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Original article

Fish oil decreases the severity of treatment-related adverse events in gastrointestinal cancer patients undergoing chemotherapy: A randomized, placebo-controlled, triple-blind clinical trial

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SUMMARY

Background and aims: Due to its high peroxidizable characteristics, n-3 fatty acids, present in fish oil, could increase tumor cells sensitivity to conventional cancer treatment while non-neoplastic cells remain unaffected, this may lead to an increase in cancer treatment response with no increase on adverse effects. The aim of this study was to evaluate anti-cancer treatment response, performance status and adverse events in gastrointestinal cancer patients supplemented with fish oil. Oxidative stress parameters were investigated in blood non-neoplastic cells as an indicator of cytotoxicity. Methods: This is a randomized, triple-blind, placebo-controlled clinical trial. Fish oil group (FOG) received two capsules of fish oil containing 1.55 g of EPA + DHA a day for nine weeks, placebo group (PG) received two capsules containing olive oil. Baseline was set right before the administration of the first chemotherapy, oxidative stress parameters, adverse events presence and grading and performance status were assessed at baseline and after nine weeks of supplementation. Tumor markers, response to treatment and survival were evaluated at baseline and after one year of study inclusion. Results: 76 patients were considered eligible, 56 were randomized, and 51 remained for analysis. After nine weeks, although there were no differences between groups for treatment response and presence of adverse events, PG patients were graded with more severe diarrhea than FOG patients ($p = 0.03$) and with higher (worse) performance status score ($p = 0.02$). No differences in lipid peroxidation and activity of antioxidant enzymes were observed between groups. Conclusions: Fish oil may lead to a better performance status for gastrointestinal cancer patients undergoing chemotherapy while does not seem to increase treatment-related toxicity. Registered under ClinicalTrials.gov Identifier no. NCT02699047, www.clinicaltrials.gov.

1. Introduction

Gastrointestinal cancers, represented by tumors of esophagus, stomach or colon and rectum, are among the most common cancers worldwide [1]. Conventional treatment for these types of diseases consists of therapies such as surgery, chemotherapy, and radiation [2]. However, currently approved treatments for these tumors result in only modest improvement in overall survival (OS),

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especially when dealing with advanced diseases [3]. Development of new therapeutic strategies with higher efficacy or new approaches for treatment are encouraged and needed [3,4].

Direct or indirect reactive oxygen species generation is one of the mechanisms of action behind the majority of chemotherapeutics used in gastrointestinal cancer treatment to induce neoplastic cell death [5]. Increased cellular oxidative stress, characterized by a higher concentration of reactive oxygen species, favors the formation of toxic compounds derived from a process called lipid peroxidation [6,7]. N-3 polyunsaturated fatty acids, found in fish oil (mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)), when ingested are incorporated into cells membranes, independently if cancerous or not. These fatty acids, have a higher number of double bonds and are highly peroxidizable. Therefore, n-3 fatty acids are more susceptible to reactive oxygen species and the onset of lipid peroxidation [8].

Studies show that cancerous cells exposed to n-3 fatty acids (or tumors of animals supplemented with) presented a higher production of compounds derived from lipid peroxidation and, as a consequence, increased susceptibility to cell death or tumor size reduction [9,10]. In addition, when supplementation occurs concomitantly with treatment, it seems to induce synergistic or additive effects, increasing death induction in some cell lines [11–13].

Besides lipid peroxidation, other mechanisms mediated by n-3 fatty acids are shown to enhance the efficacy of anti-cancer drugs. Such mechanisms include improvement of drug uptake and modulation of intracellular targets including cyclooxygenase-2, nuclear factor kappa B, peroxisome proliferator-activated receptor gamma, mitogen-activated protein kinase, AKT, and BCL-2/BAX [14].

However, although n-3 fatty acids demonstrate the capacity to amplify chemotherapy effects, they seem to have “a selective” effect. “Selective sensitization” was the term attributed to the observation of distinct effects when exposing different cell types (e.g., malignant and non-malignant) to n-3 fatty acids. This hypothesis suggests that n-3 fatty acids do not increase treatment sensitivity (e.g., side-effects) in healthy cells. This difference is possibly related to cells' oxidative stress response, which is more consistent and efficient in healthy cells/tissues when compared to neoplastic cells/tumors [8]. Such observation might suggest that n-3 fatty acids can be used as adjuvants to improve the efficacy of anti-cancer treatments without increasing adverse effects, as observed in two studies performed in human subjects [15,16].

This is the first randomized, triple-blind, placebo-controlled clinical trial to evaluate treatment response and adverse effects after combining anti-cancer agents and fish oil supplementation. In addition, this clinical trial also evaluates the effect of fish oil on oxidative stress of non-neoplastic cells during anticancer treatment. We hypothesize that fish oil will increase treatment response without increasing adverse events and lipid peroxidation on healthy cells.

2. Materials and methods

This is a randomized, triple-blind, placebo-controlled clinical trial, registered at ClinicalTrials.gov (NCT02699047) and Brazilian Register of Clinical Trials (RBR-2XCD99).

