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Omega-3 fatty acid supplementation attenuates intestinal mucositis and tumor growth in a murine model of breast cancer

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ABSTRACT

The potential use of Omega-3 fatty acid for its anti-inflammatory properties has been proposed to alleviate the side effects of cancer therapeutics. Fifty female BALB/c mice were randomly assigned to five treatment groups. Mice were injected with Murine 4 T1 breast cancer cells, and 5-fluorouracil (5-FU) to induce mucositis. The mice were provided control or chow supplemented with Omega-3 fatty acid (fish oil with 15 % EPA and 7 % DHA, respectively). Our results showed that, the use of Omega-3 prevented intestinal mucositis by reducing intestinal permeability and restoring histological parameters. Additionally, Omega-3 supplementation enhanced the antineoplastic effect of 5-FU, as evidenced by a greater reduction in tumor growth compared to the other groups. Furthermore, the combined administration of 5-FU and Omega-3 significantly reduced the formation of lung metastasis. Collectively, these findings suggest that Omega-3 supplementation, particularly in conjunction with 5-FU, may contribute to the treatment of cancer by decreasing induced intestinal mucositis.

1. Introduction

According to the World Health Organization (WHO), about 19 million new cancer cases are diagnosed yearly (Sung et al., 2021) with breast cancer being amongst the most prevalent in women. Breast cancer treatment may include surgical resection, immunotherapy, chemotherapy, and radiotherapy (INCA-BRASIL, 2019). These last two approaches might cause side effects such as dysphagia, pain, nausea, vomiting, diarrhea, and mucositis impairing the nutrition status and quality of life of the patients (Ariyawardana et al., 2019; Mercadante et al., 2015). Intestinal mucositis (IM) is observed in 40–100 % of patients and can lead to increased intestinal permeability (IP) and bacterial translocation, enhancing the risk of sepsis (Dahlgren et al., 2021; Sougiannis et al., 2021). All these factors may increase the morbidity and mortality of patients (Dahlgren et al., 2021).

Our group is interested in finding alternative approaches that improve the patients intestinal health following chemotherapy. Currently the treatment strategies for IM remain limited. Laser therapy is effective for oral lesions but leaves the gut leasions vulnerable (Elad et al., 2020). One strategy to overcome this clinical challenge is through the dietary supplementation with Omega-3 fatty acids due to its anti-inflammatory properties that have already been reported in intestinal bowel disease and some side effects of cancer (Gorjao et al., 2019; Morsy et al., 2023; Wawrzyniak et al., 2021). There are two main families of polyunsaturated fatty acids (PUFA), that are relevant to human health, as they are not synthesized in animal metabolism yet are considered

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essential fatty acids. The first is linoleic acid or omega-6 (18:2-W6), which is more abundant in Western diets, and the second is alpha-linolenic acid (18:3-W3), which is consumed in smaller quantities (Djuricic & Calder, 2021). Both fatty acids are found in the phospholipids of cell membranes, influencing fluidity, flexibility, and inflammatory signaling pathways(Freitas & Campos, 2019; Huang et al., 2017). They compete for the same desaturase enzymes to form the final metabolites, icosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). They are both final products of Omega-3 metabolism and can release resolvins, protectins, and maresins, which improve the inflammatory process (Calder, 2017; Serhan, 2014). Furthermore, PUFAs can produce several signaling molecules called pro-resolvin lipid mediators (Ishihara et al., 2019). These molecules can decrease oxidative stress and inflammatory cytokines produced by tumor cells, reducing deleterious effects observed in cancer (D'Eliseo & Velotti, 2016; Li et al., 2017; Song et al., 2019).

Previous data from our research group confirmed the beneficial effects of Omega-3 fatty acid (Omega-3 FA) supplementation in the experimental model of IM induced by the chemotherapy drug 5-fluorouracil. The results showed that Omega-3 prevented weight loss, reduced the increase in IP resulting from IM, and increased intestinal cell proliferation (Generoso et al., 2015). However, to date, it is unknown whether the contribution of the tumor burden would alter the action of Omega-3 FA on IM and whether the interaction between these fatty acids and chemotherapy would affect tumor growth and metastasis (Cechinel-Zanchett et al., 2019; Generoso et al., 2015). Therefore, the present study aims to evaluate the effect of Omega-3 FA (fish oil with 15 % and 7 % of EPA and DHA respectively) supplementation in a murine model of breast cancer (4 T1) on both intestinal mucositis and tumor progression. We hypothesize that Omega-3 could act as an agonist to chemotherapy, improving both the antitumor efficiency while concurrently reducing intestinal mucositis.

2. Material and methods

2.1. Animals

Female BALB/c AnNCrl mice (N = 50), aged 6–8 weeks (18–22 g), were obtained from "Universidade Federal de Minas Gerais's animal facility (CEBIO/UFMG)". Mice were kept in a light and dark environment with controlled temperature, with free access to food and water. All studies were approved by the "Ethics Committee on the use of animals from UFMG (CEUA/UFMG)" under protocol number 158/2018 (approval date: 29/04/2019). Hence, the standards proposed by the *Animal Research: Reporting In Vivo Experiments*- ARRIVE Guidelines and the Guide for the Care and Use for Laboratory Animals were followed (du Sert et al., 2020; National Research Council (U.S.), 2011).

2.2. Cell culture

Murine breast cancer (4 T1) cells were grown in Dulbecco's modified Eagle's medium (D-MEM; Gibco, Waltham, Massachusetts, USA), supplemented with 10 % (v/v) fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml). Cells were maintained in humidified air containing 5 % CO_2 at 37 °C. For tumor initiation studies, aliquots (100 μL) containing 1.0×10^6 4 T1 cells in Phosphate Buffer Saline (PBS) were injected subcutaneously into the right flank of female BALB/c mice. Tumor cells were allowed to grow *in vivo* for 10 days(Fernandes et al., 2018). Tumor-bearing BALB/c mice were used for further *in vivo* studies (de Oliveira Silva et al., 2023; Fernandes et al., 2022).

2.3. Diet

The control standard diet was based on the AIN-93G diet (Reeves et al., 1993) that contains 7 % of soy oil as lipids. The Omega-3 FA diet was developed as previously described. The total amount of lipids was divided into two parts, 50 % (35 g/kg of chow) of soy oil and 50 % (35 g/kg of chow) of fish oil. The content of EPA and DHA in the Omega-3 FA diet was previously analyzed (15.2 g of EPA and 7.0 g of DHA per 100 g of chow). The new formulation is isocaloric compared to AIN93G (Generoso et al., 2015). The animals were fed with the respective diets throughout the experiment and were provided fresh food and water every 2 days.

