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A double-blind randomized controlled trial of the effects of eicosapentaenoic acid supplementation on muscle inflammation and physical function in patients undergoing colorectal cancer resection.

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Abbreviations:

colorectal cancer: CRC; eicosapentaenoic acid EPA; peroxisome proliferator activated receptor gamma: PPAR-γ; nuclear factor kappa-light-chain-enhancer of activated B-cells: NFκB; tumor necrosis factor alpha: TNF-α; V1: visit 1; V2: visit 2; hand-grip strength: HG; dual energy X-ray absorptiometry: DXA; cardiopulmonary exercise testing: CPET; length of hospital stay: LoS; advanced life support: ALS; lean muscle mass: LMM; respiratory exchange ratio: RER; anaerobic threshold: AT; post-operative days: POD.

Abstract

Background: Resection of colorectal cancer (CRC) initiates inflammation, mediated at least partly by NFκB (nuclear factor kappa-light-chain-enhancer of activated B-cells), leading to muscle catabolism and reduced physical performance. Eicosapentaenoic acid (EPA) has been shown to modulate NFκB, but evidence for its benefit around the time of surgery is limited.

Objective: To assess the effect of EPA supplementation on muscle inflammation and physical function around the time of major surgery.

Design: In a double-blind randomized control trial, 61 patients (age: 68.3±0.95 y; 42 male) scheduled for CRC resection, received 3g per day of EPA (n=32) or placebo (n=29) for 5-days before and 21-days after operation. Lean muscle mass (LMM) (via dual energy X-ray absorptiometry (DXA)), anaerobic threshold (AT) (via cardiopulmonary exercise testing (CPET)) and hand-grip strength (HG) were assessed before and 4-weeks after surgery, with muscle biopsies (*m. vastus lateralis*) obtained for the assessment of NF-κB protein expression.

Results: There were no differences in muscle NFκB between EPA and placebo groups (mean difference (MD) -0.002; 95% confidence interval (CI) -0.19 to 0.19); p=0.98). There was no difference in LMM (MD 704.77g; 95% CI -1045.6g to 2455.13g; p=0.42) or AT (MD 1.11 mls/kg/min; 95% CI -0.52 mls/kg/min to 2.74 mls/kg/min; p=0.18) between the groups. Similarly, there was no difference between the groups in HG at follow up (MD 0.1; 95% CI -1.88 to 2.08; p=0.81). Results were similar when missing data was imputed.

Conclusion: EPA supplementation confers no benefit in terms of inflammatory status, as judged by NFκB, or preservation of LMM, aerobic capacity or physical function following major colorectal surgery.

61

62 **Words:** 263

63

64 **Keywords:** cancer, colorectal, muscle, eicosapentaenoic acid, surgery, inflammation

Introduction

With an estimated 1.4 million cases per annum worldwide [1] colorectal cancer (CRC) represents a major clinical burden, often resulting in morbidity and death. Surgical resection remains the only known cure for CRC. Despite an increased proportion of resections being performed laparoscopically, in the United Kingdom 39.2% are still resected via open surgery with a further 8.5% of laparoscopic procedures converted to open surgery [2]. This exposes an often elderly and frail patient population to a major physiological challenge. To exemplify the magnitude of this challenge, surgery for CRC confers a risk of death of 3.8% at 90 days [2] and considerable morbidity, with length of hospital stay typically five or more days [3]. Moreover, 30 day re-admission rate following CRC surgery remains high at 10% [2].

The inflammatory responses associated with major body cavity surgery have been implicated in the initiation and maintenance of an acute phase response, and in countering the cellular processes important in the preservation of skeletal muscle [4]. This has led to research attempting to modulate these inflammatory responses through immuno-nutritional supplementation in the perioperative period. However, to date these efforts have reported variable results [5-8]. Eicosapentaenoic acid (EPA), an omega-3 long chain polyunsaturated fatty acid, has been advocated as one such nutritional supplement that may diminish the stress response and lessen the stress-related burden of operation in a cachectic cancer population. EPA has been proposed as an agent which may ameliorate post-operative inflammation and subsequent muscle catabolism via a variety of mechanisms [9]. EPA supplementation has been shown to reduce serum concentrations of pro-inflammatory agents [7, 10] and the activity of tumour derived proteolysis inducing factor [7, 11]. In addition, EPA is thought

to exert anti-inflammatory activity through its activation of the inflammatory regulatory gene peroxisome proliferator activated receptor (PPAR)-γ which can inhibit nuclear factor kappa-light-chain-enhancer of activated B-cells (NFκB) activity [12], and also via its inhibition of tumour necrosis factor (TNF)-α induced activation of caspase pathways of muscle catabolism [11].