2.1. Patients

Patients with lower gastrointestinal cancer (stomach, intestine, rectum, and anal canal) assisted at a cancer-specialized public hospital and outpatient clinic (Oncology Research Center - Florianópolis, Santa Catarina, Brazil) between March 1st, 2015 and March 31st, 2016 were enrolled in this study. Eligibility criteria were: age between 18 and 70 years, no previous chemo or radiotherapy treatment, Eastern Cooperative Oncology Group (ECOG) performance status [17] of less than or equal to 2 and a prescription for chemotherapy initiation. Exclusion criteria were: diagnosis of inflammatory or infectious disease; Diabetes Mellitus; pregnancy; inability to take capsules orally; patients in palliative care; allergy to the dietary supplements used; use of statins; chronic use of anti-inflammatory drugs or use on the day prior to baseline assessment; use of polyunsaturated fatty acids supplements in the six months prior to inclusion; continuous use of supplements containing antioxidants and cognitive limitations to understand the study protocol and answer the questions asked.

2.2. Study design

Eligible patients were randomized into two groups: experimental group, which received two capsules per day of fish oil containing 1.55 g/day of EPA and DHA (Omega 3 Concentrate®, Tiaraju, Rio Grande do Sul, Brazil) or control group, which received 2 capsules/day of placebo supplement containing extra-virgin olive oil (Cápsulas de oliveira®, Tiaraju, Santo Ângelo, Rio Grande do Sul, Brazil), in addition to the habitual diet. Supplementation started on the same day of the first chemotherapy and continued for nine weeks (Fig. 1). Patients were instructed to ingest capsules right before a substantial meal, both at the same time or divided into two meals. The nutritional information of the supplements is presented in Table 1.

Fish oil was extracted from sardines, anchovies, and mackerel and fatty acids are presented in the form of ethyl ester. The placebo consisted of pure extra-virgin olive oil. Olive oil was used because it has no detectable traces of DHA nor EPA. Patients who received radiotherapy in addition to chemotherapy were not excluded, but the analysis of oxidative stress parameters was also performed separately. The study protocol was carried out in accordance with the ethical standards of the World Medical Association's Declaration of Helsinki and was reviewed and approved by the Ethics Review Board of the Hospital. Written informed consent was obtained from each patient prior to participation in the trial.

2.3. Randomization and allocation concealment

Sequence generation (1:1) was created using the computer software STATA® version 13.0 (StataCorp LP, College Station, Texas, USA) and was stratified by gender. Three sequences were generated in accordance with the cancer type and with the incidence prospection at the hospital, to evenly distribute tumor types (colorectal, gastric and other cancers) and males or females within groups. One researcher not involved in the study codified the name of the groups using different numeric codes (eight digits each) from a table containing random numbers. No code was repeated. These codes were used to identify the supplements by the same researcher. Randomization was planned to provide a proportion of 1:1 in both strata (male or female). Researchers responsible for recruitment had access to the list with the codified randomization sequence, but it was not possible to predict to which group the patient would be allocated.

2.4. Blinding

Fish oil and olive oil were provided in soft gel capsules with the same appearance and color. Both supplements were stored in an equal plastic white bottles identified with a numeric code (the same of the randomization sequence list). The patients and their caregivers, medical staff and researchers involved in the recruitment, data collection, and analysis (biochemical and statistical analysis) were blinded. To minimize the residual taste that could

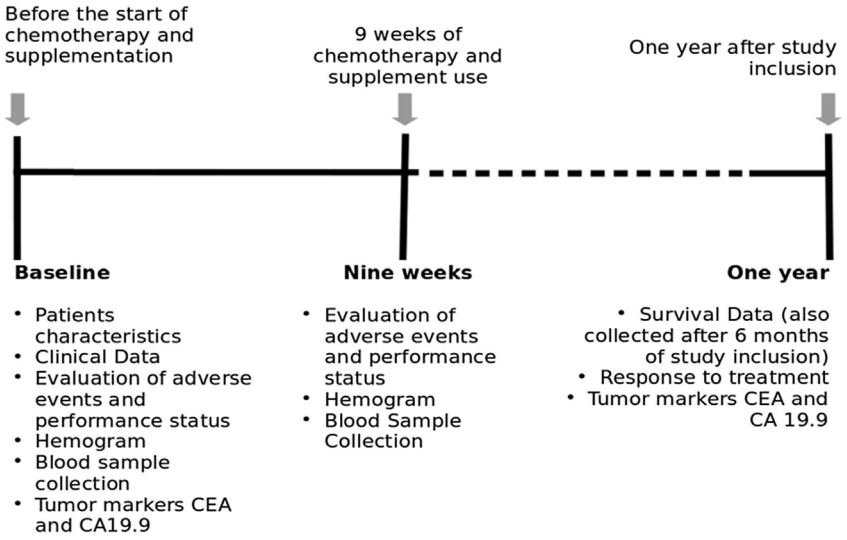

Fig. 1. Study timeline.

compromise patient blinding, we instructed the consumption of the supplements just before any main meal. After the end of nine weeks, we asked some of the patients if they knew what group they were assigned to. Some subjects in the placebo group (6 of 20, 30%) reported perceiving a fishy flavor as some in the fish oil group did not notice this.

2.5. Data collection

Clinical data (tumor type and stage, antineoplastic treatment protocol, associated comorbidities) and date of birth were obtained from patient's charts. Tobacco use, medications and body weight change in the past six months were reported by the patient. All outcomes were collected before the intravenous chemotherapy session (baseline) and after nine weeks of treatment and supplementation, except for tumor markers, response to treatment and survival, which were recorded at baseline, 6 months (for survival) and one year after study inclusion.

