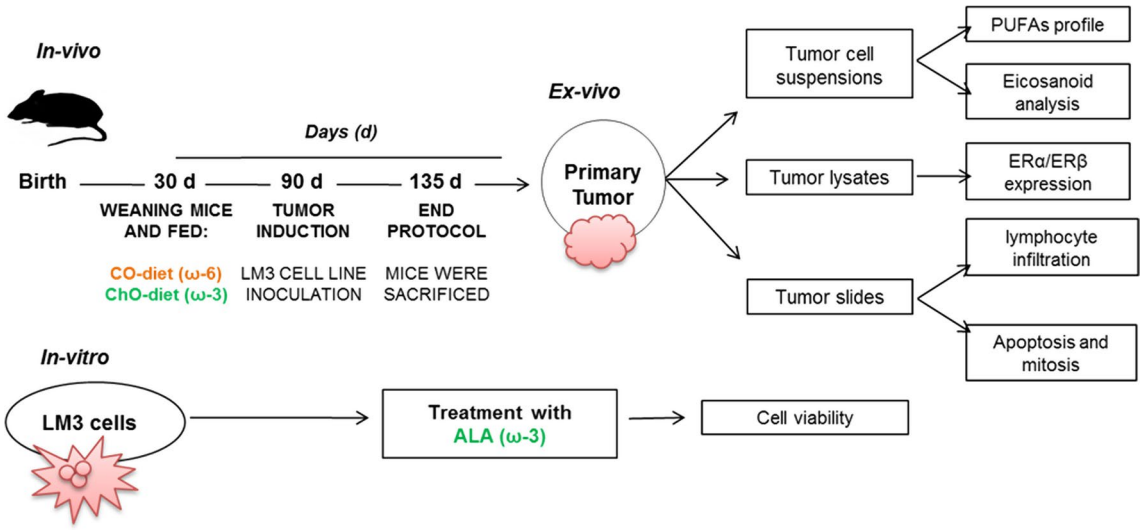

Fig. 1 A schematic line drawing the experimental approach of the protocol described in the methods

were inspected daily; food consumption and weight were recorded weekly.

Tumor and cancer cell culture

LM3 cells were isolated from mouse mammary gland tumors that exhibited moderate metastatic behavior [14]. LM3 cells were maintained in MEM, supplemented with 10 % (v/v) fetal bovine serum and 1 % antibiotics solution (10.000 U/mL penicillin/10 mg/mL streptomycin), and incubated at 37 °C under 5 % CO_2.

In order to determine the number of cells suitable for tumor induction, M3 tumors were induced by intradermal inoculation on the left flank of animals. Three suspensions with different LM3 densities (2.5 or 5 × 10^5 or 10^6 cells) in 0.2 mL MEM were used in four mice per each condition. In LM3-bearing mice, the latency time (days) and volume (mm^3) were determined. During tumor development, LM3-bearing mice were fed with a standard commercial diet (Cargill, S.A.C.I., Argentina) that was composed of 20 % protein, 64 % carbohydrate, 4 % fiber, and 6 % fat containing 10 % of LA (18:2 ω-6) and 3 % of ALA (18:3 ω-3).

Once the cell density for tumor induction was decided and mice had been fed for 3 months, the mice were inoculated with a murine cancer cell line (LM3), in order to obtain a syngeneic breast cancer murine model. After tumor induction by inoculation with the LM3 cells, mice were fed with their respective diets. Meanwhile, the latency time (days) for palpable tumors was recorded. Animals were killed at 45 days after inoculation.

Ex vivo experiments:

During necropsy, tumor weight (g), volume (mm^3), and the number of grossly macroscopic metastasis were recorded in all organs of the host fed animals with the aid of magnifying lens, as published in previous studies [9].

Tumor cell suspensions were obtained quickly from primary tumors free of necrotic areas, blood clots, and connective tissue. Enzymatic digestion was carried out with 0.01 % pronase and 0.24 % Type1-deoxyribonuclease in fresh medium. Samples from tumor tissues were fixed in paraformaldehyde 4 % and dehydrated and embedded in paraffin for histological analyses [9]. To analyze the ER protein expression, 200 mg of tumor tissue was homogenized in lysis buffer, supplemented with complete protease inhibitor mixture and stored at −80 °C.

Plasma membrane separation and fatty acid determination

Gas–liquid chromatography (GLC) was used to determine the extent to which each dietary formula modified the PUFA profile in the tumor cell membranes. Tumor cell plasma membrane purification was carried out as described elsewhere [9]. Briefly, tumor cells (10^7/mL) were placed in a homogenization solution (hypotonic Hepes–Mannitol buffer) and homogenized using a Polytron (7 s. at setting 7). The homogenate was treated with 10 mM $CaCl_2$ and centrifuged at 3000*g* for 15 min. The supernatant was centrifuged at 48000*g* for 30 min. The pellet containing the plasma membrane fragments was retained overnight in a phosphate-buffered saline (PBS) at 20 °C. Total lipids were extracted from tumor cells membranes and partitioned according to Folch [15]; methyl esters were obtained from the lower phase containing phospholipids. Fatty acids methyl esters were analyzed using a polyethylene glycol capillary column (30 m × 320 µm id × 0.50 µm) on a gas–liquid chromatograph equipped with flame

ionization detector. Fatty acids were identified by comparing their retention times with those of authentic standards. Percentages were estimated by calculating the basis of the peak area in reference fatty acid concentrations of linear curves obtained from standards [9].

