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HEALTH SCIENCES

Dietary lipids management: are there any benefits from the prevention and treatment of 4T1 murine breast carcinoma?

NICOLLE CAMILLA R. DA SILVA, YASMIM DE O.B. SILVA, JACQUELINE A. TAKAHASHI, SILVIA P. ANDRADE, GEOVANNI D. CASSALI & DIRCE R. DE OLIVEIRA

Abstract: This study investigated the effect of vegetable and fish oils with different n-3 / n-6 PUFAS ratios on the lipoprotein profile and on the development of murine breast cancer 4T1. Female Balb/c mice (6-7 weeks) received diets containing 4.0% fat during seven weeks. On the fourth week, animals were inoculated into the posterior left flank with 2.5×10^6 4T1 cells. Body weight and food intake were registered and the profile serum lipoproteins was determined. Tumor volume, histopathological and immunohistochemical studies, myeloperoxidase and N-acetylglucosaminidase activities, TNF-α, hemoglobin and VEGF levels were analysed. The highest n-3 / n-6 ratio was found in fish oil (15.8:1), followed by linseed (2.4:1), canola (1:2.1) and soybean (1:9.4) oils. Body weight, food and caloric intake, lipoprotein profile, tumor weight, tumor evolution and histopathological analysis were not different. Canola oil increased cell proliferation when compared to soybean oil, and fish oil changed the inflammatory response and increased VEGF in tumors compared to other groups. The type of fatty acid and the high ratio of n-3 / n-6 PUFAs in the diet influenced cell proliferation and inflammation in the tumor differentially, highlighting the increase of neutrophils and VEGF levels in animals fed on fish oil.

Key words: n-3/n-6 ratio, 4T1 murine breast cancer model, angiogenesis, inflammation, lipoprotein.

INTRODUCTION

Breast cancer is the leader cause of cancer-associated mortality in women in Brazil and the estimate number for each year of the 2020-2022 triennium points out that there will be about 625,000 new cases of cancer, of which 66,000 are new cases of breast cancer (Ministério da Saúde 2019). Several risk factors contribute to the incidence of breast cancer, including advanced age, woman's reproductive life (early menarche, nulliparity, oral contraceptives), family and personal history, lifestyle and environmental influences (Momenimovahed & Salehiniya 2019). Among them, dietary factors can be important modifiers in the risk of that neoplasm.

Previous studies have shown that high-fat diets can increase breast tumor growth, metastasis and mortality in obese resistant mice (Kim et al. 2011). On the other side, diets rich in n-3 polyunsaturated fatty acids (PUFA n-3) can reduce the rate of tumor growth and metastasis in experimental models (Cho et al. 2010). Nindrea et al. (2019) have shown that a high consume ratio of n-3/n-6 PUFAs is associated with lower risk of breast cancer in women from Western and Asian countries (Nindrea et al. 2019) and Yang et al. (2014) reported the same findings in adult women (Yang et al. 2014). Both studies

suggested that increasing proportion of n-3/n-6 PUFAs dietary intake would provide benefits for breast cancer prevention.

Experimental models are useful tools to evaluate the effects of certain drugs and / or nutrients on neoplastic development. Few studies have shown the influence of dietary lipid content modification in a 4T1 breast carcinoma model. Kim et al. (2011) showed that a high-fat diet (45 or 60% of total calories) increases breast tumor 4T1 growth, and the number of metastases, in addition to decreasing the survival rate of obesity-resistant mice (Kim et al. 2011).

Caloric restriction (40% deficit of kcal / week) reduced the growth and the total number of metastases of 4T1 tumors, decreased cell proliferation and angiogenesis, increased apoptosis, reduced levels of insulin, leptin, IGF-1 and IGF-1 type 3 binding protein and increased levels of adiponectin in tumors (De Lorenzo et al. 2011).

In the current study, we hypothesized that the consumption of a dietary intake of lipids with high n-3 / n-6 ratio may reduce the development of neoplasm by inhibiting inflammatory, angiogenic and proliferative processes in breast cancer. In addition, the aim of this investigation was to determine the effects of vegetable or fish oils on lipoprotein profile and composition in mice bearing 4T1 tumors. Murine mammary carcinoma 4T1 was chosen as the experimental model due to its capacity of efficiently metastasizing to distant sites, similar to common features of the disease in humans.

MATERIALS AND METHODS

Identification and quantification of fatty acids by gas chromatography

All oils (soybean, canola, linseed and fish) were purchased at the Central Market of Belo Horizonte (Belo Horizonte city - Minas Gerais state, Brazil), properly stored under refrigeration (4-10 °C), and protected from light until used.

Hydrolysis, Lipid Methylation and Gas Chromatography

About 10 mg of oil were dissolved in 100µL of a 1 mol/L potassium hydroxide solution (5%) in ethanol / 95%. After vortexing for 10 s, the oil was hydrolyzed in a microwave oven (Panasonic Piccolo) for 5 min (80 W power). After cooling, 400 µL of 20% hydrochloric acid, approximately 4 µg NaCl, and 600 µL ethyl acetate were added. After vortexing for 10 s and letting it sit for 5 min, 300 µL of the organic layer were removed and dried by evaporation in order to obtain the free fatty acids. The free fatty acids were then methylated with 100µL BF_3 in methanol (14%) and heated for 10 min in a 80 °C water bath, diluted with 400 µL of methanol and stored for further analysis.

Analyzes were performed in a HP7820A gas chromatograph (Agilent) equipped with gas flame ionization detector. An INNOWAX (HP) 15 m x 0.25 mm x 0.25 µm column was used with temperature gradient: 100 °C at 0 min, increasing 7 °C/min up to 240 °C; injector split at 1/30 at 250 ºC and detector temperature at 260 ºC. Hydrogen was used as the entrainment gas (3 mL/min), with flow rate of 2 mL/min and injection volume of 1 µL. EZChrom Elite Compact (Agilent) was the program used for data acquisition. Peak identification was done by comparison with known methylated fatty acid standards analyzed in the same conditions (Supelco, 37 FAME blend) (Takahashi et al. 2015).

Animals and experimental design

All procedures were approved by the Ethics Committee on Animal Use (CEUA) of the Federal University of Minas Gerais - UFMG (protocol 126/2012).

Female Balb/c mice at 6-7 weeks of age were obtained from the animal care center of Universidade Federal de Minas Gerais (CEBIO-UFMG), randomly distributed into four groups: (1) Control (4% of soybean oil, n=14); (2) Canola (4% of canola oil, n=14); (3); Linseed (4% of linseed oil, n=14), and (4) Fish (4% of fish oil, n=14) and kept in an environmentally controlled room under a 12 h light/dark cycle at 22 ± 2 °C. All diets were prepared according to the AIN-93M maintenance diet, containing 46.7% carbohydrate, 4.0% fat, and 14% protein (Reeves et al. 1993). Food and water were supplied *ad libitum*. All experimental diets had the same calorie density (3.81 kcal/g) and the composition is summarized in Table I.