Whilst a number of pre-clinical trials have investigated the immuno-modulation properties of EPA in a cachectic cancer population, few have addressed its use in the perioperative patient [13-16]. The aims of this study were therefore, to determine the effects of perioperative EPA supplementation on perioperative inflammation, muscle loss, and functional and clinical outcomes in CRC patients.

Methods

Trial design

This was a single centre, double-blind, placebo-controlled, parallel-group study, conducted in the United Kingdom. Patients were randomly assigned 1:1, using computerised randomisation (www.randomiser.org), to receive either EPA, or near identical placebo capsules, for 5 days prior to surgery and for 21 days post-operatively. Treatment assignment was unknown to the investigators, patients, trial statistician and treating surgical team.

Patients were asked to attend the University of Nottingham Clinical, Metabolic & Molecular Physiology laboratories in Derby following an overnight fast (water *ad libitum*) one week prior to surgery (visit 1 (V1); timed to coincide with the routine care pre-operative assessment visit) and again 4 weeks after surgery (V2). The

supplementation period of 21-days determining the timing of V2 was based on previous work [6] demonstrating this time-point to be the nadir in hand-grip strength (HG), total body protein and body weight following major abdominal surgery.

At both study visits body composition was first analysed by dual-energy x-ray absorptiometry (DXA; Lunar Prodigy II, GE Medical Systems). Cardiopulmonary exercise testing (CPET) (ZAN680, nSpire Health, UK) and HG dynamometry (Hand-grip Dynamometer Digital A5401, Takei, Japan) were then performed after a standardized light breakfast. A muscle biopsy was taken under local anaesthesia (lidocaine 1%) from the *m. vastus lateralis* using our standard conchotome technique [28] on the day of surgery (within five minutes of induction of anaesthesia) and repeated 5-weeks after surgery. The second biopsy was taken one week after V2 to prevent any acute effect of intense exercise (the CPET) on muscle sample analysis.

Patients were reviewed throughout the perioperative period with vital signs and HG recorded for 5 days post-operation.

Study participants

Patients presenting to the CRC multidisciplinary team meeting at the Royal Derby Hospital, who met inclusion/exclusion criteria were approached for recruitment when they attended hospital as an out-patient prior to surgery. Eligible patients were those scheduled for open elective CRC resection surgery with curative intent; right hemi-colectomy, left hemi-colectomy or anterior resection, who had not received neo-adjuvant chemoradiotherapy. All patients had open CRC resection, with anaesthetic technique at the discretion of the anaesthetist. Exclusion criteria were: intended

laparoscopic resection, abdominoperineal resection of the rectum, previous chemo or radiotherapy, peripheral neuropathy/myopathy, unstable angina, myocardial infarction within 3 months, severe aortic stenosis, chronic heart failure, New York Heart Association class 3 or above, severe COPD, emphysema, fibrosing alveolitis, interstitial lung disease, current fish-oil derived nutritional supplement or known metastatic disease. Patients having laparoscopic surgery were expected to have a less marked stress response from surgery and those having abdominoperineal resection or neo-adjuvant chemotherapy were deemed to likely have markedly greater stress responses, hence these exclusion criteria. Patients were instructed not to undertake strenuous exercise and/or intramuscular injections within 48-hours of either study visit. Before commencing the study, all patients were screened using a medical questionnaire, physical examination and resting ECG.

The study was conducted according to the Declaration of Helsinki under the auspices of an NHS Research Ethics Committee (REC 11/EM/0066) and was registered at ClinicalTrials.gov (NCT01320319). All patients gave their written, informed consent to participate in the study.