PUFA metabolite analysis from tumor cell suspensions

We were able to make determinations based on eight samples of cell suspensions (10^7 cells/mL) taken from primary tumors of each of the dietary treatments. Cells were washed twice and suspended in phosphate-buffered saline with Ca^{2+} and Mg^{2+}. Tumor cells were stimulated with calcium ionophore A23187 for 15 min at 37 °C. Metabolites were extracted using a STRATA C-18 cartridge (1 mL) and detected by reverse-phase high-performance liquid chromatography (HPLC). Analysis was conducted with a C-18 Phenosphere-Next column (5 µm; 4.6 × 250 mm) in a Beckman System Gold Programmable Module Model 126. The metabolite separation was accomplished through the use of a time program. A linear gradient from solvent A to solvent B was conducted for 20 min. A UV Beckman System Gold Model 166 with a programmable detector linked to a computer for data processing was used. UV absorbance of the LOX metabolites such as 5 (S)-HETE, 15 (S)-HETE, and 13 (S)-HODE was read at 235 nm. Quantifications of LOX metabolites were obtained by using standard curves and were expressed as ng/10^7 tumor cells [9, 16].

Tumor lymphocytes infiltration

CD3 complex is expressed on mature T-lymphocytes [9], and CD3+ cells could reflect the immune response status against tumor since lymphocytes represent a local barrier to neoplastic dissemination. Tumor infiltration lymphocytes were evaluated by immunohistochemistry on stained slides from a 4-µm section of tumor tissue that had been fixed and embedded previously, as described above. After antigen-unmasking treatment, the sections were washed and incubated with the hamster anti-mouse CD3 (1:100) monoclonal antibody. After washing with a tris-buffered saline (TBS), the sections were incubated with avidin–biotin detection kit and finally placed on a chromogen diaminobenzidine reaction, giving a brown positive precipitate. CD3+-positive cells were counted from slides of tumor tissue from three animals for each of the dietary conditions per 10 high-power fields using a light microscope (40× magnification) Olympus BH2 in a blinded manner. CD3+-positive cells were expressed as a mean ± SEM of CD3+/mm^2.

Apoptosis and mitosis determination

Apoptotic and mitotic figures were recognized by characteristic morphological changes, as we have published previously [9, 16]. Fixed tumor tissues were stained with hematoxylin and eosin (HE). Apoptotic figures were recognized by shrinking, condensing, and fragmenting of nuclei, and mitotic as cells on metaphases. Additionally, to evaluate apoptosis and mitosis, a TUNEL assay and Ki-67 immunohistochemistry were used, respectively.

Apoptotic DNA degradation was evaluated by using the terminal deoxynucleotidyl transferase (TdT)-mediated dUDP-biotin nick end labeling (TUNEL) method. TUNEL assay was conducted in the paraffin-embedded tumor tissues for the specific detection and quantification of apoptotic cells. The in situ cell death detection kit Fluorometric TUNEL System was used in accordance with the manufacturer protocol.

To detect mitotic figures, we performed the Ki-67 staining using MIB-5 monoclonal antibody according to the manufacturer's protocol. TUNEL and Ki-67-positive cells in 10 high-power fields were counted in a blinded manner from three animals for each of the dietary conditions using a light microscope (40×) Olympus BH2. Results were expressed as a mean ± SEM of TUNEL or Ki-67-positive cells/mm^2.

Western Blot analysis

Tumor lysates from mice were used for ER expression. The protein concentration was measured by the Lowry method [17]. Lysates were denatured in the presence of β-mercaptoethanol, and 60 µg of protein was loaded onto a 10 % polyacrylamide gel. After electrophoresis, the protein was transferred to polyvinylidene difluoride membranes, which were subsequently blocked with 5 % (w/v) skim milk dissolved in a TBS buffer. The membranes were incubated with either a polyclonal anti-ERα or anti-ERβ (1:1000 dilution) for ER expression. Primary antibodies were incubated for 16 h at 4 °C. Primary anti-β-actin antibody (1:5000 dilution) was incubated at room temperature for 2 h. After washing, the secondary antibody anti-mouse IgG or anti-rabbit IgG (1:5000 dilution) was applied to the membranes and incubated for 1 h at room temperature. The secondary antibodies were detected using a chemiluminescent ECL substrate. Loading control was performed using β-actin immunodetection. The intensity of protein bands was calculated by densitometric analysis with ImageJ 1.47 version for windows (http://imagej.nih.gov/ij/ US National Institutes of Health. Bethesda, Maryland, USA) [18, 19].