Tumor cells and inoculation

4T1 neoplastic cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modification of Eagle's essential medium supplemented with 10% fetal bovine serum (FBS), 100 µg/mL penicillin and 100 µg/mL streptomycin, at 37 °C in a humidified atmosphere of 5% CO_2. Four weeks after diet initiation, seven animals from each group were randomly inoculated into the posterior left flank with 2.5×10^6 4T1 cells in a total volume of 100 µL to obtain solid tumors growth (Reis et al. 2014). Then, the animals were randomly regrouped into four other groups: (5) Control 4T1 (4% of soybean oil, n=7); (6) Canola 4T1 (4% of canola oil, n=7); (7);

Table I. Ingredient composition (grams per kilogram) and energy content of the diets.

INGREDIENT	Control	Canola	Linseed	Fish
Casein[a]	140	140	140	140
Corn starch	467	467	467	467
Sucrose	100	100	100	100
Maltodextrin	15.5	15.5	15.5	15.5
Experimental oils				
Soy[b]	40	0	0	0
Canola	0	40	0	0
Linseed	0	0	40	0
Fish	0	0	0	40
Cellulose	50	50	50	50
Mineral mixture[c]	35	35	35	35
Vitamin mixture[c]	10	10	10	10
Choline bitartrate	2.5	2.5	2.5	2.5
Tert-butylhydroquinone	0.08	0.08	0.08	0.08
L-Cystine	1.8	1.8	1.8	1.8
Energy content[d] (kcal/g)	3.81	3.81	3.81	3.81

[a]Isofar (Duque de Caxias, Rio de Janeiro, Brazil), containing 85 % protein.
[b]Brasil Foods SA (São Paulo, Brazil).
[c]Mineral and vitamin mixture as recommended by the AIN-93M rodent diet (Reeves et al. 1993).
[d]Conversion factors: protein, 4 kcal/g; fat, 9 kcal/g; carbohydrate, 4 kcal/g.

Linseed 4T1 (4% of linseed oil, n=7), and (8) Fish 4T1 (4% of fish oil, n=7). The experimental design is illustrated in Figure 1.

Body weight and food intake were registered weekly. Tumor volume was measured every 48 hours using a caliper ruler (Mitutoyo, MIP/E-103) and calculated using the equation: tumor volume (mm^3) = (length × $width^2$)/2, with length and width given in millimeters (Souza et al. 2013, Reis et al. 2014).

At the end of the experiment (7 weeks), mice were fasted for 12 h and were anesthetized with 130 mg/kg body weight (BW) of ketamine and 0.3 mg/kg BW of xylazine then killed by exsanguination to obtain serum. Tumor and lungs were removed of all animals in the 4T1 groups for histopathological analysis.

Lipoprotein profile and composition

The profile of circulating serum lipoproteins was determined by the gel filtration chromatography method on a Waters Fast Protein Liquid Chromatograph (FPLC) model 600 using a Superose 6 column. (Fazio et al. 1997) Pooled serum from 3 or 4 of the animals was used for lipoprotein determination. An aliquot of the pooled serum (100 µL) was loaded and separated at a flow rate of 0.5 ml/min with a buffer containing 0.15 M NaCl, 0.01 M Na_2HPO_4, 0.1 mM EDTA, pH 7.5. Forty fractions were collected (0.5 mL each), with the lipoproteins (high density lipoprotein) being collected in tubes 16–32. The cholesterol and triglycerides levels were determined in fractions using commercial kits (Doles, Goiás, Brazil) according to the manufacturer's recommendations.

Histopathological and immunohistochemical studies

Samples of tumors and lungs were fixed in formalin (10% w/v in phosphate-buffered saline - PBS pH 7.4), and serial sections (4 µm) were stained with hematoxylin and eosin (HE) for morphologic and morphometric assessments (de Souza Garcia et al. 2014).

Tumor cell proliferative activity was evaluated by immunohistochemical study using the cell proliferation marker CDC47 – clone 47DC141 (NeoMarkers, CA, USA) (Souza et al. 2011). Blades stained by the immunohistochemistry technique were initially visualized under a magnification of 100x to identify intrateciduous regions with a higher density of nuclear positive immunoblot ("hot spots"). Five high-resolution photomicrographs of these areas were obtained at 40x magnification in an optical microscope (final magnification = × 1000) (Ferreira et al. 2007) Serial sections of 4 µm were obtained from the tumors and the antigen retrieval was performed through the use of retrieval solution (Dako, CA, USA) for 20 min. The primary antibody CDC47 (clone 47DC14, 1: 300) was applied for 60 min and peroxidase activity was developed with liquid chromogenic solution of 3,3'diaminobenzidine (DAB, Dako, Carpinteria, USA). The slides were counterstained with Harris hematoxylin. For negative control, either nonimmune mouse or rabbit serum was used instead of the primary antibody. The positive control was performed on the tissue section itself in the basal layer of the epidermis. The proliferative index was obtained by calculating the percentage of positive cells in 500 tumor cells.

Tissue extraction and determination of myeloperoxidase and N-acetylglucosaminidase activities

Infiltration of neutrophils in the tumors was evaluated by assaying myeloperoxidase (MPO) as previously described (Marques et al. 2011). Tumors were weighed, homogenized in pH 4.7 buffer (0.1 M NaCl, 0.02 M $NaPO_4$, 0.015 M NaEDTA), centrifuged at 12,000 × *g* for 10 min. The pellets were then resuspended in

0.05 M $NaPO_4$ buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide (HTAB) followed by three freeze–thaw cycles using liquid nitrogen. MPO activity in the supernatant samples was assayed by measuring the change in absorbance (optical density; OD) at 450 nm using tetramethylbenzidine (1.6 mM) and H_2O_2 (0.3 mM). The reaction was terminated by adding 50 µL of H_2SO_4 (4 M). Results were expressed as change in OD per g of wet tissue. The infiltration of mononuclear cells into the tumors was quantified by measuring levels of the lysosomal enzyme N-acetylglucosaminidase (NAG) present in high levels in activated macrophages (Marques et al. 2011). The tumors were homogenized in NaCl solution (0.9% w/v) containing 0.1% v/v Triton X-100 (Promega) and centrifuged (3,000 ×g; 10 min at 4 °C). Samples (100 µL) of the resulting supernatant were incubated for 10 min with 100 µL of p-nitrophenyl-N-acetyl-beta-D-glucosaminide (Sigma-Aldrich, St. Louis, MO, USA) prepared in citrate/phosphate buffer (0.1 M citric acid, 0.1 M Na_2HPO_4; pH 4.5) to yield a final concentration of 2.24 mM. The reaction was stopped by adding 100 µL of 0.2 M glycine buffer (pH 10.6). Hydrolysis of the substrate was determined by measuring the absorption at 400 nm. Results were expressed as nmol/mg wet tissue.

Hemoglobin extraction

Vascularization of the carcinoma was assessed by the amount of hemoglobin (Hb) using the Drabkin method (Drabkin & Austin 1932). Mammalian tumor fragments were homogenized with Ultra-Turrax, (Schlappmuhler, Usingen, Germany) in 2 mL of reagent of Drabkin (Labtest, São Paulo, Brazil), and centrifuged at 10,000 ×*g* for 40 min. The supernatants were filtered through a 0.22 µm Millipore filter. Hemoglobin concentration in the samples was determined at 540 nm compared with a standard hemoglobin curve. The results were expressed as µg Hb per mg wet tissue.