2.4. Experimental design

Fifty mice were randomly assigned into five groups (N = 10): 1. Control (CTL), fed standard chow diet and non-tumor bearing; 2. Control – tumor bearing (CTLTU), fed standard chow diet; 3. Tumor bearing plus Omega-3FA supplement (TUW3); 4. Tumor bearing plus 5-FU induced mucositis (TU5FU), fed standard chow diet; and 5. Tumor bearing plus 5-FU induced mucositis plus Omega-3FA supplement (TU5FUW3).

As illustrated in Fig. 1, all groups received a standard chow diet from day 1 to 10. Food consumption and animals weight were checked every two days. On Day 1, murine breast cancer 4 T1 cells were injected into the right flank of the mice within treatment groups 2–5 (1×10^6cell/animal)(de Oliveira Silva et al., 2023). Once the tumor was palpable, (day 10) the animals of groups TU5FU and TU5FUW3 started receiving the Omega-3 FA supplemented diet for an additional 10 consecutive days, while the other animals were maintained on the standard chow diet (Nunes et al., 2022). On the 17th day, animals from groups TU5FU and TU5FUW3 received an intraperitoneal injection of 5-FU (300 mg/kg) to induce IM, while the other animals received an injection of saline solution (Trindade et al., 2018). After 72 h, all animals were anesthetized with intraperitoneal administration of ketamine (80 mg/kg) and xylazine (10 mg/kg), and a blood sample was collected. Subsequently, the mice were euthanized for collection of liver, intestine, lung and tumor tissues.

2.5. Analysis of intestinal integrity

2.5.1. Intestinal permeability

To determine intestinal permeability, we measured the absorption of a radiopharmaceutical from the intestine to the blood using an automated gamma counter (Wallac Assistant 1470–020 Gamma Counter; PerkinElmer, Waltham, MA, EUA). Prior to sacrifice (20th day) mice received, by gavage, an aliquot of 0.1 ml (18.5 MBq) of dietilenetriaminepentactic acid labeled with technetium–99 m (^{99m}Tc-DTPA). After 4 h, mice were anesthetized, and 300 μL of blood was collected and placed into appropriate tubes for radioactivity determination. The data are expressed as % dose, using the following equation (Generoso et al., 2011; Trindade et al., 2021):

$$\%bloodDoses = [(cpmofbloodX100)/cpmadministereddoses]$$

$$^{*}cpm = countsperminute$$

2.5.2. Histopathological analysis

Following euthanasia, sections of the ileum, tumor, and lung of all animals were removed for histopathological analysis. The intestines were opened longitudinally, and the luminal content was gently removed and washed with phosphate buffer saline (PBS). The tissue was rolled up to form '*Swiss rolls*' and fixed for 24 h in 4 % buffered formaldehyde. The material was processed for paraffin embedding, and 4 μm thick slices of each sample were prepared and stained with hematoxylin and eosin (H&E). The intestinal samples were coded and then scored by a trained pathologist who was blinded to the treatment modalities, and classified according to the histopathological grading proposed by Soares et al (Soares et al., 2008). The histological score was presented on a scale of 0–3, where 0 represents normal histological findings; score 1 signals of villi shortening, loss of crypt architecture, inflammatory cell infiltration, vacuolization in the intestinal mucosa and the normal muscle

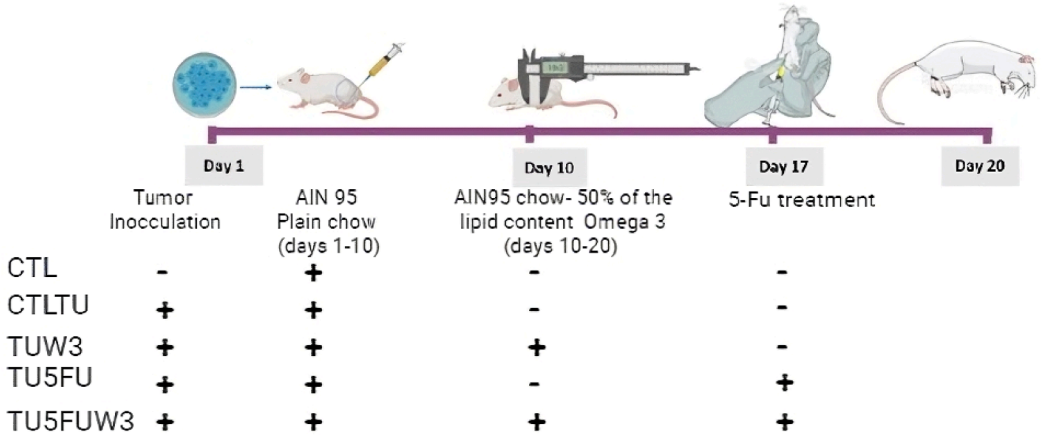

Fig. 1. Experimental Design. Female BALB/c mice were randomly assigned to into 5 groups (N = 10). On day 1, animals from CTLTU, TUW3, TU5FU, and TU5FUW3 were injected with breast cancer cells on their right flank, while animals from CTL group received saline solution (non-tumor bearing controls). All animals were fed with standard diet chow (AIN93) for ten days. On day 10, the tumor volume was measured and groupsTU5FUW3 and TUW3 groups began Omega-3FA supplementation. On the 17th day, animals from groups TU5FU and TU5FUW3 received the IP 5FU injection, while animals from CTL, CTLTU, and TUW3 received the saline solution. On day 20th, animals were anesthetized and euthanized. Groups: CTL- Control, CTLTU- Tumor Control, TUW3 – Tumor + Omega-3 FA diet, TU5FU – Tumor + 5-FU, TU5FUW3 – Tumor + 5FU + Omega-3 FA diet. Figure was created with Biorender™.

layer; score 2, in addition to the shortened villi, there was the presence of vacuolated cells and crypt necrosis. Finally, score 3 represented villus shortening, increase in crypt depth, intense inflammatory cell, infiltration in the villi, lamina propria, and submucosa, ulceration, edema, vacuolization, and a decrease in goblet cells. For morphometric analysis, images of ten independent fields of each sample were obtained with a 40 × magnification objective. The villus height (from villus tip to villus-crypt junction) and crypt depth (defined as the depth of invagination between adjacent villus) were analyzed and classified by light microscopy using a calibrated micrometer (Soares et al., 2008).