2.6. Nutritional status

Weight, height, mid-upper arm circumference and triceps skinfold were measured by a nutritionist with experience in anthropometry and standardized for these measures, according to the techniques proposed by WHO [18]. An electronic platform scale with a coupled vertical stadiometer (Toledo®, Toledo Company of Brazil, São Paulo, Brazil; scale: 100 g and 0.5 cm), an inelastic tape measure and a skinfold compass (Lange Skinfold Caliper®, Beta Technology, Santa Cruz, California, USA; scale: 1 mm) were used.

Table 1
Nutritional composition of supplements used.

	Olive Oil- 2 g (2 capsules)	Fish oil – 2.5 g (2 capsules)
Calories (kcal)	20	26
Protein (g)	0.6	0.7
Total Fat (g)	2.0	2.50
Monounsaturated Fat	1.4	–
Oleic Acid (mg)	1100	0
Polyunsaturated Fat (mg)	200	1500
Docosahexaenoic Acid – DHA (mg)	0	550
Eicosapentaenoic Acid – EPA (mg)	0	1000

Information provided by the producer (Tiarajú, Santo Ângelo, Rio Grande do Sul, Brazil).

2.7. Presence and grading of treatment-related adverse events

Toxicity to chemotherapy treatment was graded using Common Terminology Criteria for Adverse Events v 4.0 (CTCAE) provided by the National Cancer Institute of United States of America. Hematological and gastrointestinal toxicities most commonly associated with treatments used were graded at baseline and after nine weeks of treatment based on patients report and blood tests. Grades range from 0 (no symptoms) to 5 (death). Treatment dose reduction, discontinuation, and delay due to toxicities, as well as hospitalization and death at any given moment for 9 weeks, were also recorded.

2.8. Performance status, response to treatment and survival

As an indication of prognosis, Eastern Cooperative Oncology Group (ECOG) performance status [17] was evaluated at baseline and after nine weeks. After one year since study initiation, data on response to treatment (disease progression, relapse and complete response to treatment) was collected on the patient's charts. Also, six months and one-year-survival were recorded. After 27 months of study initiation (June 2017), we collected data on survival and follow-up time again. Tumor markers CEA (ng/ml) and CA 19.9 (U/ml) were obtained in patient's charts. Baseline values were those right before the initiation of chemotherapy; final values were the ones closest to the date marking one year of patient's study inclusion.

2.9. Blood collection

Venous blood samples were collected into tubes containing lithium-heparin as anti-coagulant. Blood was centrifuged, plasma was collected, leukocytes and erythrocytes were isolated, and all were frozen immediately at −80 °C for further analysis. The lysate obtained from erythrocytes was used for antioxidant enzymes activities assays.

2.10. Erythrocyte catalase activity

This assay was adapted from the procedures described by Aebi [19]. Data were expressed as units normalized by hemoglobin concentration.

2.11. Erythrocyte Superoxide Dismutase activity

SOD activity was determined by the ability of the enzyme to inhibit the autoxidation of pyrogallol [20]. The principle of this method is based on the competition between the pyrogallol autoxidation by $O2\bullet^{-}$ and the dismutation of this radical by SOD. SOD activities are expressed as units/mg of hemoglobin. One unit of SOD activity is defined as the amount of enzyme required to cause 50% inhibition of pyrogallol autoxidation.

2.12. Erythrocyte Glutathione Peroxidase activity

GPx activity was measured by monitoring the oxidation of β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt (NADPH) in the presence of hydrogen peroxide, as previously described by Wendel [21] and the specific activity was represented as U/mg hemoglobin.

2.13. Plasma and leukocyte lipid hydroperoxides

The method described by Nourooz-Zadeh et al. [22] was used. The plasma was homogenized with methanol and then centrifuged. The supernatant was transferred to six wells of a 96-well plate, in 3 of these wells was added triphenylphosphine (TPP) and only methanol in the remaining three. The samples were incubated for 30 min and then added a FOX-2 solution and incubated again. The absorbance was read at 550 nm. The value of absorbance of the samples treated with TPP was subtracted from the absorbance value of the samples without TPP, a standard curve was made with known concentrations of hydrogen peroxide (H1009 Hydrogen Peroxide Solution, Sigma–Aldrich, St Louis, MO, USA). Total lipids were measured according to Cheng, Zheng and Vandergheynst [23] and the lipid hydroperoxides data normalized to this variable. The results were expressed in nmol/mg of lipids.

2.14. Erythrocyte fatty acid analysis

The erythrocyte fatty acid profile was determined by HPLC according to the method described by Nishiyama-Naruke et al. [24]. Cells fatty acid constituents of phospholipids, triacylglycerols, cholesterol esters and free fatty acids were extracted using chloroform: methanol (2 : 1, v/v), adapting the method described by Folch et al. [25]. Fatty acids were derivatized with 4-bromomethyl-7-coumarin and acetonitrile as described by Abushufa et al. [26] and separated on a reversed phase analytical column (Discovery BIO Wide Pore, C8, 5 microns particles, 250 9 4.6 mm; Supelco/Sigma–Aldrich, St Louis, MO, USA). The chromatographic analysis was performed with an Alliance BIO Separation Module e2796 (Waters Corp., Milford, MA, USA). Compounds were detected by fluorescence detection (Waters 2475 Multi k Fluorescence Detector; Waters Corp.). Chromatographic data were recorded and integrated into EMPOWER PRO, version 2.0 (Waters Corp.). The fatty acids investigated were: DHA, EPA, arachidonic, stearic, oleic, linoleic, a-linolenic, palmitic, myristic and lauric. Data are expressed as a percentage of total fatty acids.