Measurement of VEGF and TNF-α in the tumors

Tumors were weighed and homogenized in PBS (1 mL for 100 mg of the tissue) pH 7.4 containing 0.05% Tween and centrifuged at 10,000 ×*g* for 30 min. The cytokines VEGF and TNF-α were measured in 50 µL of the supernatant using the standard protocols of the Duo Set Murine Immunoassay Kits supplier (R & D Systems, USA). Briefly, dilutions of cell-free supernatants were added in duplicate to ELISA plates coated with a specific murine monoclonal antibody against the cytokine, followed by addition of a second horseradish peroxidase-conjugated polyclonal antibody against the cytokine. After washing the wells, 50 µL of a 1:1 solution of hydrogen peroxide and tetramethylbenzidine 10 mg/mL in DMSO were added to them. After 20 min incubation, the color development was stopped with 50 µL 2 N sulphuric acid and color intensity was measured at 540 nm on a spectrophotometer (E max-Molecular Devices). Standards were 0.5-$\log_{10}$ dilutions of recombinant murine cytokines from 7.5 $pgmL^{-1}$ to 1000 pg mL^{-1} (100 µL). The results were expressed as pg cytokine per mg wet tissue.

Statistical analysis

Kolmogorov-Smirnov test was used to verify normality and Grubbs' test was used to detect outliers. Data were expressed as means ± S.E.M. Data were submitted to one-way or two-way analysis of variance (ANOVA) followed by the Tukey post hoc analysis. $P<0.05$ was considered significant. Statistical analysis was performed using GraphPadPrism v.7.0 statistical software (La Jolla, CA, USA).

RESULTS

Fatty acids composition of oils

As shown in Table II, soybean, canola, linseed and fish oils presented 5.2%, 8.1%, 36.9% and 20.5% of n-3 PUFA and 48.9%, 17.3%, 15.6% and 1.3% of n-6 PUFA, respectively. Thus, the highest n-3 / n-6 ratio was found in fish oil (15.8:1), followed by linseed (2.4:1), canola (1:2.1) and soybean (1:9.4) oils. The major fatty acids of soybean, canola, linseed and fish oils were n-6 PUFA (48.9%), n-9 MUFA (61.6%), n-3 PUFA (36.9%) and SFA (35%), respectively. Fish oil was the only one that contained eicosapentaenoic (EPA, C20:5, 12.4%) and docosahexaenoic (DHA, C22:6, 7.4%) acids.

Food and caloric intake, body weight evolution and kinetics of 4T1 tumor growth

None of the groups showed differences in body weight (BW) (Figure 2a) and in food intake (Control: 3.9 ± 0.1; Canola: 4.1 ± 0.2; Linseed: 4.0 ± 0.2; Fish: 4.1 ± 0.2; Control 4T1: 4.1 ± 0.2; Canola 4T1: 4.0 ± 0.2; Linseed 4T1: 3.9 ± 0.2; Fish 4T1: 4.0 ± 0.2 g/animal/day; $P > .05$) during the experimental period. Likewise, regarding caloric intake, there were no differences among the groups. Control, Canola, Linseed, Fish, Control 4T1, Canola 4T1, Linseed 4T1 and Fish 4T1 groups showed, respectively, the following means of caloric intake: 14.8 ± 0.5, 15.59 ± 0.8, 15.4 ± 0.7, 15.57 ± 0.6, 15.67 ± 0.9, 15.37 ± 0.8, 14.9 ± 0.7 and 15.1Kcal/day ± 0.8. All results are expressed as mean ±

Table II. Fatty acid composition of oils incorporated into the diets.

	Amount (%)				
Fatty acid	**Soy**	**Canola**	**Linseed**	**Fish**	**RT(min)**
SFA	16.8	8.6	14.8	35.0	
C12:0	0.0	0.0	0.0	0.2	2.994
C14:0 (miristic acid)	0.0	0.0	0.0	8.2	5.111
C15:0	0.0	0.0	0.3	0.6	6.254
C16:0 (palmitic acid)	12.1	5.0	7.9	19.0	7.454
C17:0	0.0	0.0	0.0	0.6	8.611
C18:0 (stearic acid)	4.3	2.1	6.1	3.7	9.789
C20:0	0.4	1.5	0.5	1.4	11.988
C24:0	0.0	0.0	0.0	1.3	15.949
n-9 MUFA	**27.1**	**61.6**	**28.4**	**26.4**	
C16:1 (palmitoleic acid)	0.1	0.3	0.2	11.2	7.697
C18:1 (oleic acid)	27.0	61.3	28.2	15.2	10.002
n-6 PUFA	**48.9**	**17.3**	**15.6**	**1.3**	
C18:2 (linoleic acid)	48.9	17.3	15.6	1.3	10.555
n-3 PUFA	**5.2**	**8.1**	**36.9**	**20.5**	
C18:3 (linolenic acid)	5.2	8.1	36.9	0.7	11.213
C20:5 (EPA)	0.0	0.0	0.0	12.4	13.917
C22:6 (DHA)	0.0	0.0	0.0	7.4	16.247
Other	**2.0**	**4.4**	**4.3**	**16.8**	
n-3/n-6 ratio	**1:9.4**	**1:2.1**	**2.4:1**	**15.8:1**	

SFA: Saturated fatty acid; MUFA: Monounsaturated fatty acid; PUFA: n-3 polyunsaturated fatty acids; EPA: eicopentaenoic acid; DHA: docosahexaenoic acid
RT(min): Retention time (minutes).

standard error of the mean. Likewise, the type of oil consumed did not change the tumor weight (Figure 2b) and the tumor evolution during 20 days after inoculation (Figure 2c).

Lung metastases were found in one or two animals of each group, presenting the same morphological characteristics of the primary tumors, with mononuclear and polymorphonuclear cell infiltrates (data not shown).

Different oils consumption did not change lipoprotein profile and composition in mice bearing 4T1 tumors

All groups showed similar lipoprotein profiles with serum cholesterol mainly carried by high-density lipoprotein (HDL) (Figure 3a). The contents of cholesterol (Figure 3b) and triglycerides (Figure 3c) found in lipoproteins were also similar among the groups.

Dietary lipids did not change histopathological findings, but canola oil increased cell proliferation in tumors

Histological analysis in all groups revealed atypical mitoses (Figure 4). Extensive areas of necrosis and inflammatory infiltrate were also observed (not shown). The peritumoral infiltrate present in the diverse groups varied from mild to moderate, with a mononuclear predominance of the lymphoplasmohistiocytic type (not shown).

Representative immunohistochemistry sections of CDC 47 are illustrated in Figures 5a and 5b. A significant increase in the percentage of positive CDC 47 tumor cells was observed in the mice treated with Canola oil (Canola 4T1 group) compared to those treated with soybean oil (Control 4T1 group), shown in Figure 5c.