2.5.3. mRNA expression of tight junction proteins

The mRNA expression for tight junction proteins zonula occludens-1 (ZO-1) and occludin were measured using real-time PCR, as previously described (Andrade et al., 2019). The total RNA from the ileum was extracted using the Trizol™ reagent according to the manufacturer's protocol. All samples were analyzed and standardized according to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. The values were expressed as the number of amplified genes as compared to the control group ($2^{-\Delta\Delta CT}$). The PCR results were analyzed using the StepOne™ Software v2.2.2 (Applied Biosystems, CA, USA). The sequences of the primers used were as follows: ZO-1: 5′CC AGCTTATGA AG GGTTGTTC3′ and 5′TCCTCTCTTGCCAACTTTTCTC3′, Occludin: 5′ATGTCC GGCCGA TGCTCTC3′ and 5′TTTGCTGCTCTTG GGTCTGT AT3′, GAPDH: 5′CTCA AGATTGTCAGCAA TGC3′ and 5′CAGGATGCCCTTTAG TGGGC3′.

2.6. Analysis of tumor development

2.6.1. Tumor volume measurement

Tumor dimensions were measured every two days using a caliper (Digimess™, model 100.170) starting on day 10 until the end of the experiment (D20) along with the animals' total body weight. The tumor volume (V) was calculated by the ellipsoidal adapted equation, where d1 and d2 are the smaller and larger diameter of the tumor, respectively

$$V = (d_1)^2 \times d_2 \times 0.5$$

To determine the Relative Tumor Volume (RTV), on day 20th (D20), the total volume was divided by the initial volume (D10), as the following equation(Fernandes et al., 2022):

RTV = relative tumor volume D20 / relative tumor volume D10.

In addition, to calculate the inhibition rate (IR), the following equation was employed:

$$IR = [100 - (RTVgroup/controlgroup - CTLTU) \times 100].$$

2.6.2. Immunohistochemistry analysis

Immunohistochemical analyses were performed for the expression of caspase 3 (apoptosis) and CDC47 (cell proliferation) in the tumor tissues. Negative control sections were assessed in parallel in the absence of immune rabbit serum. The tissues were incubated with the primary antibody for 60 min, and peroxidase activity was assessed using diaminobenzidine (DAB- Dako). For CDC47, the marker expression was obtained by estimating the percentage of positive cells per 500 mucosal cells. Caspase-3 expression was determined by counting the number of positive cells in 10 fields, thoroughly enclosing the histological section with a 40 × objective (Fernandes et al., 2018).

2.6.3. Metastasis analysis

Lung metastasis is well documented in the murine 4 T1 breast cancer model(Pulaski & Ostrand-Rosenberg, 2001). Lung sections were fixed in formalin (10 % w/v in phosphate-buffered saline (PBS), pH 7.4) and incorporated in paraffin blocks. Tissues were evaluated for the formation of distal metastasis. All specimens were evaluated by a pathologist and were classified according to the number of neoplasia foci (de Oliveira Silva et al., 2019; Yang et al., 2020).

2.6.4. Evaluation of cytokine expression in the tumor

The mRNA expression for inflammatory cytokines in tumor samples was measured using real-time PCR, as previously described by Andrade et al.(2015). The total RNA from the tumors was extracted using the Trizol™ reagent according to the manufacturer's protocol. All samples were analyzed and standardized according to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. The values were expressed as the number of amplified genes as compared to the control group ($2^{-\Delta\Delta CT}$). The PCR results were analyzed using the StepOne™ Software v2.2.2 (Applied Biosystems, CA, USA). The sequences of the primers used were as follows: **TNF**: 5′-GAG ATA GCA AAT CGG CTG ACG-3′, 5′CGT CGT AGC AAA CCA AG-3; **IL6**: 5′-CAC GAT TTC CCA GAG AAC ATG TG-3′, 5′ACA ACC ACG GCC TTC CCT ACT T-3′, **IL10**: GGT TGC CAA GCC TTA TCG GA, ACC TGC TCC ACT GCC TTG CT, **IL1b** GCA ACT GTT CCT GAA CTC AACT, ATC TTT TGG GGT CCG TCA ACT.

2.7. Statistical analysis

Statistical analyses were performed using *GraphPad Prism* 5.0 software (GraphPad Software, La Jolla, CA), and the data were evaluated for normal distribution using the ShapiroWilk normality test. Parametric data were analyzed using one-way analysis of variance (ANOVA) followed by *Newman-Keuls* multiple comparison test. For non-parametric data, the *Kruskal-Wallis* and *Dunn's* post-test was used. The level of significance was set at $p < 0.05$. Power analysis for sample size was calculated according to the formulas we have used for previous works on our group for intestinal permeability measurements ($n = 1 + [2C^*(s/d)2]$) (Andrade,et al.,). For c, we used the formula: $C = (z\alpha + z\beta)2$. A power analysis of 90 % with a significant level of 0.05, and a maximum deviation of 0.3 (30 %) was employed. The expected difference between the groups was (d) de 0.5 (50 %). The result was = 8.56 but we used 10 per treatment group.

3. Results

3.1. Supplementation with the Omega-3 FA diet alleviates IM effects on the gut and protects the mucosal barrier

We evaluated the effect of Omega-3 FA supplementation on food consumption and total body weight changes on the mice throughout the study. Fig. 2A shows that all treatment groups except the controls (CTL and CTLT) displayed a decrease in their food consumption that was measurable on Day 17. In contrast, mice within the IM induced treatment groups (TU5FU and TU5FUW3) showed significant weight loss when compared to those within the CTL, CTLTU, and TUW3 groups on the last day of investigation (Fig. 2A, $P < 0.05$). Fig. 2B shows the changes in food consumption along the experimental days.

Changes in intestinal mucosal health were evaluated by immunohistochemistry. Fig. 3A-E represent the histopathological analysis and Fig. 3F shows the corresponding histological score classified by Soares Score (Soares et al., 2008). Damage to the mucosal lining was not observed in the following treatment groups: CTL, CTLTU, and TUW3 ($p > 0.05$). This was anticipated given the animals did not receive 5FU to induce mucositis. However, damage was observed in the intestines of the TU5FU treatment group (Fig. 3D, 3F). Specifically, mice in this group displayed villus shortening, discrete infiltration of inflammatory cells in the basal layer, and flattened or vacuolated cells when compared to the other groups ($p < 0.05$). Conversely, supplementation with Omega-3 FA was protective. Animals within the TU5FUW3 treatment group (Fig. 3E, 3F), showed maintenance in the architecture of the villi, in addition to the absence of inflammatory infiltrate and vacuolated cells when compared to the TUW3 group ($p < 0.05$) (Fig. 3C). Consistent with these results, we observed a decreased in the villi/crypt ratio in TU5FU group with compared to TU5FUW3 (Fig. 3G, 3H, 3I, $p < 0.05$).

The intestinal disruption due to IM was evaluated by measurement of intestinal permeability (IP). IP was evaluated on day 20th, exactly 3 days following mucositis induction by 5FU treatment (Fig. 4A). Data showed the IP of CTLTU and TU5FU were increased compared to the CTL group ($p < 0.05$). In contrast, the treatment groups supplemented with Omega-3 FA (TUW3 and TU5FUW3) showed reduced IP when compared to the animals in groups CTLTU and TU5FU ($p < 0.05$) (Fig. 4A).