2.15. Statistical analysis

Determination of data symmetry was based on Shapiro–Wilk test results and analyses of the coefficient of variation. Quantitative variables are presented as median and interquartile and average ± standard deviation according to symmetry. Categorical variables are presented in categories and frequencies. Mann–Whitney or Student's t-test were used to compare groups and Wilcoxon matched-pairs test to compare moments in the same group. For categorical variables, Chi-squared test, Chi-squared with Yates correction and Fisher's Exact Test were used according to data distribution. ANCOVA was performed to test differences in continuous variables between groups using baseline data as a covariate, for these analyses asymmetrical data was transformed into a natural logarithm. For the survival data, we performed a Kaplan–Meier survival estimate using the log-rank test to compare differences between groups. Differences were considered statistically significant at $p < 0.05$. For effect sizes of non-parametric tests, results on eta-squared ($\eta 2$) were transformed to Cohen's d, effect sizes of 0.2 were considered small, 0.5 were considered medium, and 0.8 were considered large. Statistical analyses were performed using STATA® statistical software version 11.0 for Windows (StataCorp, Texas, USA).

3. Results

During the recruitment period, 76 patients were considered eligible for the study. Of these, six patients denied participation, while researchers were unable to contact 14 individuals before they underwent the first chemotherapy session. A total of 56 patients were randomized into two study groups, 28 on placebo group and 28 on fish oil group. Two patients withdrew from the placebo group; both did not want to attend the evaluations anymore. Two on fish oil group changed their treatment to another clinic, and one patient in fish oil group started using an immunomodulatory nutritional supplement enriched with n-3 fatty acids. Therefore, it was excluded from the analysis (Fig. 2). For the remaining 51 patients, characteristics are presented in Table 2.

Both study groups were similar regarding baseline characteristics, with no significant difference in sex, age, tumor location, disease stage, performance status, treatment protocol, smoking habit and nutritional status. The most common diagnosis was colon cancer, in 29 patients (56.86%), and the most used chemotherapy protocol was oxaliplatin plus capecitabine. Although patients in both groups presented weight loss in the six months prior to study inclusion, at baseline, both groups were at overweight classification

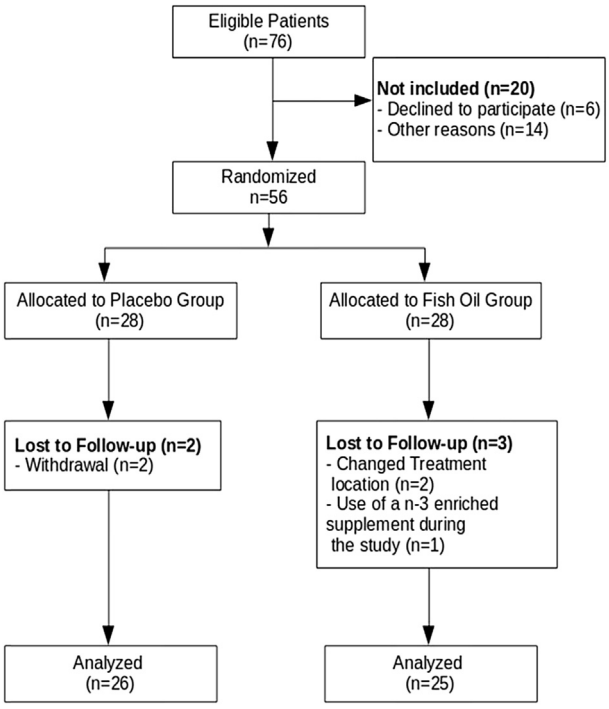

Fig. 2. Flowchart of participants.

Table 2
Patients characteristics at baseline (part 1).

	Placebo Group (n = 26)	Fish Oil Group (n = 25)	p
Sex (n)			0.49[a]
Female	11 (42.31%)	13 (52.00%)	
Male	15 (57.69%)	12 (48.00%)	
Age (years)	51 [41; 60]	58 [46; 63]	0.12[f]
Tumor Location			0.91[a]
Gastric	6 (23.08%)	5 (20.00%)	
Colon	14 (53.85%)	15 (60.00%)	
Rectum/sigmoid	6 (23.08%)	5 (20.00%)	
Disease Stage[g]			0.69[a]
2	6 (24.00%)	5 (20.00%)	
3	9 (36.00%)	12 (48.00%)	
4	10 (40.00%)	8 (32.00%)	
Performance Status			>0.05[b]
0	10 (38.46%)	12 (48.00%)	
1	11 (42.31%)	11 (44.00%)	
2	5 (19.23%)	2 (8.00%)	
Comorbidities[h]			0.41[d]
Yes	9 (34.62%)	6 (24.00%)	
No	17 (65.38%)	19 (76.00%)	
Chemotherapy Treatment			>0.05[c]
Oxaliplatin + capecitabine	11 (42.31%)	12 (48.00%)	
Oxaliplatin + fluorouracil	6 (23.08%)	2 (8.00%)	
Fluorouracil + leucovorin	5 (19.23%)	5 (20.00%)	
Others[i]	4 (15.38%)	6 (24.00%)	
Radiotherapy			0.41[c]
Yes	4 (15.38%)	2 (8.00%)	
No	22 (84.62%)	23 (92.00%)	
Smoking Habit			0.19[a]
Never Smoked	8 (30.77%)	14 (56.00%)	
Ex-Smoker	8 (30.77%)	5 (20.00%)	
Smoker and Passive Smoker	10 (38.46%)	6 (240.00%)	
Nutritional Status			
Body mass index (Kg/m^2)	25.56 (±4.16)	26.46 (±4.60)	0.47[e]
% Weight Loss	11.34 (±12.88)	7.23 (±8.73)	0.19[e]
Mid-Upper Arm circumference adequacy (%)	93.54 (±13.15)	92.17 (±12.81)	0.71[e]
Triceps Skinfold adequacy (%)	116.78 (±53.08)	105.67 (±43.32)	0.47[f]
Mid-arm muscle circumference adequacy (%)	90.90 (±9.72)	91.25 (±9.79)	0.84[f]