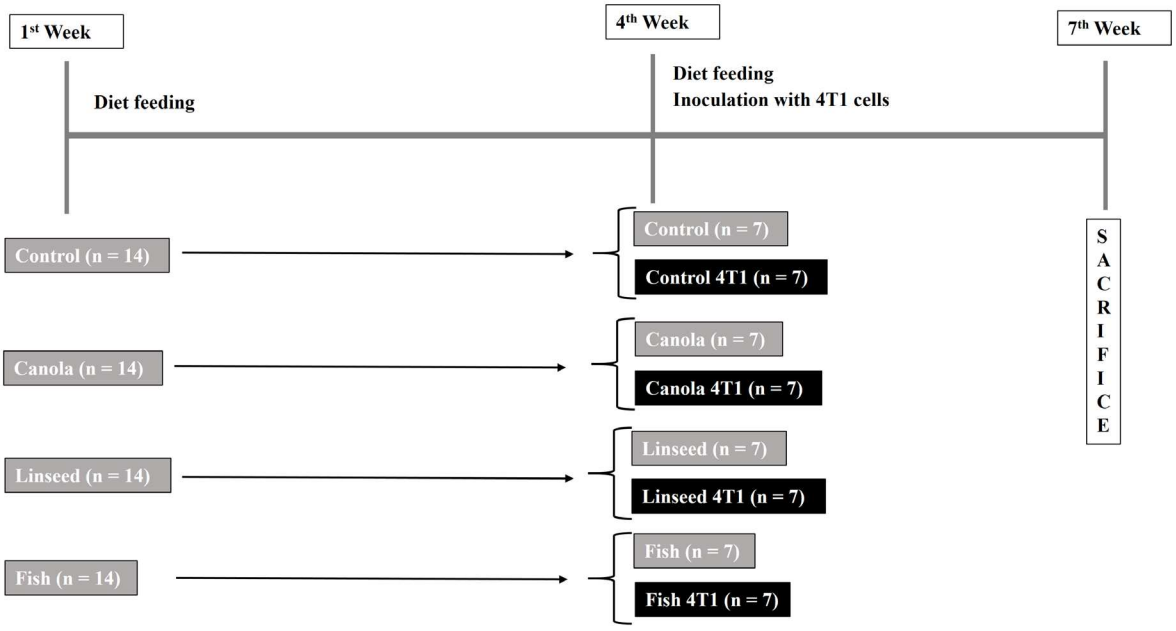

Figure 1. Experimental design. Mice were fed AIN-93 diet containing 4% soybean oil (Control), 4% of canola oil (Canola), 4% of linseed oil (Linseed), or 4% fish oil (Fish) for 7 weeks. On the fourth week, animals were randomly inoculated into the posterior left flank with 2.5 × 10^6 4T1 cells and randomly regrouped into four other groups: Control 4T1 (4% of soybean oil), Canola 4T1 (4% of canola oil), Linseed 4T1 (4% of linseed oil), and Fish 4T1 (4% of fish oil).

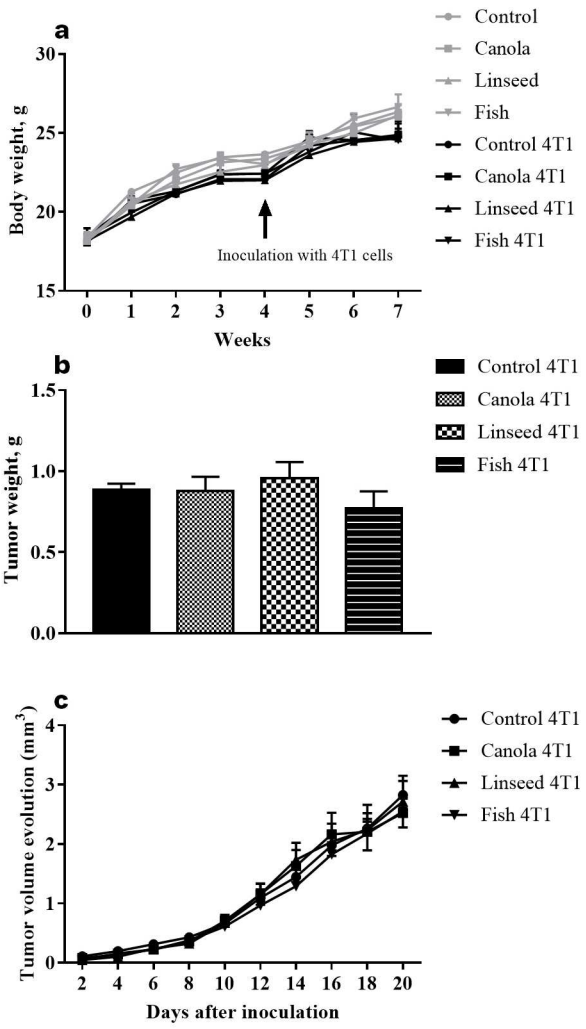

Figure 2. Effects of different oils on body weight evolution and kinetics of 4T1 tumor growth. a) Body weight evolution; b) Tumor weight; c) Tumor volume evolution after inoculation. Data represent means + S.E.M. of 7 animals per group, using one or two-way ANOVA followed by post hoc Tukey test.

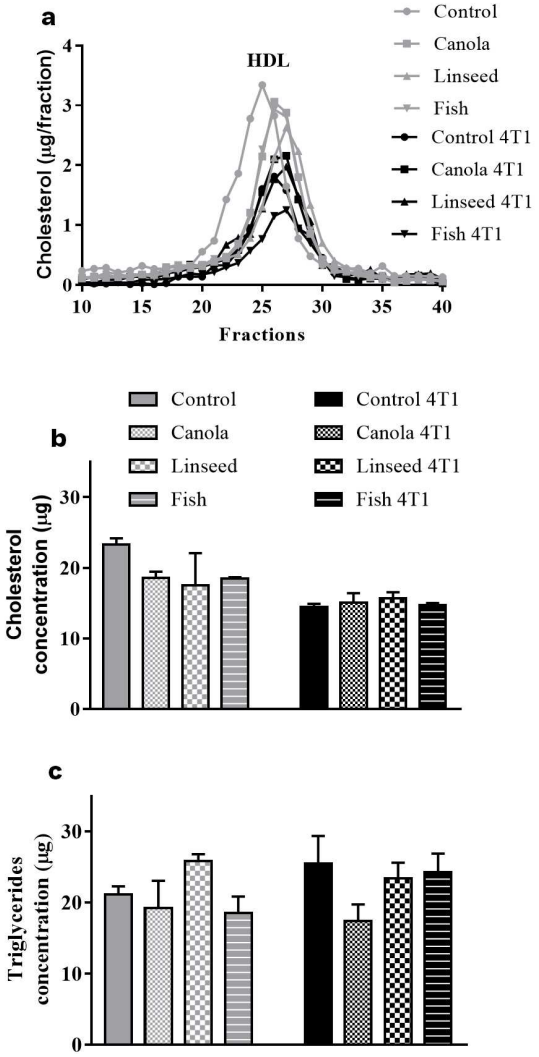

Figure 3. Profile and composition of circulating lipoproteins of fast performance liquid chromatography (FPLC) fractions. Lipoprotein profile (a); mean values of the sum of cholesterol (b), and triglyceride concentration (c), found in the different experimental groups. Gel filtration chromatography was performed using a Superose 6 column (Pharmacia) on a Waters 600 FPLC system. The pooled serum (a 100µL aliquot) was loaded and separated as described above. Cholesterol determinations were measured in the microplate assay. Data points represent mean values + S.E.M. for cholesterol from three pooled serum from each group (n=3), using one-way ANOVA followed by post hoc Tukey test.