Changes in the expression levels of some proteins within the tight junctions of the intestine are hallmarks of damage to the intestinal mucosa. Using mRNA analysis, we evaluated changes in zonula occludens-1 (ZO-1) and occludin. ZO-1 mRNA expression levels were decreased in the intestinal tissue of animals in the TU5FU group when compared to the CTL group ($p < 0.05$). However, the TU5FUW3 treatment group showed a significant increase in ZO-1 mRNA expression when compared to the other groups ($p < 0.05$) (Fig. 4B). There were no statistically significant differences among the CTL, CTLTU, TUW3, and TU5FU groups ($p > 0.05$) in the analysis of the mRNA expression of the tight junction protein occludin, (Fig. 4C). However, the occludin protein expression increased in animals from the TU5FUW3 treatment group (Fig. 4C) ($p < 0.05$).

3.2. Supplementation with Omega-3 FA enhanced the antineoplastic effect of 5-FU

We evaluated the impact of omega-3 supplementation on tumor burden. CDC47 is a marker for tumor cell proliferation. Pathological evaluation of CD47 expression in tumors was represented in Fig. 5A-D. As predicted, the expression levels of CD47 within the CTLTU treatment group showed a higher percentage of proliferating tumor cells compared to the other groups ($p < 0.05$) (Fig. 5E). Markers of proliferation within the tumors from the treatment groups TUW3, TU5FU, and TU5FUW3 did not show significant statistical differences (Fig. 5E) ($p > 0.05$).

Analysis of the relative tumor volume showed a reduction in the tumor size in the TUFU group compared to the CTLTU group (19.23 %) ($p < 0.05$) (Fig. 5F). Interestingly, the TU5FUW3 treatment group had a significant reduction in the tumoral volume when compared to the other groups (38.12 %) ($p < 0.05$) (Fig. 5F). There was no statistical difference between the tumor volume in the treatment groups, TUW3 to CTLTU ($p > 0.05$) (Fig. 5F).

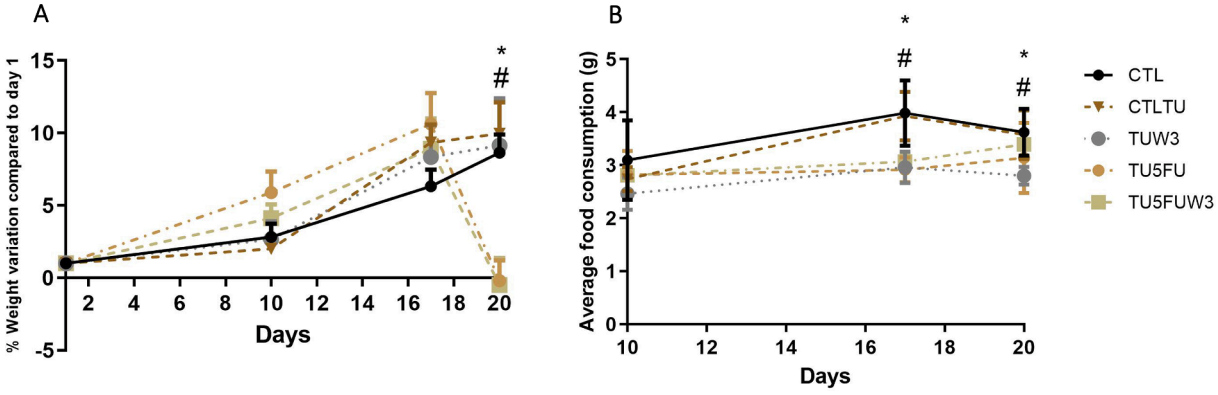

Fig. 2. Effect of Omega-3 FA supplementation on food consumption and changes in total body weight. (A) Weight variation for the animals were compared to day 1. Changes in weight are expressed as the mean ± SEM (ANOVA One-Way and post-test *Neuman-Keuls* for multiple comparisons). **(B)** Differences in the average food consumption (g) throughout the experiment was compared to day 1. Data are expressed as the median (*Kruskal-Wallis* and post-test *Dunn* for multiple comparisons). Symbols # and * represent the statistical difference among the groups. Day 17th ($p < 0,05$). #: CTLTU ≠ TUW3, TU5FU, TU5FUW3 and *: CTL ≠ TUW3, TU5FU, TU5FUW3. Day 20th ($p < 0,05$). #: CTLTU ≠ TUW3, TU5FU and *: CTL ≠ TUW3, TU5FU. N = 10 Groups: CTL- Control, CTLTU- Tumor Control, TUW3 – Tumor + Omega-3 FA diet, TU5FU – Tumor + 5-FU, TU5FUW3 – Tumor + 5FU + Omega-3 FA diet.