Data presented as median [interquartile range] for age, average (±standard deviation) for nutritional status variables and absolute frequencies (relative frequencies) for other variables.
[a] Chi-squared.
[b] Chi-squared and Fischer's Exact Test, categories were tested as two at once.
[c] Fischer's Exact Test, categories were tested as two at once.
[d] Chi-squared, with Yates correction.
[e] T test.
[f] Mann-Whitney Test.
[g] For one individual, there was no record of disease stage.
[h] Related comorbidities: Systemic arterial hypertension (n = 11), hypothyroidism (n = 4), pre-diabetes (n = 1), osteoarthritis (n = 1), heart murmurs (n = 1) e arrhythmia (n = 1).
[i] Irinotecan + cisplatin; oxaliplatin + capecitabine + bevacizumab; epirrubicin + cisplatin; cisplatin + capecitabine; folfiri + cetuximab.

according to BMI. Patients presented similar values of skinfolds adequacy, indicating eutrophy except for triceps skinfold which also indicated overweight.

3.1. Presence and grading of treatment-related adverse events

There were no differences between groups at baseline for any of the investigated adverse events. After nine weeks of treatment, the most common gastrointestinal adverse events were nausea (40.4%), anorexia (34%) and diarrhea (28.8%). Regarding hematological adverse events, after nine weeks, only one patient presented grade higher than 2 for lymphocyte count reduction. Anemia, neutrophil, and lymphocyte count reductions were the most common events, although each only had 4 (8.6%) individuals. There were no differences between groups for the presence of adverse events after nine weeks of treatment. However, diarrhea grades were different between groups. Of the six patients that presented this adverse event in the placebo group, five were graded 2 or 3. As for fish oil group, of the eight patients that presented diarrhea after nine weeks, only one received grade 2, none received grade 3 and 7 were classified as grade 1 and this was statistically significant ($p = 0.03$). For the other gastrointestinal and hematological adverse events, there were no differences related to grading after nine weeks (Supplementary Tables 1 and 2).

Adverse events consequences and outcomes were monitored during supplementation period of nine weeks and are presented in Table 3. Chemotherapy administration delay, interruption and dose reduction due to adverse events occurred in both groups with no statistically significant differences. In each group, one individual died because of treatment-related adverse events. Hospitalization caused by adverse events also occurred in both groups, but in the placebo group, six individuals were hospitalized compared to one subject in fish oil group, and this difference was statistically significant ($p = 0.048$). However, considering the fact that the p-value was very close to not being significant, we decided to perform a stratified analysis to identify possible confounding factors. As a result, the crude analysis overestimated the true association between hospitalization and fish oil intervention due to the greater

Table 3
Adverse Events Consequences during supplementation for each study group.

	Placebo Group (n = 26)	Fish Oil Group (n = 25)	p	RR	95% CI	p
Chemotherapy Administration Delay	5 (19,23%)	10 (40.00%)	0.10[a]	2.08	0.83–5.23	0.12
Chemotherapy Interruption	3 (11.54%)	2 (8.00%)	0.67[b]	0.69	0.13–3.81	0.67
Chemotherapy dose reduction	1 (3.85%)	3 (12.00%)	0.28[b]	3.12	0.35–28.03	0.31
Hospitalization	6 (23.08%)	1 (4.00%)	0.048[b]	0.17	0.02–1.34	0.09
Death	1 (3.85%)	1 (4.00%)	0.98[b]	1.04	0.07–15.74	0.98

RR = Relative Risk; CI= Confidence Interval.
Data presented as absolute frequencies (relative frequencies).
[a] Chi-squared Test.
[b] Chi-squared Test, with Fisher correction.

proportion of individuals with higher performance status grades in the placebo group at baseline. Therefore we did not consider this result to be associated with the intervention alone.

3.2. Performance status, response to treatment and survival

Performance status was evaluated at baseline in order to verify if groups had the same indication of prognosis related to this parameter. No differences were found between groups (Table 2). After nine weeks, there were also no differences in presence or absence of performance restriction. But, there were differences when grades were considered (Table 4). Although fish oil group had a significantly higher number of patients classified as grade 1 compared to placebo group only when considering grades 0 and 1 ($p = 0.01$), the placebo group had a significantly higher number of individuals classified as 2 or 3 compared to fish oil group considering grades 1 and 2 or 3 ($p = 0.02$). This means that even though there were no differences between groups regarding presence or absence of performance restriction, individuals in the placebo group were classified with higher grades, indicating that they had severe performance restrictions when compared to fish oil group individuals (Table 4).

We were not able to obtain data for tumor markers (CEA and CA 19-9) for all the patients included in this study as these tests were performed only as a request of the assigned oncologist. The data regarding tumor markers presented in this study were obtained from patients' chart records. According to the collected data, there is a statistically significant difference at final values for tumor marker CA 19-9 when comparing placebo and fish oil supplemented groups. As shown in Table 4, higher values were presented by individuals in placebo group when compared to final levels presented by fish oil group ($p = 0.04$). However, after adjusting the final values for baseline values, there are no differences between groups ($p = 0.23$). For the remaining analysis of the response to treatment and survival (six months survival (Table 4), one-year survival (Table 4), and Kaplan–Meier survival estimates (Fig. 3)), there were no differences between groups after six months and one year of follow-up.