In vitro experiments:

Viability assay

Cell viability was determined by a fluorimetric technique using resazurin. The activity of the intracellular enzymes converting resazurin into resorufin can be detected as an

increase in fluorescence. LM3 cells were plated at a density of 10^5/mL in a 96-well plate. The cells were incubated overnight to allow for adhesion. The following day, the cells were treated for 24 h with ALA at concentrations of 10, 50, or 100 µM. Four hours before the end of the protocol, 10 µL of a resazurin solution (0.1 mg/mL) was added into each well. Plates were incubated at 37 °C, and the fluorescent signal was measured at 560 Ex/590 Em wavelength with in a FL X800 Microplate Reader (BIO-TEK Instruments, Inc.)

Data analysis

Data are expressed as mean ± standard error (SEM) of at least three independent experiments. Statistical analysis was performed using *t* test (two-tailed probability value) to compare two groups or ANOVA for multiple groups using GraphPad Prism version 5.00 for Windows (San Diego, CA, USA). Each of the $p \leq 0.05$ changes was considered statistically significant.

Results

ALA-enriched diet inhibits BC growth and metastasis

M3 tumors induced by LM3 cell suspensions differed according to the inoculated cell density. 10^6 cells resulted in the most convenient condition, as bearing mice demonstrated an acceptable tumor dimension (48.75 ± 0.48 mm^3) after 45 days of tumor induction: The general status was optimal. Latency time did not differ with cell density (approximately 10 days after inoculation).

In our experiment, the fatty acid composition of chia oil used for dietary oil supplementation showed approximately a 60 % ALA (18:3 ω-3) and 20 % LA (18:2 ω-6), whereas corn oil contains around 50 % of LA and trace amounts of ALA (Table 1). Values for the oils agree with those published by the American Oil Chemists Society (AOCS) [20].

Diet and water consumption were similar for both groups. There were no significant differences in survival at 45 days after host inoculation and in weight gain between the two groups (data not shown). Tumor incidence was higher in CO-fed mice. Chia and corn oil diet consumption caused a statistically significant difference in latency time, tumor weight, size, and the metastases number. In particular, chia-fed guests had significantly fewer metastases when compared to corn-fed mice group. The highest percentage of metastases were located in the lung (2.5 vs. 0.9 metastases/mouse), liver (1.4 vs. 0.6 metastases/mouse), and retroperitoneum (1.5 vs. 0.5 metastases/mouse) (Fig. 2a)

Table 1 Fatty acid composition (%) of corn and chia oil added to the diet

	Major fatty acids (%)				
	Saturated FA		MUFAs	PUFAs	
			ω-9	ω-6	ω-3
	14:0	16:0	18:1	18:2	18:3
Corn oil	1.04	30.50	13.43	53.01	2.02
Chia oil	6.40	4.42	2.29	23.65	63.24

Values represent mean of each fatty acid. Fatty acids' common names: 14:0 myristic acid, 16:0 palmitic acid, 18:0 stearic acid, 18:2 linoleic acid, 18:3 α-linolenic acid

($p < 0.05$). Moreover, latency time was longer in mice fed with chia oil, while tumor weight as well as tumor volume was significantly lower with respect to the corn oil group (Table 2).

ALA supplementation enhances lymphocytes infiltration, apoptosis, and lowers mitosis in tumor tissues

Tumor–stromal interactions involve continuous cross talk among different cell types and play pivotal roles in tumor progress. Tumor-infiltrating lymphocytes are important components of these tumor–stromal interactions and are significantly associated with a good prognosis in breast carcinoma [21]. Our experiments show that an ALA-rich diet increases the number of CD3+-infiltrating lymphocytes when compared to the corn oil group (238 ± 43 vs. 103 ± 14 cell/mm^2, $p < 0.001$; $n = 3$). This finding is in accordance with the decrease in the tumor metastases number, and the increased CD3 indicates a good prognosis (Fig. 2b).

Besides CD3+ cells, an imbalance between cell proliferation and apoptosis may contribute to tumor progression. The rate of tumor growth depends, in part, on an excess of proliferation over apoptosis [22]. ALA-fed mice diminished the mitotic figures (5.88 ± 0.54 vs. 9.90 ± 0.46, $p < 0.001$; $n = 5$) and significantly increased the number of apoptotic cells (2.27 ± 0.19 vs. 1.42 ± 0.29, $p < 0.001$; $n = 5$) in tumor tissue stained with HE. Moreover, TUNEL-positive cells significantly increased in tumor tissue from ChO-fed mice compared to the corn oil group (31.49 ± 0.57 vs. 17.59 ± 2.60 cell/mm^2, $p < 0.05$; $n = 3$) (Fig. 3a) and Ki-67-positive cells were lower in ChO-fed mice with respect to CO-fed mice (89.88 ± 16.07 vs. 155.17 ± 20.34 cell/mm^2, $p < 0.05$; $n = 3$) (Fig. 3b). These findings are in accordance with the small tumor dimension found in mice that received the chia oil diet.