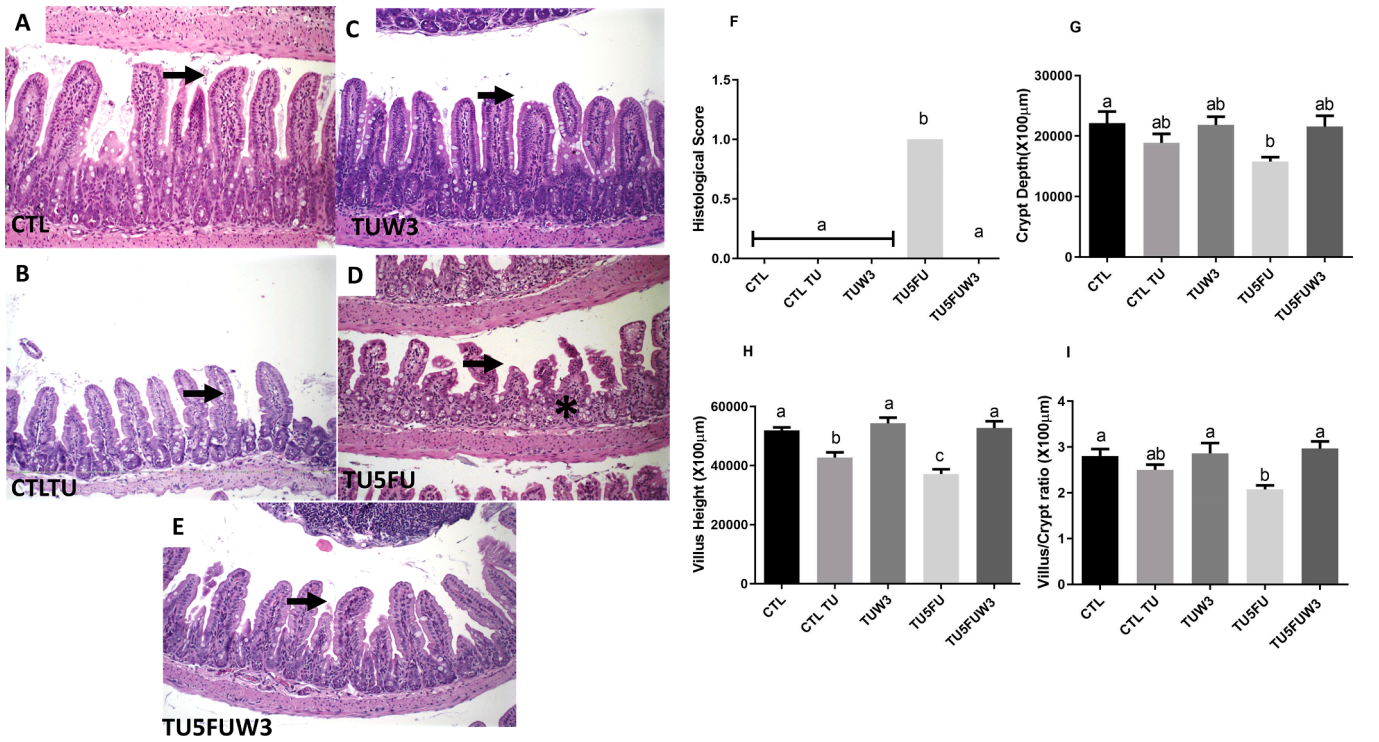

Fig. 3. Omega-3 FA supplementation decreased gut damage due to 5-FU. (A-E) Histopathological analysis of the ileum 72 h after mucositis induction. The arrows represent the height of the villi and the star represents the infiltrate of inflammatory cells in the basal layer. HE staining, 40x magnification. **A**)CTL, **(B)** CTLTU, (**C**) TUW3, **(D)** TU5FU and (**E**) TU5FUW3. **(F)** Histological Score after 72 h of mucositis induction. Data expressed in mean ± SEM (*Kruskal-Wallis* and post-test *Dunn* for multiple comparisons). **(G)** Cript Dept- Data expressed in mean mean ± SEM (*ANOVA One-Way* and post-test *Neuman-Keuls* for multiple comparisons). **(H)** Vili Height - Data expressed as the mean ± SEM (*ANOVA One-Way* and post-test *Neuman-Keuls* for multiple comparisons). **(I)** Vili/cripta ratio. Data was expressed as the mean ± SEM (*ANOVA One-Way* and post-test *Neuman-Keuls* for multiple comparisons). N = 10. Groups: CTL- Control, CTLTU- Tumor Control, TUW3 – Tumor + Omega-3 FA diet, TU5FU – Tumor + 5-FU, TU5FUW3 – Tumor + 5FU + Omega-3 FA diet.

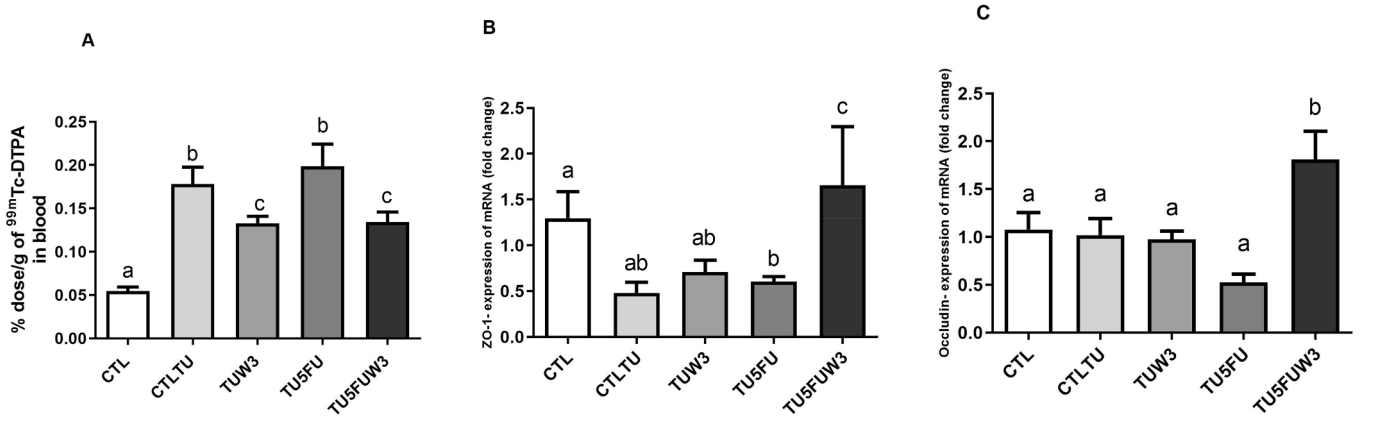

Fig. 4. Omega-3 FA improves intestinal permeability and tight junction expression. (A) Analysis of intestinal permeability 72 h after mucositis induction. Data expressed as the mean ± SEM (*Kruskal-Wallis* and post-test *Dunn* for multiple comparisons). **(B)** Tight junction analysis- ZO-1 **(C)**. Tight junction analysis- Occludin. For both analyses, data was expressed as the mean ± SEM (*ANOVA One-Way* and post-test *Neuman-Keuls* for multiple comparisons). For all results, different letters indicate the statistical difference. N = 10. Groups: CTL- Control, CTLTU- Tumor Control, TUW3 – Tumor + Omega-3 FA diet, TU5FU – Tumor + 5-FU, TU5FUW3 – Tumor + 5FU + Omega-3 FA diet.

Metastasis in the lung tissue is characteristic of 4 T1 breast cancer models. Metastatic foci were quantified in the lung tissue of each animal (Fig. 7A-D). There were no significant differences between the CTLTU, TUW3, and TU5FU treatment groups ($p > 0.05$) (Fig. 7E). Interestingly, the TU5FUW3 treatment group showed a significant reduction of lung metastasis when compared to the CTLTU group ($p < 0.05$) (Fig. 7E).

Finally, mRNA analysis was used to assess changes in cytokine expression levels within the tumor tissue (Fig. 6A- 6D). All treatment groups showed a reduction in tumoral IL-1β cytokine expression (Fig. 6A) when compared to the CTLTU group ($p < 0.05$). Increased expression of IL-10, IL-6, and TNF-α (Fig. 5B, 5C, and 5D, respectively) was limited to the TU5FU treatment groups when compared to the other groups ($p < 0.05$).