Fish oil changes the inflammatory response in tumors

The effects of dietary oils were investigated considering neutrophil and macrophage activation (MPO and NAG activities, respectively) and the levels of the pro-inflammatory cytokine TNF-α in tumors. Figure 6a shows an increase in neutrophil numbers (detected as MPO activity) in the Fish 4T1 group compared with the Control 4T1, Canola 4T1 and Linseed 4T1 groups. Conversely, Fish 4T1 group had a decrease in macrophage numbers in relation to Canola 4T1 and Linseed 4T1 groups (Figure 6b). The ratio between MPO

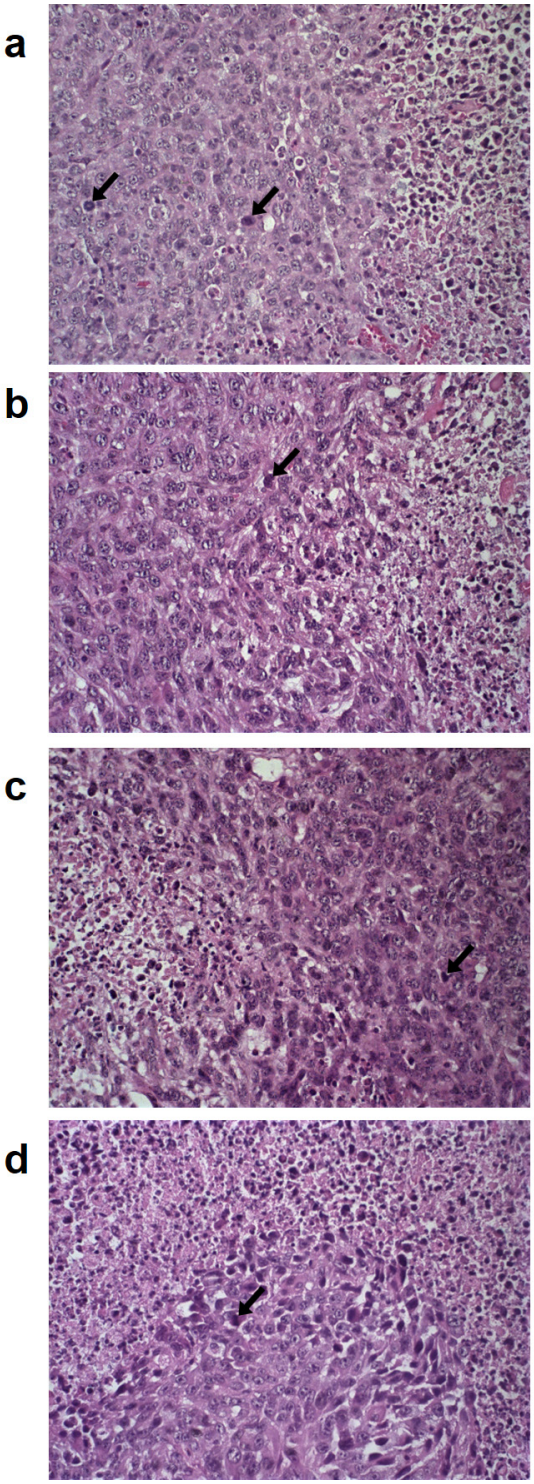

Figure 4. Photomicrographs of 4T1 murine breast carcinoma of the Control 4T1 group, evaluated 20 days after inoculation. Detail of the primary tumor showing atypical mitoses (arrow) in Control 4T1 (a), Canola 4T1 (b), Linseed 4T1 (c) and Fish 4T1 (d), 400 x. Hematoxylin and eosin staining.

and NAG activities was calculated (Figure 6c). The results were similar to those found for MPO activity, confirming that, compared to the other groups, neutrophil infiltration in the tumor was more significant in animals fed on fish oil. The level of the pro-inflammatory cytokine TNF-α did not change with the dietary oils (Figure 6d).

Fish oil increased VEGF levels in tumors

Tumor neovascularization, assessed by the amount of hemoglobin in the tumors, did not change with the different dietary oils (Figure 7a). However, VEGF levels were significantly higher in the Fish 4T1 group compared to other groups (Figure 7b).

DISCUSSION

In this study, we investigated the effect of oils (soybean, canola, linseed or fish oil) with different n-3 / n-6 PUFAS ratios on the lipoprotein profile and on the development of murine breast cancer 4T1. We found that the consumption of those oils at 4% in the diet did not change the body weight evolution, and food and calorie intake, in accordance to previous studies (Rosa et al. 2012, Borsonelo et al. 2013). Likewise, differences were not observed in the tumor growth follow-up and in the number of lung metastases among the groups. Compared to vegetable oils, fish oil contained the highest n-3 / n-6 ratio (15.8:1), being the only one containing EPA (12.4%) and DHA (7.4%) in its composition. Canola oil increased cell proliferation in tumor, compared to soybean oil. Fish oil changed the inflammatory response and increased vascular endothelial growth factor (VEGF) in tumors, compared to the other groups.

Dietary lipids can modify the progression and aggressiveness of breast cancer through different mechanisms that can influence gene expression, modulation of inflammation, cell

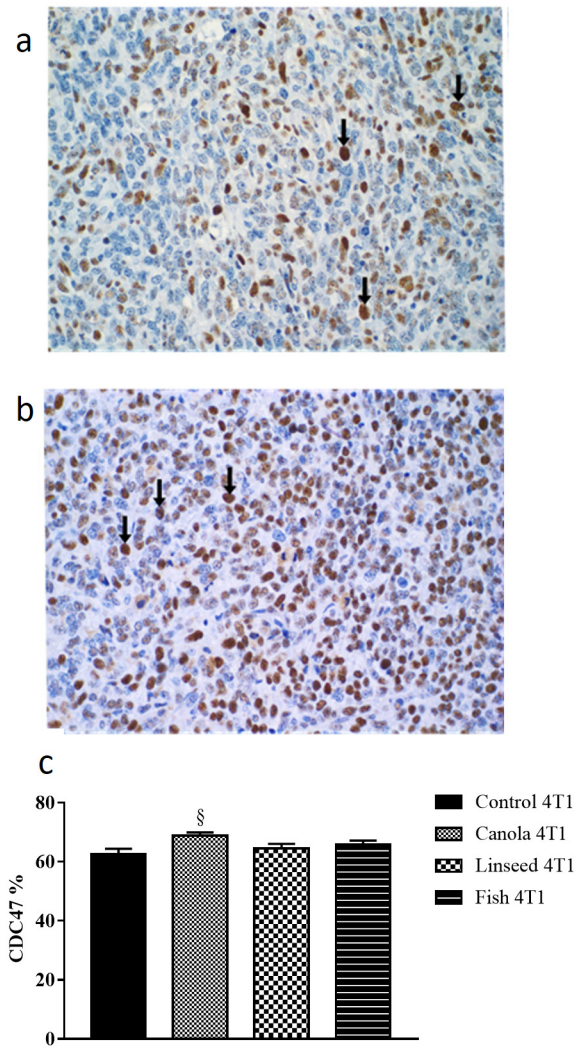

Figure 5. Immunohistochemical reaction for CDC47. Representative micrographs of tumor cross sections in (a) Control 4T1 group and (b) Canola 4T1 group; nuclear immunolabeling (arrows), where a higher percentage of positive cells may be observed in Canola 4T1 group. 400 ×. (c) Percentage of positive CDC 47 tumor cells in 500 tumor cells. Data represent mean ± S.E.M. of 7 animals for each group. § P < 0.05 vs. Control 4T1, using one-way ANOVA followed by post hoc Tukey test.