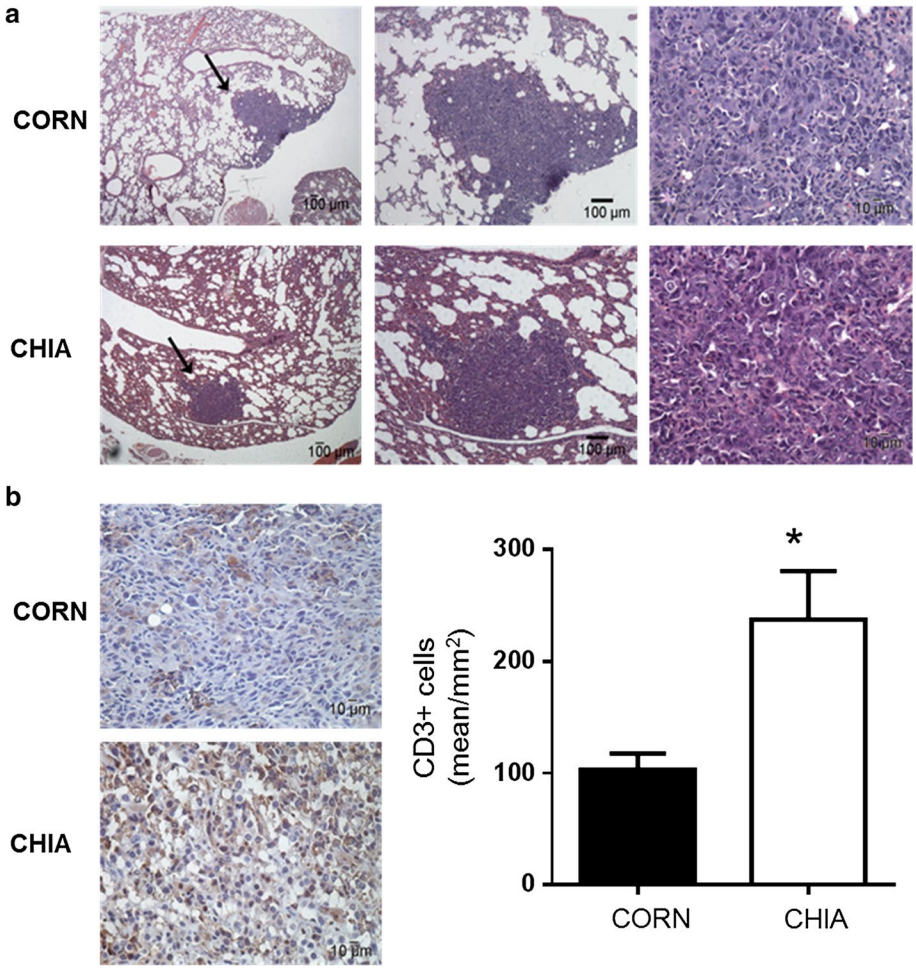

Fig. 2 Lung metastases from tumor-bearing mice from corn and chia oil groups, and CD3 detection in tumor slides of corn- and chia oil-fed mice. **a** Representative power microscopic images of lung metastasis from corn- and chia-fed mice (4×, 10×, and 40× magnifications). **b** Immunolabeling for CD3+ cell infiltration in tumor tissue from corn- and chia oil-fed mice. Tumor tissues were collected from mice and fixed in paraformaldehyde 4 % for immunostaining. CD3+ cells were counted from tumor tissues (three mice for each dietary condition). Representative images from tumor samples are shown (40× magnification). *Bars* show CD3+ cells expressed as mean ± SEM/mm2, $*p \leq 0.05$. Corn versus chia. *t* test. $n = 3$

Table 2 Tumor parameter comparison between corn- and chia oil-fed mice

Tumor parameters	Corn diet	Chia diet
Tumor incidence (*n*)	20	17*
Tumor weight (g)	2.2 ± 0.2	1.0 ± 0.2*
Tumor volume (mm^3)	7.2 ± 1.0	4.4 ± 0.4*
Metastasis number (*n*)	10 ± 1.0	7.0 ± 0.8*
Latency time (days)	15 ± 2.0	22 ± 1.0*

LM3 cells (10^6 in 200 µl fresh MEM) were injected subcutaneously into the left flanks of the mice. Tumor incidence was considered as the number of mice with tumors (*n*). Tumor weight was measured using a precision scale (g). Tumor volume was calculated using a digital caliper (mm^3). The number of macroscopic metastasis (loci) was recorded in all organs (*n*). Latency time was determined as the period between tumor induction and when the tumor became palpable (days). Values are mean ± SEM, * $p \leq 0.05$; Corn versus chia. *t* test, $n = 20$–17