4. Discussion

The use of Omega-3 fatty acids due to its anti-inflammatory action has been proposed as an alternative strategy for the prevention of

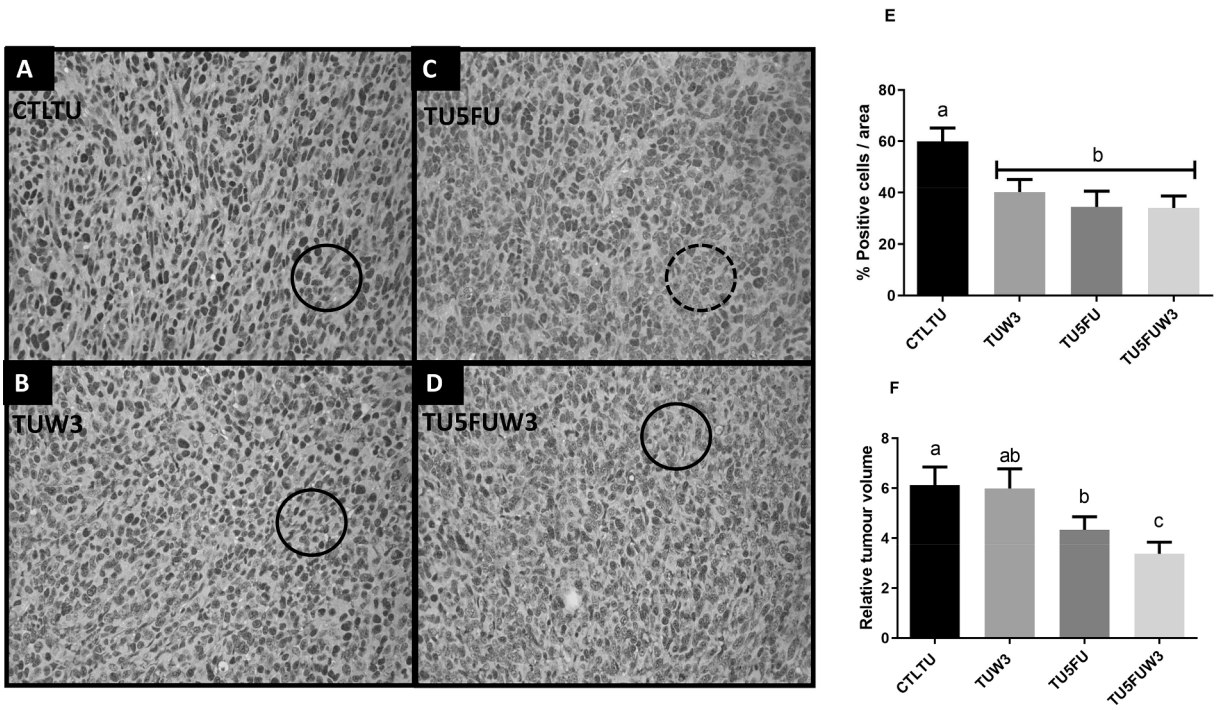

Fig. 5. Omega-3 FA supplementation effects on tumor cell proliferation and tumor volume.(A-D) Immunohistochemistry evaluation of the cell proliferation marker CDC47 in tumor sections. Harri's Haematoxilin Staining. Positive cells are stained in brown meanwhile, negative cells are in blue. Representative positive and negative cells indicated by a continuous and dotted circles, respectively. **A)** CTLTU, (**B**) TUW3, **(C)** TU5FU and (**D**) TU5FUW3. **(E)** Graphic representation of the % positive cells/ area. Data are expressed as the mean ± SEM (*ANOVA One-Way* e post-test *Neuman-Keuls* for multiple comparisons). **(F)** Relative Tumoral Volume was calculated between days 10 and 20. Data was expressed as the mean ± SEM *ANOVA One-Way* e post-test *Neuman-Keuls* for multiple comparisons). Different letters indicate a statistical difference. N = 10. Groups: CTL- Control, CTLTU- Tumor Control, TUW3 – Tumor + Omega-3 FA diet, TU5FU – Tumor + 5-FU, TU5FUW3 – Tumor + 5FU + Omega-3 FA diet.

intestinal diseases and side effects of cancer chemotherapy (Morsy et al., 2023; Song et al., 2019). Based on these observations, our research group conducted a study with Omega-3 FA supplementation on IM induction by 5-FU previously. Our results showed that Omega-3 FA could prevent weight loss and decrease intestinal damage due to IM (Generoso et al., 2015). However, the effect of this supplementation in the context of tumor burden has not yet been evaluated. Thus, the present work explored the effects of Omega-3 FA (fish oil with 15 % and 7 % of EPA and DHA, respectively) on 5-FU-induced IM associated with a breast cancer tumor model in mice. Our results showed that the use of Omega-3 FA was able to prevent intestinal damage caused by chemotherapy and acted to enhance the antineoplastic effect of 5-FU. Specifically our data showed that Omega-3 FA supplementation led to a reduction in tumor volume, cell proliferation, and lung metastases *in vivo*.

Chemotherapy-induced mucositis contributes to weight loss and can be observed within 72 h after treatment (Generoso et al., 2015; Soares et al., 2008; Trindade et al., 2018). In the present study, Omega-3 FA supplementation did not alter weight loss and decreased food consumption in tumor-bearing animals with or without chemotherapy treatment. These results should be interpreted with caution since the tumor mass interferes with the animal's final weight (Hajjaji, et al., 2012). Nevertheless, these changes can be justified by the metabolic alterations caused by the tumor, such as cachexia, which contributes to weight loss (Talbert and Guttridge, 2022). In addition, chemotherapy also can decrease appetite/food consumption due to intestinal damage caused by mucositis, which leads to a decrease in villi, crypts, nutrient absorption capacity and also contributes to weight loss (Sougiannis et al., 2021).

In this context, when evaluating intestinal histological alterations and the intestinal barrier, we observed that the TU5FU treatment group had intestinal lesions with a higher histological score, as well as increased IP and lower expression of tight junction proteins ($p < 0.05$). Collectively, these alterations are attributed to the accumulation of reactive oxygen species (ROS) and the production of pro-inflammatory cytokines, such as interleukin-1b (IL-1b), IL-6, tumor necrosis factor-a (TNF-a) and Nuclear factor k-B (NFk- B) in the intestine due the 5-FU action (Chang et al., 2012). Interestingly, the tumor bearing control group (CTLTU) also showed an increase in IP similar to the TU5FU group. This finding reinforces the fact that the tumor's inflammatory process can favor an increase in IP (Bischoff et al., 2014) independent of chemotherapy treatment. However, the histological findings of the animals in the TU5FUW3 treatment group showed that omega-3 supplementation prevented IM alterations in tumor-bearing animals that received chemotherapy ($p < 0.05$). This beneficial effect was also observed in IP and tight junctions since an increase in ZO-1 tight junction and occludin gene expression was observed in this treatment group. Similar findings have been reported that showed a reduction in IP after Omega-3 FA supplementation in animals with 5-FU-induced mucositis (Generoso et al., 2015). A study conducted by Rodrigues et al. (2023) evaluated the acute effect of diets enriched with saturated and unsaturated fatty acids. The results showed that the consumption of a diet enriched with Omega-3 fatty acids had a slight effect on protecting the intestines against inflammation leading to an increase in the expression of tight junctions (Rodrigues et al., 2023; Hashemipour, 2024). On a molecular level, it has been suggested that the incorporation of Omega-3 fatty acids into the enterocyte membrane can promote the release of anti-inflammatory mediators, which may be associated with the effects of recovery of the gut epithelium as part of inflammation resolution processes (Kimura et al., 2019; Serhan, 2014). Specifically, it has been postulated that DHA plays a role in the modulation of the tight junction proteins, ROS production, and inflammatory cytokines secretion (Beguin et al., 2013; Vargas-Robles et al., 2019; Zhao et al., 2015) which leads to a decreased IP. Complementarily, some clinical trials are in agreement with these preclinical experimental data and support that