3.3. Oxidative stress variables

Catalase, Superoxide Dismutase and Glutathione Peroxidase activity were measured at baseline and after nine weeks of treatment. Activity values for all the enzymes tested remained unchanged for both groups after nine weeks of treatment. Also, when

Table 4
Performance Status after supplementation, six-months and one-year survival, and one-year response to treatment for each study group.

	Placebo Group		Fish Oil Group		Effect Size (Cohen's d)	p
Performance Status after nine weeks	n = 24	95% CI	n = 23	95% CI		0.09[c,g]
0	12 (50.00%)[a]	–	6 (26.09%)[a]	–	–	–
1	5 (20.83%)[b,d]	–	14 (60.87%)[b,d]	–	–	–
2	3 (12.50%)[a,b]	–	3 (13.04%)[a,b]	–	–	–
3	4 (16.67%)[a]	–	0 (0.00%)[a]	–	–	–
Carcinoembryonic antigen (CEA) (ng/mL)						
Baseline	3.35 [0.84; 171], n = 14	−49.86–425.89	3.82 [0.8; 24.1], n = 19	−31.39–291.08	0.08	0.83[e]
1 year	2.4 [1.08; 37], n = 11	−202.53–658.61	2.92 [0.72; 6.57], n = 18	−34.27–116.21	0.19	0.60[e]
p	0.89[f]		0.57[f]			0.77[i]
Δ	−0.5 (−79.36; 145.45), 8		0.6 (−2.88; 3.30), 15			0.70[e]
Cancer antigen 19-9 (CA-19-9) (U/ml)						
Baseline	12.40 [4.22; 36.5], n = 13	−123.99–949.61	12.80 [7.00; 79.56], n = 13	18.44–99.36	0.17	0.66[e]
1 year	17.90 [15.96; 420], n = 11	−358.21–1894.43	11 [6.54; 37.08], n = 15	−15.79–103.32	0.85	0.04[e]
p	0.31[f]		0.96[f]			0.23[i]
Δ	10.63 (−1.21; 383.5), 7		1.86 (−16.58; 4.22), 10			0.14[e]
Six months Survival	22 (88.00%), n = 25	–	20 (83.33%), n = 24	–	–	0.64[h]
One year Survival	18 (72.00%), n = 25	–	20 (77.55%), n = 24	–	–	0.34[g]
Complete response/Follow-up	10 (40.00%), n = 25	–	12 (50.00%), n = 24	–	–	0.48[g]
Disease progression/Relapse	6 (24.00%), n = 25	–	5 (20.83%), n = 24	–	–	0.79[g]

CI = Confidence Interval.
Data presented in median [interquartile range] or absolute frequencies (relative frequencies).
[a,b]Groups assigned with the same letters are not significantly different.
[c] Difference between groups regarding presence (grades 1, 2 or 3) and absence (grade 0) of performance restriction.
[d] There were differences between grades 1 and 0 ($p = 0.01$), grades 1 and 3 ($p = 0.01$) and grades 1 and 2 or 3 ($p = 0.02$) between groups.
[e] Mann-Whitney Test.
[f] Wilcoxon matched-pairs test.
[g] Chi-squared Test.
[h] Chi-squared test with fisher correction.
[i] ANCOVA using baseline values as a covariate to test group differences in final values.

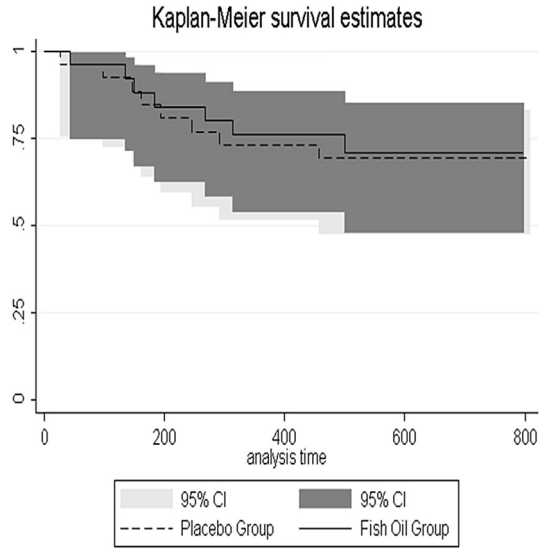

Fig. 3. Kaplan–Meier Survival curve for both study groups.

baseline values and final values are compared between groups, there were no statistically different changes. For plasma and leukocyte lipid peroxidation, no differences were observed between groups and comparing baseline to final values in each group (Table 5). When individuals that were also submitted to radiotherapy were excluded from the analysis, the results remained the same (data not shown).

3.4. Compliance

N-3 fatty acids percentage in total erythrocytes fatty acids was used analysis as a marker of compliance. The fish oil group showed a 35% increase in EPA on erythrocytes total fatty acids after supplementation, and this was statistically significant ($p = 0.03$) even after adjustments for baseline values ($p = 0.04$). The placebo group did not present any statistically significant differences for EPA incorporation. DHA levels did not show any change in both groups.

Individuals in both groups were followed throughout the period between blood collections. None of the patients included in the study received blood transfusions or other interventions that could interfere with the results of the blood sample analysis.