ALA decreases AA- and LA-derived LOX metabolites

Our findings show that PUFAs alter membrane stability. They modify cellular signaling in breast cancer cells via modifications to the cell membrane structure that increase the degree of fatty acid unsaturation and exert an anti-carcinogenic effect [23]. An ALA-rich diet induced a ω-3 PUFA increment in tumor membranes. ALA, as well as EPA and DHA, was higher, while cell membranes from the corn oil-fed mice showed a higher percentage of LA and AA, correlating fairly well with the exogenous PUFAs provided by that diet (Table 3). One of the key cellular functions of PUFAs is their enzymatic conversion into eicosanoids. The inhibition of the synthesis of metabolites derived from LA and AA through the activity of LOXs enzymes is recognized as the most salient mechanism by which PUFAs

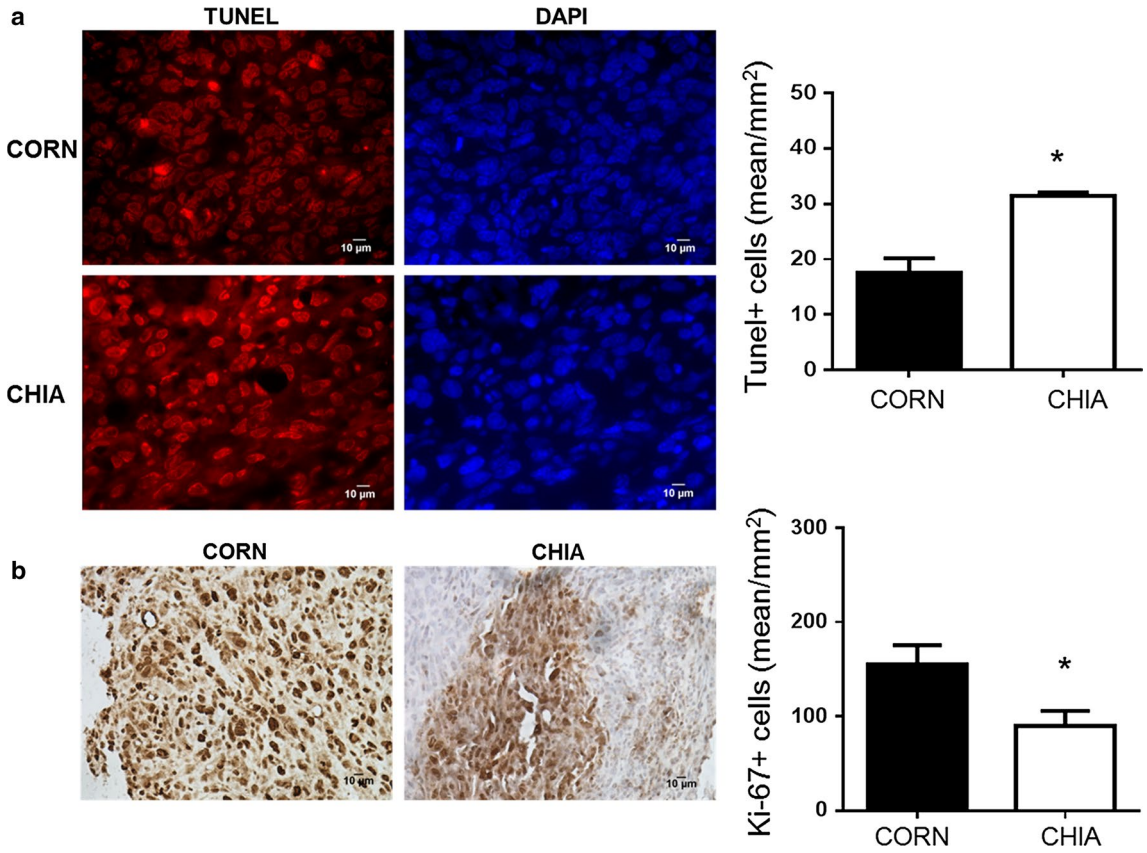

Fig. 3 TUNEL and Ki-67 assay in tumor slides of corn- and chia oil-fed mice. **a** Apoptosis evaluation in primary tumor section tissues by TUNEL assay. TUNEL-positive cells in the nucleus are stained in *red*, and total nuclei are stained with DAPI in *blue* (40× magnification). Experiments were repeated in tumor tissue sections from three mice for each dietary condition. *Bars* show TUNEL-positive cells expressed as mean ± SEM/mm2, $*p \leq 0.05$. Corn versus. chia. *t* test. $n = 3$. **b** Mitosis evaluation in primary tumor section tissues by Ki-67 Assay. Ki-67-positive cells are stained in brown (40× magnification). Experiments were repeated in tumor tissue sections from three mice for each dietary condition. *Bars* show Ki-67-positive cells expressed as mean ± SEM/mm2, $*p \leq 0.05$. Corn versus chia. *t* test. $n = 3$