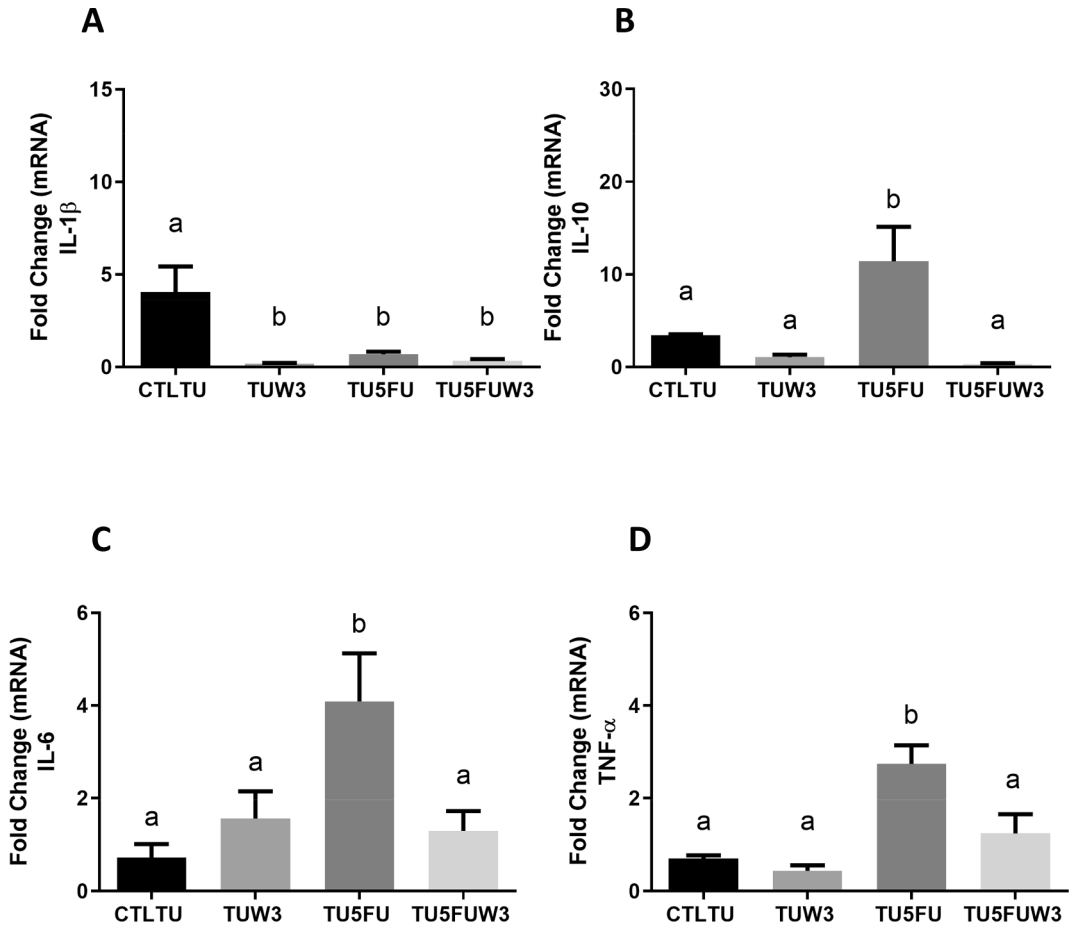

Fig. 6. Tumor cytokine expression levels. (A - D) The fold change (mRNA) of cytokine's expression within the tumor, IL-1β, IL-10, IL-6, and TNF- α, respectively. Data are expressed as the mean ± SEM. (*ANOVA One-Way* e post-test *Neuman-Keuls* for multiple comparisons). Different letters indicate a statistical difference (n = 10). Groups: CTL- Control, CTLTU- Tumor Control, TUW3 – Tumor + Omega-3 FA diet, TU5FU – Tumor + 5-FU, TU5FUW3 – Tumor + 5FU + Omega-3 FA diet.