proliferation, angiogenesis and metastases (Peck & Schulze 2019). Some vegetable oils and fish oil have stood out for having beneficial effects for the body, as they are important sources of n-3 and n-6 polyunsaturated fatty acids (PUFA). The main n-3 PUFA is alpha-linolenic (C18:3) acid, found in linseed, canola and soybean oils, and eicosapentaenoic (EPA, C20:5) and docosahexahenoic (DHA, C22:6) acids, found in considerable amounts in fish oil (Ashfaq et al. 2020, Kontogianni et al. 2013, Cho et al. 2010). The most important n-6 fatty acids are linoleic (C18:2) and arachidonic (C20:4) acids present in vegetable oils such as sunflower oil, corn, soybean, cotton, among others (MacLennan & Ma 2010).

It is believed that before the advent of industrialization, people consumed a ratio of n-3 / n-6 in the diet around 1: 1 to 1: 2, due to the abundant intake of vegetables and marine foods. Current dietary habits include the average intake around 1: 10 to 1: 20, with records up to 1: 50 (Dierge & Feron 2019, Simopoulos 2004). Several studies have shown beneficial effects of n-3 polyunsaturated fatty acids on tumor progression. Some mechanisms include suppression of arachidonic acid-derived eicosanoid biosynthesis, which results in a significant reduction of tumor cell proliferation and metastatic potential, inflammatory response modulation, apoptosis, free radicals generation and oxidative stress (Koundouros & Poulogiannis 2020, Almeida et al. 2019, Ashfaq et al. 2020). Other authors performed a meta-analysis to examine the link between dietary intake of oils rich in n-3 and n-6 PUFAs and cancer risk and concluded that only n-3 PUFAs are inversely associated with the risk of breast cancer (Liu et al. 2021). On the other hand, n-6 PUFAs are described as promoters of cell proliferation (Koundouros & Poulogiannis 2020). For this reason, the recommendation of an n-3 / n-6 fatty acid ratio of 1:2 to 1:4 assumes great importance in human nutrition (Riediger et al. 2008).

Recent studies have directed the understanding of fatty acids and their metabolites, emphasizing the importance of strategies to use the 1:1 ratio of fish oil and soybean oil to reduce not only the tumor

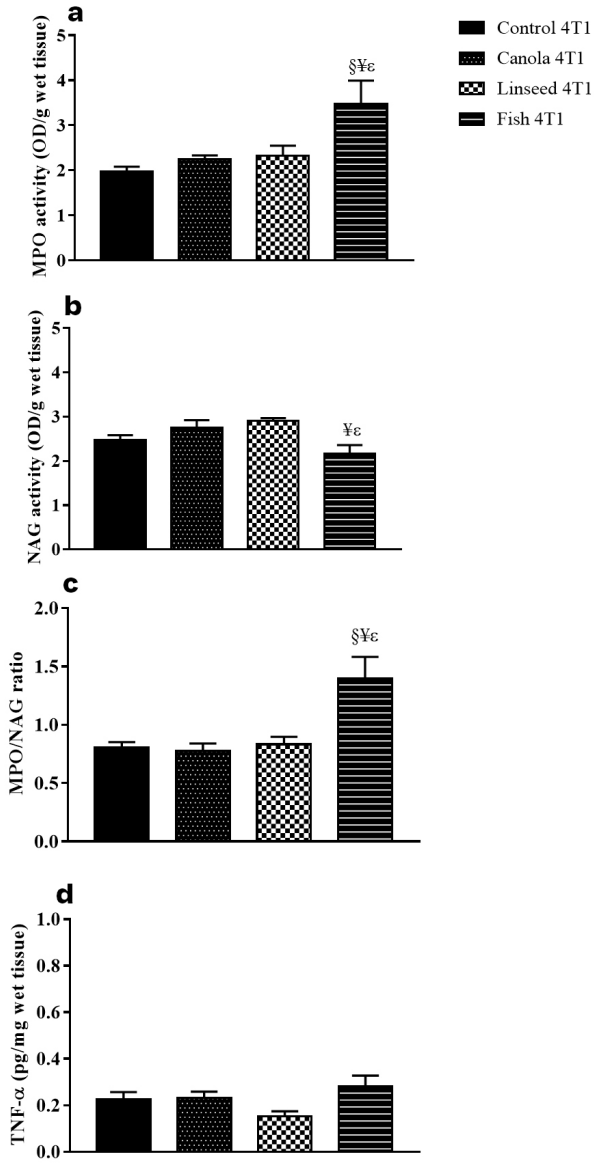

Figure 6. Effects of different treatments on inflammatory markers. Myeloperoxidase (MPO) activity (a), N-acetyl-β-Dglucosaminidase-NAG-activity (b), MPO/NAG ratio (c) and TNF-α (d). Bars represent mean ± S.E.M. of 7 animals for each group. § P < 0.05 vs Control 4T1; ¥ P < 0.05 vs Canola 4T1; ε P < 0.05 vs Linseed 4T1; using one-way ANOVA followed by post hoc Tukey test.

growth, but also the levels of pro-inflammatory mediators and chemokines in the tumor microenvironment (Khadge et al. 2020, Almeida et al. 2019). After evaluating the chemopreventive effect of regular lipid diets containing 4% olive, fish, flaxseed, or soybean oils, Rosa et al. (2012) found that fish oil improved the lipid profile and reduced inflammatory cytokines in Wistar rats (Rosa et al. 2012). Liu et al. (2018) has also showed that feeding rats with a diet containing 3% of fish oil down-regulated the expression of genes involved in eicosanoid synthesis and inflammation on pubertal mammary gland, and tumor development in rats (Liu et al. 2018).

During the process of inflammation, pro-resolving lipid mediators from n-3 PUFAs (EPA and DHA) metabolism, such as protectins, resolvins and maresins, are involved in the active resolution of inflammation (Christie & Harwood 2020, Dierge & Feron 2019). Those mediators exert anti-inflammatory actions limiting the recruitment of neutrophils to the site and promoting the macrophage switching from the M1 phenotype to a pro-resolving M2 phenotype (Moro et al. 2016, Serhan et al. 2015). On the other hand, n-6 PUFAs generates prostaglandins and leukotrienes, potent pro-inflammatory mediators (Dierge & Feron 2019, Serhan et al. 2015).