Table 3 Fatty acid composition (%) of membrane cells isolated from tumors from corn or chia oil-fed mice

Major ω-6 and ω-3 PUFAs (%)					
	ω-6 PUFAs		ω-3 PUFAs		
	18:2	20:4	18:3	20:5	22:6
Corn diet	23.76 ± 0.2	15.69 ± 0.1	11.72 ± 0.1	2.13 ± 0.2	2.33 ± 0.3
Chia diet	12.96 ± 0.2*	5.45 ± 0.3*	24.26 ± 0.2*	9.05 ± 0.1*	4.09 ± 0.1

Cells from primary tumors from three host mice fed by each of the diets were homogenated. Lipids were extracted from cells membranes and were methylated for further analyses in GLC. Values are mean ± SEM, * $p \leq 0.05$ Corn versus chia *t* test. $n = 3$. Fatty acids' common names: 18:2 linoleic acid, 18:3 α-linolenic acid, 20:4 arachidonic acid, 20:5 eicosapentaenoic acids, 20:6 docosahexaenoic acid

reduce tumor development [24]. The chia diet affected LOX-derived metabolites released from tumor cells after stimulation with calcium ionophore A23187. Tumor cells obtained from the chia diet generated lower amounts of ω-6 pro-tumor metabolites, such as 15 (S)-HETE, 5 (S)-HETE, and 13 (S)-HODE, when compared with tumor cells obtained from the corn oil-fed mice (Fig. 4).

ALA-rich diet decreased ERα expression

One mechanism in the ω-3 PUFA anticancer effect is the alteration of cell membrane microdomain composition that affects the distribution and function of numerous receptors and other signaling molecules [25]. We assessed the expression of ER isoforms to determine whether ALA treatment might alter ERs in tumor lysates from mice. The

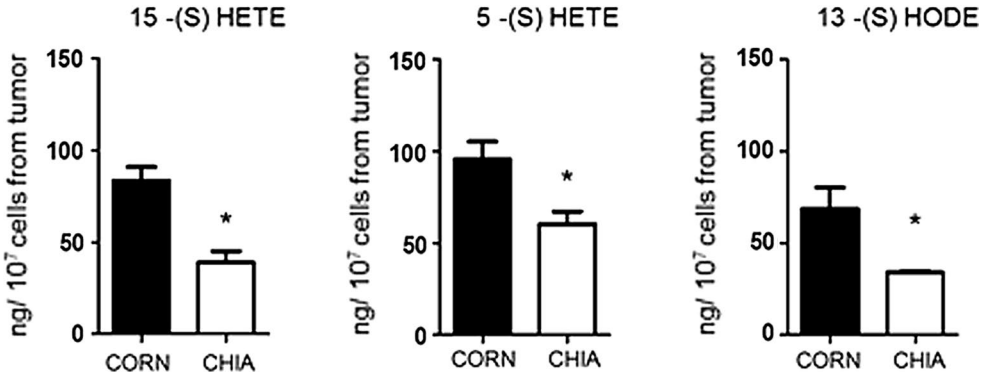

Fig. 4 Lipoxygenase metabolites determination in isolated tumor cells from corn- and chia oil-fed mice. Tumor cells were suspended in PBS with Ca2+ and Mg2+. Tumor cells were stimulated with the ionophore A23187 to induce metabolite release. Values are mean ± SEM, $*p \leq 0.05$. Corn versus chia. *t* test. $n = 8$