4. Discussion

This study evaluated adverse events presence and grading in order to assure fish oil supplementation's safety. The hypothesis that supports this approach is that fish oil supplementation would increase tumor sensitivity to treatment without increasing toxicity to normal tissues. But, not only fish oil did not increase gastrointestinal and hematological adverse events, it also showed increased protection regarding diarrhea. Patients receiving fish oil had less severe diarrhea when compared to patients receiving placebo. Other studies conducted in animals also observed a protective effect of n-3 fatty acids (especially DHA) on increment of intestine permeability and structural changes induced by chemotherapy [27–29], which could reduce the severity of diarrhea. In human trials, increase in toxicity on healthy tissues or worse outcomes were not observed in patients receiving n-3 fatty acids in addition to chemotherapy [15,16,30]. In fact, in one study a decrease in anemia and thrombocytopenia in individuals with high plasma n-3 incorporation was observed [15].

One proposed mechanism is that normal tissues are able to effectively handle the exacerbation of chemotherapy-induced oxidative stress promoted by n-3 fatty acids by enhancing its antioxidant defenses. This would guarantee no increase in toxicity already caused by the anti-cancer agent itself, but the decrease in

Table 5
Oxidative stress variables before and after supplementation for each study group.

	Placebo Group		Fish Oil Group		Effect Size (Cohen's d)	p
Catalase (U/mg of hemoglobin)	Median [interquartile range], n	95% CI	Median [interquartile range], n	95% CI		
Baseline	0.77 [0.55; 1.13], n = 25	0.66–0.99	0.79 [0.39; 1.23], n = 24	0.62–1.05	0.03	0.91[a]
9 weeks	0.81 [0.45; 1.11], n = 21	0.60–1.12	0.60 [0.33; 0.88], n = 19	0.43–0.84	0.42	0.18[a]
p	0.64[b]		0.14[b]			0.54[d]
Delta	0.14 (−0.28; 0.45), n = 21		−0.27 (−0.71; 0.15), n = 19			0.24[c]
Superoxide Dismutase (U/mg of hemoglobin)						
Baseline	0.74 [0.55; 1.04], n = 25	0.62–0.93	0.67 [0.37; 1.03], n = 24	0.56–0.94	0.11	0.69[a]
9 weeks	0.68 [0.42; 0.87], n = 21	0.52–0.92	0.76 [0.48; 0.94], n = 19	0.60–0.90	0.28	0.39[a]
p	0.54[b]		0.63[b]			0.41[d]
Δ	−0.14 (−0.37; 0.25), n = 21		0.14 (−0.37; 0.45), n = 19			0.77[c]
Glutathione Peroxidase (U/mg of hemoglobin)						
Baseline	9.57 [3.57; 19.21], n = 25	7.26–16.45	8.18 [4.06; 13.10] n = 24	7.03–15.43	0.01	0.97[a]
9 weeks	7.72 [3.86; 13.04], n = 21	6.09–13.41	7.50 [4.50; 13.21], n = 19	6.06–14.69	0.09	0.78[a]
p	0.97[b]		0.55[b]			0.68[d]
Δ	0.49 (−3.38; 1.44), n = 21		−0.90 (−5.42; 3.19), n = 19			0.82[a]
Leukocyte lipid peroxidation (nmol/mg of lipids)						
Baseline	0.84 [0.50; 1.88], n = 23	0.78–1.88	0.72 [0.30; 2.49], n = 21	0.83–2.57	0.10	0.75[a]
9 weeks	0.61 [0.20; 2.67], n = 19	0.65–2.81	0.57 [0.48; 2.09], n = 14	−3.78-13.59	0.31	0.38[a]
p	0.84[b]		0.51[b]			0.63[d]
Δ	−0.17 (−0.89; 1.30), n = 18		−0.10 (−1.42; 0.27), n = 14			0.62[a]
Plasma lipid peroxidation (nmol/mg of lipids)						
Baseline	2.01 [0.94; 3.17], n = 24	1.30–6.55	2.43 [1.18; 5.22], n = 24	−0.41-13.87	0.23	0.43[a]
9 weeks	3.33 [1.92; 6.21], n = 21	2.94–9.29	2.53 [1.49; 5.32], n = 18	1.99–6.14	0.39	0.24[b]
p	0.19[b]		0.95[b]			0.26[d]
Δ	0.89 (−1.43; 3.43), n = 20		−0.52 (−2.47; 1.69), n = 18			0.36[a]

CI = Confidence Interval.
Data presented as median [interquartile range].
[a] Mann-Whitney Test.
[b] Wilcoxon matched-pairs test.
[c] Student's t-test.
[d] ANCOVA using baseline values as a covariate to test group differences in final values.

toxicity in one specific tissue may be due to differential response. According to a study by Hajjaji et al. [31]even though normal tissues from animals receiving DHA and epirubicin had an effective response and showed no increase in lipid peroxidation generated by concomitant treatment on tumor tissues, each tissue analyzed (heart, liver, and intestines) presented different changes in antioxidant defenses. We evaluated antioxidant enzymes in our study, without significant changes between groups and comparing baseline to nine weeks. However, this was not a direct measure of redox status of colonocytes or other cells present in the organ originally affected by cancer. Such direct measure could indicate a different local response the treatment or supplementation. Another mechanism referred by Hardman et al. [29]would be the suppression of PGE2 and TXA2 synthesis by the ingestion and incorporation of n-3 fatty acids. The increase of n-3 fatty acids in the phospholipids of cells membranes, decreasing n-6 eicosanoids substrates, leads to the synthesis of n-3 derived eicosanoids, as PGE3 and TXA3. This could reduce diarrhea caused by eicosanoid-mediated chlorine secretion and lessen this treatment-related adverse event severity.