As found in the present study, high-density lipoprotein (HDL) is the main lipoprotein that transports cholesterol in mice (Kaabia et al. 2018). There is no similar reverse cholesterol transport pathway with minimal amounts of apoB-containing lipoproteins, compared to humans (Zhao et al. 2020). The high lipid demand by malignant cells is related to rapid growth and cell division (Gomaraschi 2020, Cruz et al. 2013) and supports cancer aggressiveness (Peck & Schulze 2019). Lipoproteins can favor tumor development and progression, providing cancer cells with cholesterol and fatty acids (Gomaraschi 2020). The present study evaluated the effect of dietary oils on the profile and lipoprotein composition and found similar profiles among groups with no changes in contents of cholesterol and triglycerides, showing that they did not modify the lipoprotein

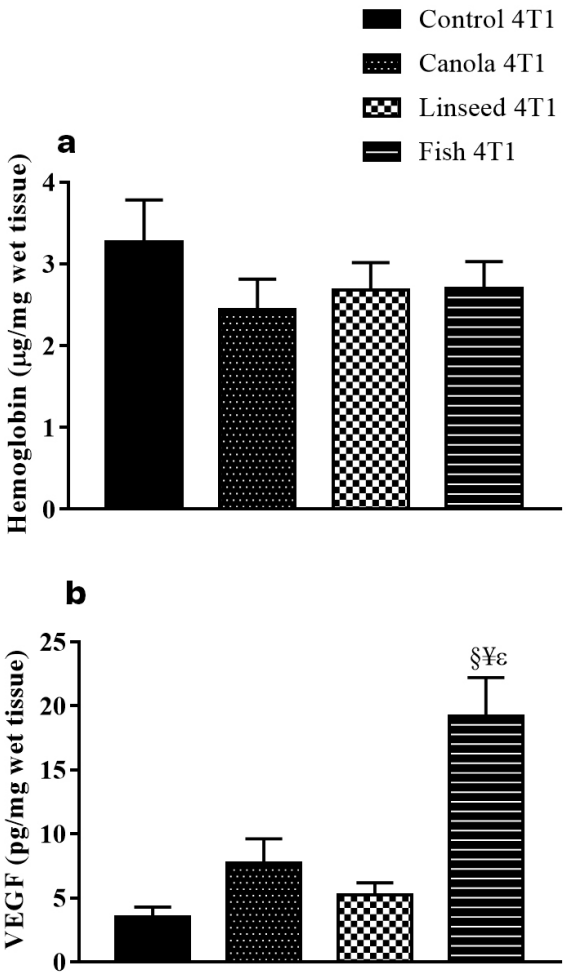

Figure 7. Effects of treatments on angiogenic markers of tumors. Hemoglobin content (a) and VEGF levels (b); Data represents means + S.E.M. of 7 animals per group. § P < 0.05 vs Control 4T1; ¥ P < 0.05 vs Canola 4T1; ε P < 0.05 vs Linseed 4T1; using one-way ANOVA followed by post hoc Tukey test.

metabolism and tumor development. On the other hand, studies suggest that lipoproteins' availability in the tumor microenvironment is likely more relevant than their circulating levels (Gomaraschi 2020).

The abnormal proliferation of cells resulting from dysregulation of the cell cycle plays a fundamental role in tumorigenesis. The antitumorigenic effects of n-3 PUFA are probably mediated by altering fatty acid composition in target tissues and modulating the expression of tumor proteins involved in cell proliferation (Liu et al. 2018). Although the mechanisms involved in the uncontrolled cell proliferation of breast tumors are not well understood, the increase in cell proliferation is a characteristic event of malignant neoplasms (Klopfleisch et al. 2011). The quantitative description of tumor cell proliferation can be used to predict the biological behavior of a particular neoplasm (Gambichler et al. 2008). The cell proliferation index can be determined by markers related to the cell cycle, with significant prognostic value in women and bitches with breast carcinomas (Araújo et al. 2016, Rakha et al. 2010). In the present study, animals in the Canola 4T1 group had a higher rate of cell proliferation when compared to the Control 4T1 group, however, this result did not lead to greater tumor growth or increased number of metastasis. This effect of canola oil in tumors could be associated to its high oleic acid (n-9 MUFA, C18:1) content (61.3%), as the literature points to a correlation between this fatty acid and invasion process and also with metastasis in breast cancer condition (Soto-Guzman et al. 2010). On the other hand, Cho et al. (2010) observed that canola oil inhibited the proliferation of cancer cells both *in vitro* and *in vivo*, but this effect was due to the association of this oil with tamoxifen and cerulenin, antineoplastic drugs (Cho et al. 2010).

Inflammatory cells have potent effects at different stages of tumor development, including initiation, promotion, malignant conversion, invasion, and metastasis (Grivennikov et al. 2010). At the beginning of the neoplastic process, these cells are powerful promoters, producing an attractive environment for tumor growth, facilitating and promoting genomic instability and angiogenesis. Subsequently, in the process of tumorigenesis, neoplastic cells also bypass inflammatory mechanisms, in order to favor

neoplastic dissemination and metastasis (Han et al. 2019).

The inflammatory component of the tumors was quantified by the specificity of myeloperoxidase (MPO) and n-acetyl-β-D-glucosaminidase (NAG) activities for the assessment of neutrophils or macrophages recruitment/activation, respectively, as described by others (Marques et al. 2011). Tumor cells and the surrounding microenvironment recruit neutrophils, which are under the influence of chemokines, cytokines, cell adhesion molecules, growth factors, prostaglandins, and leukotrienes (Khadge et al. 2020, Powell & Huttenlocher 2016, Fridlender & Albelda 2012). Neutrophils have very few mitochondria, and rely basically on anaerobic glycolysis. However, during tumor progression, they use diverse metabolic pathways, which involves oxidative phosphorylation, fatty acid oxidation, aerobic glycolysis, glutaminolysis, pentose phosphate pathway, and tricarboxylic acid (TCA) cycle to perform distinct functions (Hsu et al. 2020, Kumar & Dikshit 2019). Fatty acids are metabolized by TCA and oxidative phosphorylation, generating a higher amount of ATP molecules, and some studies reveal the important role of mitochondria and TCA cycle in neutrophil chemotaxis (Kumar & Dikshit 2019). N-3 PUFAs can upregulate PPAR-γ and stimulate mitochondrial oxidative phosphorylation (Khadge et al. 2020), modulating lipid oxidation, glucose metabolism and inflammation in both tumor cells (Grygiel-Górniak 2014) and neutrophils (Kumar & Dikshit 2019). In the present study, among other factors, we believe that the uptake of exogenous lipids (mainly EPA and DHA) in the tumor microenvironment of animals receiving fish oil enhanced the metabolic and functional reprogramming of tumor-associated neutrophils, characterized by increased oxidative metabolism. In this way, the neutrophils could conduct a positive feedback loop, by secreting chemoattractants to recruit more neutrophils into the tumor.