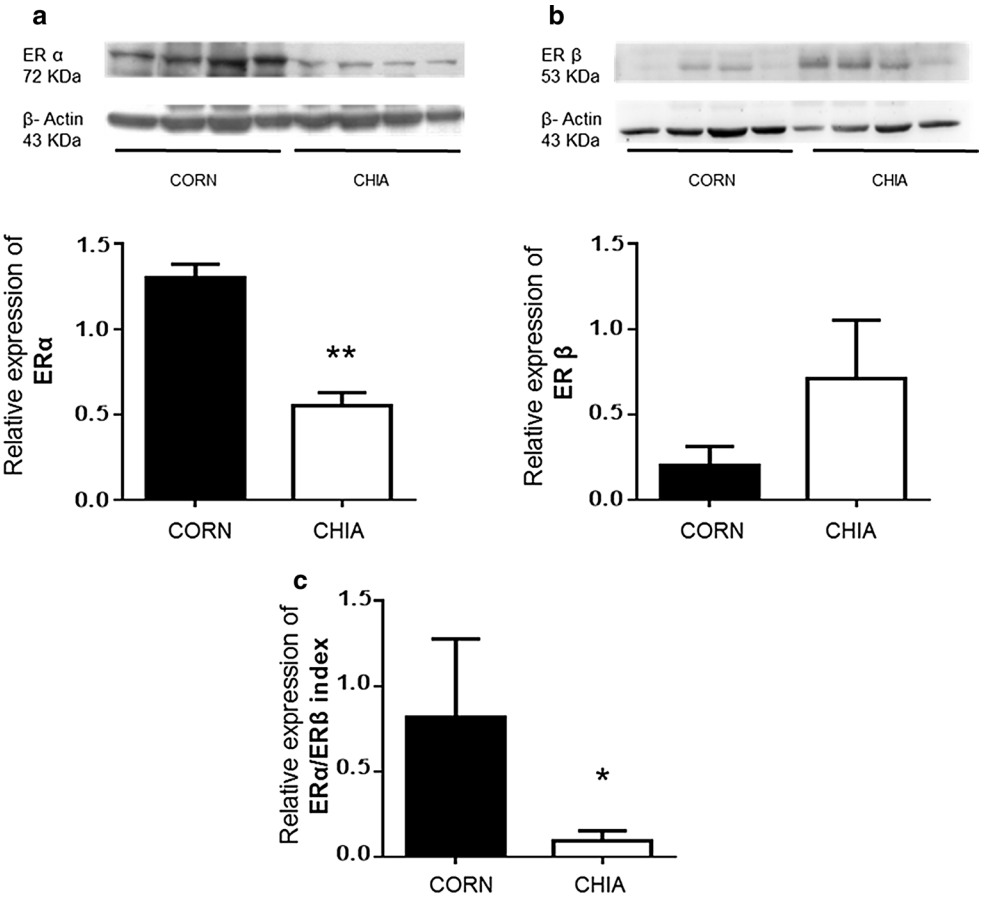

Fig. 5 ERα and ERβ expressions in tumor lysates of corn- and chia oil-fed mice. Tumor tissues were collected from mice, and total proteins were extracted for Western blot analysis. Representative images of four tumor samples from each dietary condition are shown. **a** ERα levels; **b** ERβ levels; and **c** ERα/ERβ index. *Bars* show densitometric analysis of bands expressed as mean ± SEM, $*p \leq 0.05$, $**p \leq 0.001$. Corn vs. chia. *t* test. $n = 4$

ERα amount was downregulated by ω-3 PUFAs (−65 %, in comparison with corn oil diet, $p < 0.001$) in tumor lysates (Fig. 5a); conversely, the ERβ expression was higher in tumor lysates from mice fed a chia oil diet (Fig. 5b). Accordingly, the ERα/ERβ ratio is significantly decreased in tissue lysates from ChO-fed mice (Fig. 5c). We also performed preliminary experiments to evaluate ERα expression in LM3 cells treated for 24 or 48 h with 10 or 50 μM ALA. We found that ERα is expressed in LM3 cells, and that the ER levels appear to be reduced in the ALA-treated cells (data not shown). Overall, we show, for the first time, that an ALA-enriched diet with chia seed oil downregulated ER expression in breast cancer in vivo, suggesting that downregulation of ERα expression by ALA can, at least in part, account for its anti-tumorigenic effect in a syngenic mouse model of ER-positive breast cancer. This suggests that ALA could also decrease ER-positive breast tumorigenesis through a downregulation of ERα.

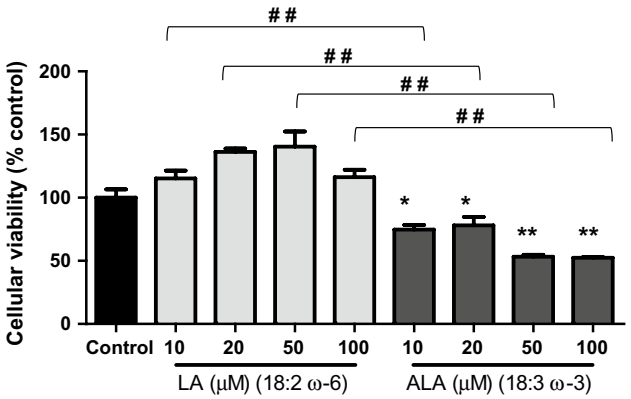

Fig. 6 Effects of ALA (ω-3) and LA (ω-6) on LM3 cell viability. Cells were incubated for 24 h with ALA or LA (10–100 μM). Cell viability was measured by resazurin test. *Bars* show the mean ± SEM (%) Anova Dunnet, Control versus Treatment $^{*}p < 0.05$ $^{**}p < 0.001$. LA versus ALA, *t* test: $^{\#\#}p \leq 0.001$ $n = 3$

ALA inhibits murine breast cancer cell growth in vitro

The effect of ALA on the viability of cultured LM3 cells was determined. Treatment with 10 or 20 μM of ALA for 24 h inhibited the viability of LM3 cells by 30 %, and this effect was accentuated up to 50 % when cells were treated with 50 or 100 μM of ALA (Fig. 6). Taking together the lower cell viability rate after ALA treatment and the higher apoptosis and lower mitotic figures found in ex vivo tumor tissues from ChO-fed mice, we suggest that ALA may drive tumor regression. Moreover, as reported above, ALA downregulated ERα expression in tumor cells as well as on tumor lysates from primary tumor. This suggests that ERα could be the mechanism responsible for tumor regression and in tumor cell viability reduction.