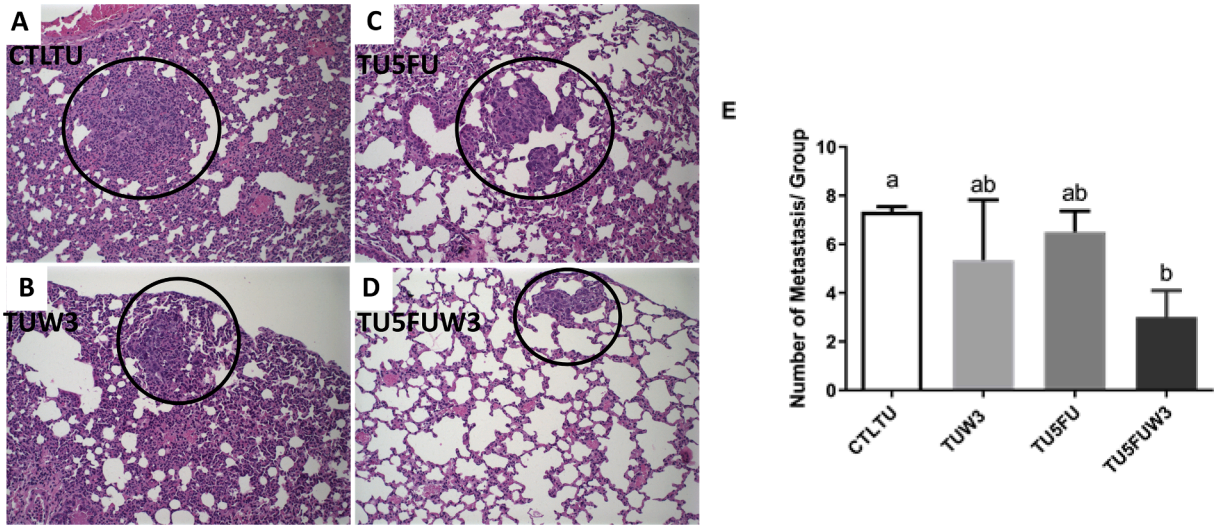

Fig. 7. Tumor metastasis to lung tissue (A-D) Lung photomicrographs were evaluated for the presence of metastasis after treatment. (A) CTLTU, (B) TUW3, (C) TU5FU, and (D) TU5FUW3. HE staining of lung tissue is represented at 40X magnification. Representative areas of metastasis are demarcated by circles. **(E)** Quantification of metastases. Data are expressed as the mean ± SEM (*ANOVA One-Way* e post-test *Neuman-Keuls* for multiple comparisons). Different letters indicate a statistical difference (n = 10). Groups: CTL- Control, CTLTU- Tumor Control, TUW3 – Tumor + Omega-3 FA diet, TU5FU – Tumor + 5-FU, TU5FUW3 – Tumor + 5FU + Omega-3 FA diet.

Omega-3 fatty acids supplementation could alleviate mucositis during cancer therapy, improving the quality of life of patients (de van der Schueren et al., 2018; Hashemipour et al., 2017; Mizumachi et al., 2019). This contributes to recognizing the safety of using Omega-3 fatty acids supplementation as an adjuvant in cancer therapy.

The tumor's inflammatory microenvironment plays an important role in growth, invasiveness, and resistance to therapy (Grivennikov & Karin, 2011). Therefore, the use of imunutrients such as Omega-3 fatty acids, can have an impact on tumor growth, sensitizing cancer cells to chemotherapy and increasing the resistance of normal cells to toxic effects (Laviano et al., 2013). Therefore, our data is consistent with the benefits of chemotherapy combined with Omega-3 fatty acids supplementation. Here we show a significant reduction in the proliferative index of tumor cells, both in immunohistochemical assays and in tumor volume. Others have showed the synergistic effect of Omega-3 fatty acids associated with doxorubicin on breast cancer cells (MCF-7), inhibiting cell proliferation and invasion (Gurav et al., 2024). We hypothesize that this adjuvant effect of Omega-3 FA in contributing to reduced cell proliferation and decreased tumor volume may be explained by omega-3′s ability to increase the sensitivity of cancer cells to chemotherapy, reducing chemoresistance (Colas et al., 2005; Fernandes et al., 2018). Some studies have shown that Omega-3 fatty acids supplementation can lead to tumor reduction by decreasing cancer cell proliferation and activating the apoptotic pathway (Chénais & Blanckaert, 2012; D'Eliseo & Velotti, 2016).

The impact of Omega-3 FA supplementation on tumor burden and metastatic potential was evaluated in our study. The literature shows that the murine 4 T1 breast cancer model spontaneously metastasizes to the lungs (Yang et al., 2020). Our results showed a reduction in tumor metastasis after consuming a Omega-3 FA diet in combination with the chemotherapy drug 5-FU. Corroborating our findings, a study by Saraswoti Khadge (2018) showed a reduction in tumor metastasis to different organs, including the lungs, which was related to a lower inflammatory index in the tumor due to Omega-3 fatty acids anti-inflammatory effects (Khadge et al., 2018). In addition, chronic inflammation can often lead to the development of tumors by providing bioactive molecules from cells that infiltrate the tumor microenvironment, including growth factors, cytokines, and chemokines, and by promoting immune evasion (Li et al., 2017). Specifically it was demonstrated that increasing Omega-3 fatty acids decreases systemic lymphocyte proliferation, interleukin (IL) 1β, INF-α, and IL-6 (Hashemipour, 2017). Our data showed that animals in the TU5FU treatment group showed an increase in mRNA expression of the inflammatory cytokines IL-6 and TNF, as well as the anti-inflammatory cytokine IL-10. In our study, omega-3 supplementation was able to reverse this increase. Interestingly, IL-10 is a regulatory cytokine of the immune system and its role in tumorigenesis remains controversial (Zhou et al., 2023). We propose that the increase or decrease in this cytokine is related to the stimulus for the anti-inflammatory response since the TU5FU treatment group showed a worsening in histological parameters, intestinal barrier, and tumor burden. It has been shown that tumor cells produce cytokines, improving inflammation and the resistance of cancer cells to antitumor therapies(Esquivel-Velázquez et al., 2015; Grivennikov & Karin, 2011). In this case, the increase in IL-6 and TNF may favor the increase in IL-10 to repress pro-inflammatory responses and limit unnecessary tissue disruption caused by inflammation (Ouyang et al., 2011).

5. Conclusion

Our finding suggests that supplementation with Omega-3 FA (fish oil with 15.2 % and 7.0 % of EPA e DHA, respectively) in combination with 5-FU chemotherapy treatment reduces tumor volume, cell proliferation, and lung metastases in a breast tumor model (4 T1). Importantly, Omega-3 FA supplementation was able to blunt 5-FU induced intestinal mucositis via regulating the immune response as shown by alterations in inflammatory cytokine release. Further studies are needed to clarify the mechanism of action of Omega-3 FA and extend to other cancer models, prior to clinical trials.

Data available statement

All data generated and analyzed during this study are included in this published article. The datasets generated during the current study are available from the corresponding author upon reasonable request.

Ethical statement

All studies were approved by the "Ethics Committee on the use of animals from UFMG (CEUA/UFMG)" under protocol number 158/2018 (approval date: 29/04/2019). Hence, the standards proposed by the *Animal Research: Reporting In Vivo Experiments*- ARRIVE Guidelines and the Guide for the Care and Use for Laboratory Animals were followed.

CRediT authorship contribution statement

Aline Luiza A. Souza: Writing – original draft, Validation, Resources, Methodology, Formal analysis, Data curation. **Luísa Martins Trindade:** Writing – review & editing, Investigation, Data curation. **Amanda Dias Borges:** Writing – review & editing, Formal analysis. **Paola Caroline Lacerda Leocadio:** Writing – review & editing, Formal analysis, Data curation. **Juliana de Oliveira Silva:** Formal analysis. **Renata Salgado Fernandes:** Resources, Formal analysis. **Jaqueline Isaura Alvarez Leite:** . **Geovanni Dantas Cassali:** Resources, Formal analysis. **Diego Carlos dos Reis:** Formal analysis. **Tatiani Uceli Maioli:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Valbert Nascimento Cardoso:** Resources, Project administration, Funding acquisition, Conceptualization. **Danyelle M. Townsend:** Writing – review & editing. **André Luis Branco de Barros:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Simone de Vasconcelos Generoso:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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