It is also important to discuss the different phenotypes of neutrophils that infiltrate the tumor, where they can change their function from a pro-tumor phenotype (N2) to an antitumor (N1) phenotype, depending on the tumor-derived factors (Hsu et al. 2020, Kumar & Dikshit 2019, Mackey et al. 2019, Fridlender & Albelda 2012). Thus, the microenvironment seems to play a defining role in the neutrophil diversity (Kumar & Dikshit 2019). One question that needs to be answered is the type of neutrophil that is infiltrating the tumor of animals in the Fish 4T1 group. This gap shows the need to develop more investigations focused on discriminating the phenotypic differentiation of neutrophils and their functional role in this experimental model.

At the tumor site, activated macrophages are recruited, thus suffering a decrease in their immune function and an exacerbation of their trophic function. Elevated macrophage densities in neoplastic tissue have been linked to a worse prognosis, as macrophages are involved in chronic inflammation processes and appear to be associated with tumor progression and metastasis (Nasrollahzadeh et al. 2020). Tumor-associated macrophages (TAMs) accumulate in hypoxic regions of tumors and upregulate VEGF and other proangiogenic factors, such as FGF-2 (fibroblast growth factor-2) and CXCL8 (a member of the CXC chemokine family), as well as glycolytic enzymes, in response to hypoxia (Ribatti 2017). In our study, animals from the Fish 4T1 group showed a reduction in N-acetylglucosaminidase activity in relation to the Canola 4T1 and the Linseed 4T1 groups, indicating that fish oil may be a possible therapeutic strategy to inhibit TAMs from suppressing tumor development (Chen et al. 2019). However, the mechanisms used for that need to be studied more deeply.

Considering that neutrophils in inflammatory environments recruit macrophages and that recruited macrophages affect neutrophil functions (Kim & Bae 2016), it is possible that the fish oil has changed this close link between these components of the immune system in the same tumor microenvironment, which could be confirmed by the most significant MPO/NAG ratio showed by the Fish 4T1 group. In summary, our results corroborates previous observations that fish oil has important anti-tumoral activity.

TNF-α is the most important pro-inflammatory cytokine involved in the growth, differentiation, cellular function, and the survival of many cells. It is produced by several types of cells, including macrophages, neutrophils, fibroblasts, keratinocytes and tumor cells, and has been implicated in the development and progression of the tumor (Yang et al. 2011). In the present study, the concentration of TNF-α at the tumor site was not associated with the types of fatty acids or the n-3 / n-6 ratio present in dietary oils, although some studies have shown possible role of fatty acids in regulating the production of TNF-α in tumors and pathological processes of angiogenesis (Gorjao et al. 2019, Liu et al. 2018, Weylandt et al. 2011).

The growth of tumors requires an increased supply of intratumor blood, triggered by tumor hypoxia, being one of the mechanisms of angiogenesis promotion, tumor progression and aggressiveness (Magalhães & Dias 2019, Muz et al. 2015). Several molecules have shown to play an important role in angiogenesis, including VEGF (Ribatti 2017, Kang & Liu 2013) and hemoglobin (Marques et al. 2011). Interestingly, the Fish 4T1 group showed an increase in VEGF levels in this study, without modifying the hemoglobin content (vascular index) in tumor tissue, as found by Marques et al. (2011) and Lima et al. (2014), who studied an animal model of chronic inflammation. The authors discussed that the VEGF production varied among mice strains and that a high level of VEGF was not associated with the most pronounced angiogenesis response induced by hypoxia (Lima et al. 2014, Marques et al. 2011).

In this sense, it is important to discuss the possibility of other isoforms of VEGF with antiangiogenic properties being synthesized in the group fed on fish oil, and that could exert an antitumor activity (Eswarappa & Fox 2015). This same experimental group also presented a higher TAN density into the tumor, which has been associated with better therapeutic response and better prognosis according to some authors (Masucci et al. 2019, Galdiero et al. 2016). Therefore, it is very likely that fish oil exerted actions modulating the inflammatory response that led to the structuring of a "benign" environment within the tumor.

Taking into account the evaluation of the inflammatory components of the tumors, the present work opens new perspectives on carrying out other analysis using flow cytometry or high content image system to permit better characterization of the immune cells into the tumor microenvironment. The use of RT–PCR to study VEGF isoforms and the analysis of fatty acid composition, eicosanoids, ROS production, growth factors, cytokines and chemokines levels could also be a valid goal for future studies.

CONCLUSION

The findings of this work suggest that the type of fatty acid and the high ratio n-3 / n-6 PUFAs in the diet impact cell proliferation and inflammation in the tumor site in a different way, highlighting the increase of neutrophils recruitment and VEGF levels in the Fish 4T1 group. Additional studies considering the inflammatory, oxidative and metabolic components within the tumors would further confirm the results presented.

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NICOLLE CAMILLA R. DA SILVA[1]
https://orcid.org/0000-0003-4592-0508

YASMIM DE O.B. SILVA[2]
https://orcid.org/0000-0003-3522-7750

JACQUELINE A. TAKAHASHI[3]
https://orcid.org/0000-0002-8831-1609

SILVIA P. ANDRADE[4]
https://orcid.org/0000-0001-5059-2807

GEOVANNI D. CASSALI[5]
https://orcid.org/0000-0002-5650-6743

DIRCE R. DE OLIVEIRA[6]
https://orcid.org/0000-0003-4592-0508

[1]GSM Mineração Ltda., Rodovia MG 030, 8625, Torre 3, 8º andar, B., Vale do Sereno, 34012-970 Nova Lima, MG, Brazil

[2]Santa Casa de Misericórdia de Belo Horizonte, Av. Francisco Sales, 1111, B. Santa Efigênia, 30150-221 Belo Horizonte, MG, Brazil

[3]Universidade Federal de Minas Gerais, Instituto de Ciências Exatas, Departmento de Química, Av. Pres. Antônio Carlos, 6627, Pampulha, 31270-901 Belo Horizonte, MG Brazil

[4]Universidade Federal de Minas Gerais, Instituto de Ciências Biológicas, Departmento de Fisiologia e Biofísica, Av. Pres. Antônio Carlos, 6627, Pampulha, Caixa Postal 468, 31270-901 Belo Horizonte, MG, Brazil

[5]Universidade Federal de Minas Gerais, Instituto de Ciências Biológicas, Departmento de Patologia Geral, Laboratório de Patologia Comparada, Av. Pres. Antônio Carlos, 6627, Pampulha, Caixa Postal 468, 31270-901 Belo Horizonte, MG, Brazil

[6]Universidade Federal de Juiz de Fora, Departmento de Ciências Básicas da Vida, Campus Governador Valadares, Rua São Paulo, 745, Centro, 35010-180 Governador Valadares, MG, Brazil

Correspondence to: **Dirce Ribeiro de Oliveira**
E-mail: dirce.oliveira@ufjf.br

Author contributions

Nicolle Camilla R. da Silva and Yasmim de O.B. Silva performed the experiments. Jacqueline A. Takahashi and Silvia P. Andrade performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, and reviewed drafts of the paper. Geovanni D. Cassali and Dirce R. Oliveira conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, and reviewed drafts of the paper.