Discussion and conclusion

In this work, an ALA-enriched diet obtained with chia oil dietary supplementation resulted in a protective effect on tumor growth parameters and a decreased tumor incidence (3/20 mice fed with chia oil did not develop tumors). Significant tumor regressions were described as reduced tumor weight size and number of metastasis, as well as higher latency time.

Recent studies in breast cancer cells have found that ω-3 PUFAs might change the cell membrane structure, especially in lipid rafts phospholipids. Altering the chemical and physical properties of lipid raft structures leads to a reduction in breast cancer cell proliferation, probably through different mechanisms related to acyl chain length and unsaturation, and suggests a potential mechanism underlying ω-3 PUFA anticancer effects [25, 26].

The fatty acid tumor cell membrane composition can be composed of up to 40 % of AA, which is the main precursor of eicosanoids with pro-metastatic effects [27]. In our work, after the chia oil dietary treatment, we found that the ALA-enriched diet significantly reduced AA and LA in the lipid composition of the cell membrane as well as the levels of metabolites derived from LOXs activity, such as 13 (S)-HODE from LA, and 15 (S)-HETE and 5 (S)-HETE from AA, in isolated cells from the primary tumor (Fig. 4). In line with these results, our previously published work demonstrated that metabolites derived from LOX activity, such as 13 (S)-HODE, 15 (S)-HETE, and 5 (S)-HETE, showed proliferative and anti-apoptotic activities [9, 16]. This present study demonstrates that 13 (S)-HODE has a mitogenic effect on certain cancer cells, and that 15 (S)-HETE has a similar effect on non-differentiated intestinal epithelial cells [28]. The reduction in intracellular 13 (S)-HODE and 15 (S)-HETE production in chia treatment could decrease the activity of the enzyme that phosphorylates the mitogenic epidermal growth factor pathway (EGFR/MEK/ERK1/2), thus resulting in the inhibition of DNA synthesis and cell proliferation [29]. A widely accepted theory suggests that the health-promoting effects of ω-3 PUFAs are mediated by the suppression of the metabolism of ω-6 AA giving rise to the formation of pro-tumor eicosanoids, while ω-3 PUFAs may serve as alternative substrates to generate ω-3 lipid mediators with beneficial activities [30]. In this sense, lipoxygenase metabolites, depending on the substrate, may both initiate the inflammatory response and mediate resolution of inflammation [31].

Using immunohistochemistry for CD3+ quantification, we observed an increase in CD3 +-labeled cells in tumor tissues from hosts fed with the ALA-enriched diet. These results are in agreement with previous findings obtained by using flow cytometry [9]. In some tumors, such as breast carcinomas, infiltrating lymphocytes form lymph node-like structures, suggesting that the immune response is operating in situ [32]. Moreover, T-lymphocyte infiltrates appear to be associated with an improved prognosis in many tumor types [33, 34].

With respect to the potential role of ω-3 PUFAs on estrogen receptor expression, Lu et al. [11] have proposed that DHA (22:6 ω-3) inhibits the growth of MCF-7, an ER+ breast cancer cell line, through a decrease in ERα expression. In the present work, we found a downregulation of ERα in tumor lysates isolated from ChO-fed mice, while ERβ expression was increased, even if the results did not reach statistical significance (Fig. 5b). Interestingly, while ERα has long been recognized as promoting breast cancer growth and development, a recent study reported that the presence of ERβ, if phosphorylated at Tyr 36, could predict both disease-free survival and overall survival in breast cancer [35]. Although we cannot exclude that an

ALA-enriched diet affects ER expression in non-tumoral cells (such as macrophages, lymphocytes stromal cells), it is likely that ω-3 PUFAs specifically regulate ER expression in breast cancer LM3 ERα-positive cells (data not shown).

Viability experiments were performed in order to assess the cytotoxic effect of ALA on LM3 cells, the murine cell line used to induce syngenic tumor in mice. We found that ALA (the major omega-3 PUFA in chia oil diet) decreased cell viability compared to the control and LA (ω-6), when tested in the same conditions.

Overall, we have shown that an ALA-rich diet determines incorporation into cell membranes, generating microenvironmental changes resulting in an anti-tumor effect. Diminution of AA and LA metabolites, as well as an increment of tumor-infiltrating T-lymphocytes, may contribute to cancer cell apoptosis. Overall, these findings support the role of chia oil, an ALA dietary source, as a potential nutrient in the prevention and treatment for breast cancer.

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Compliance with ethical standards

Conflict of interest The author states that there is no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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