We observed a better performance status in patients receiving fish oil, which has not only a prognostic value but also acts as an operational tool, used to indicate how well a patient is tolerating the prescribed treatment. Similar to what was observed with diarrhea grading, there were no differences between groups regarding presence or absence of performance restrictions, but the severity was higher in placebo group patients. These two parameters also may be linked to the extent that higher severity of any adverse event will ultimately lead to a decrease in general well-being and restrict activities of daily life.

Tumor markers are used in clinical practice to monitor response and help in the detection of recurrence. Although CA19.9 tumor marker is increased in multiple types of gastrointestinal cancer, it is considered insufficient and imprecise to detect disease progression alone. Therefore, its use is often combined with other markers, as CEA, to increase sensitivity to the remaining presence of cancer cells [32]. In this study, we were not able to detect changes in CEA between study groups, but final values for CA 19.9 were lower in individuals receiving fish oil. This would be an indication of better response to treatment; however, after adjusting final values for baseline, the difference was no longer significant. Other studies performed in humans using n-3 fatty acid intervention during chemotherapy treatment showed a better response to treatment, higher time to tumor progression and survival [15,16,33,34]. In our study, when diagnoses regarding treatment response are considered, which represents the sum of response evaluation methods and also the clinician's view, no differences were observed between groups as well. On the four previously mentioned human studies which also evaluated response to the treatment, two of them [15,16] included only patients with advanced disease, and one [33] had a longer follow-up (at least 36 months). This might suggest that fish oil impact in disease's course may be more relevant after a more prolonged period of time or in more advanced stages.

Many *in vitro* and animal-model based studies showed an increase in cytotoxicity of anti-cancer agents after co-treatment with n-3 fatty acids, and this was accompanied by an increase in lipid peroxidation products [35–38]. In studies performed in humans evaluating lipid peroxidation after supplementation with n-3 and anti-cancer treatments, two found a reduction of this parameter (measured in plasma and serum) [39,40] and one showed no changes (measured in polymorphonuclear cells) [41]. Our study did not find any differences regarding lipid peroxidation or antioxidant enzymes activities between groups. This set of analysis was performed in plasma, erythrocytes, and leukocytes, better representing the whole blood compartment of the redox status. Propositions that n-3 fatty acids would sensitize tumor cells to treatment due to an increase in the production of lipid peroxidation products without an effective response of the tumor cells antioxidant system, leading to cells death, are well established in *in vitro* and in animals studies. However, this was not proven yet in clinical studies. Data on better treatment response influenced by supplementation with n-3 fatty acids are starting to emerge from clinical trials. Nevertheless, these clinical changes are still not associated with biochemical changes. If, in fact, oxidative stress mechanisms are playing a role in the tumor's response, it is not possible to conclude with the available *in vivo* or *ex vivo* human data. This would probably require clinical trials aimed at finding these answers using biopsies obtained from tumor tissues. Our main focus was to investigate systemic changes, especially those occurring in normal cells, in order to verify if supplementation was altering redox status in these cells and if this could potentially lead to harmful effects. As to evaluate direct effects on the tumor, the analysis must be performed in this specific tissue, the same applies to other tissues, so inferences on normal cells based on systemic variables are limited. However, we consider the lack of differences between groups as a sign that supplementation is not increasing systemic lipid peroxidation in a damaging manner or overwhelming normal cell's antioxidant defense system.

This study presents some limitations and strengths. At planning, we aimed to minimize as possible the recognition of fish oil characteristic flavor instructing patients to ingest it right before any main meal. Even though this can still be considered a limitation, patients did not seem to know to what group they were assigned to; we believe that placebo effect was present in both groups, therefore annulled and not responsible for differences observed. We are aware of possible components of olive which might have influenced these results, but, in order to obtain an appropriate placebo we had to make some choices and ended up opting for one edible oil low on polyunsaturated fatty acids (mainly DHA and EPA), due to the fact that our main hypothesis was linked to fish oil unsaturation level. As far as we know, this is the first placebo-controlled, triple-blind clinical trial to evaluate response to treatment and adverse events after a fish oil supplementation. All the analysis (including statistical analysis and data presenting) were performed before groups disclosure.

5. Conclusion

Supplementing patients undergoing chemotherapy for gastrointestinal cancers with fish oil may lead to better performance status. This supplementation does not seem to increase treatment-related toxicity, as it can reduce the severity of diarrhea, a common adverse event of this type of treatment. Furthermore, fish oil ingestion during chemotherapy does not seem to negatively alter systemic redox status. If or how n-3 fatty acids can act to increase sensitization of tumor cells to treatment, it is still not established in human trials.

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Statement of authorship

C.Q.C.: Conceptualization, recruitment, data collection, biochemical analysis, statistical analysis, original draft; M.C.M.: recruitment, data collection, biochemical analysis, manuscript review; H.S.B. and T.R.C.: biochemical analysis, manuscript review; MF: recruitment, supervision, manuscript review; E.L.S: biochemical analysis, manuscript review; E.B.S.M.T. and E.A.N.: conceptualization, project administration, supervision, manuscript review. All authors of this article have read and approved the final version.

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Appendix A. Supplementary data

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