Nutritional intervention

500mg capsules of EPA (Minami Nutrition, Belgium) or near identical placebo capsules (Wassen Nutrition, UK) (EPA (Eicosapentaenoic Acid) 500 mg, DHA (Docosahexaenoic Acid) 0 mg, Other Omega-3s 27 mg, Omega-6 Fatty Acids 0 mg) were dispensed by the hospital pharmacy, with a daily regimen of 1g three times a day (tds) prescribed. Treatment regimen was for 5 days pre-operatively and for 21 days following surgery. Patients were supplied with a fixed number of excess capsules and

were asked to return the remaining capsules on V2 for pharmacy determination of compliance.

Outcomes

The primary outcome measures of this study were the effect of EPA supplementation on changes in i) cardiorespiratory fitness, ii) physical strength, iii) lean muscle mass (LMM) and iv) cellular markers of inflammation, in the perioperative period. Secondary outcomes were based on perioperative care data which was collected in the first 5 days following surgery. This data comprised temperature, white cell count, and post-operative complication scoring [17]. Additional data regarding length of hospital stay (LoS) was recorded following a retrospective analysis of patient notes at V2.

Assessment of Physical Function

On both study visits patients completed CPET supervised by an Advanced Life Support (ALS) certified clinician. CPET was performed using a Lode Corival cycle ergometer (ZAN680, nSpire Health, UK) and our standard ramp protocol [18]. In brief, after a two-minute warm-up, cycling workload was increased in a ramp manner of 20W per minute, with patients instructed to maintain a cadence of 60-70 rpm. Patients were encouraged to exercise to 85% or more of age-predicted maximal heart rate (220-age) and to a respiratory exchange ratio (RER; VCO_2/VO_2) above 1.1. Tests were terminated when patients were unable to maintain a cycling cadence of >60rpm, or reported volitional exhaustion. On completion of the study anaerobic threshold (AT) was calculated by two independent trained assessors using the V-slope method [19] and determined as the mean of these two values.

HG was determined by dynamometry as the highest reading of three attempts at maximal contraction with the dominant hand, using a digital hand-grip dynamometer (Takei, Japan). Measurements of HG were taken on V1 and V2, and on each of the first five post-operative days (POD), or until hospital discharge, whichever occurred first.

Assessment of Cellular Markers of Inflammation

Muscle protein expression of NFκB was determined from biopsy tissue (10–20mg) of the *m. vastus lateralis* using our standard immunoblotting techniques [20] with a primary antibody for NFκB (New England Biolabs, UK). Blots were imaged and quantified by assessing peak density after ensuring the band was within the linear range of detection using the ChemiDoc XRS system (Bio-Rad, UK).

Sample size and statistical analysis

Based on previous data [20] our power calculation suggested that to detect a clinically (>30%) important difference in NFκB muscle protein expression between the two groups (with a two-sided 5% significance level and a power of 80%), a sample size of 28 patients in each group was needed. The study was therefore powered to have 56 patients in total. Accounting for an assumed drop-out rate of 20% a target recruitment of 70 patients was established.

Normality was tested using histograms and the Shapiro-Wilk test. If normality was violated, then non-parametric tests or appropriate transformations were conducted. Data compared at one time point were analysed using t-tests or Mann-U-Whitney tests as appropriate. For primary outcomes measured at two time points, we tested the

difference between each group using ANCOVA, with baseline values as the covariate. We assessed normality of residuals using histograms. Homogeneity of variance was assessed using Levene's robust test statistic. We also tested the assumptions of linearity, homoscedasticity and homogeneity of regression slopes using the appropriate plots. For variables measured at multiple time points, we performed linear mixed models with random intercepts and slopes. An unstructured covariance structure was used. Time was fitted with a quadratic term for temperature due to non-linearity. Assumptions tested include normality of residuals, linearity and homoscedasticity. The Dindo-Clavien post-operative complication score was compared between the two groups using Fisher's Exact Test.

In addition to the main available case analysis, we also conducted a sensitivity analysis with an intention to treat analysis. This was carried out on all randomised patients with baseline data. The mechanism of missingness was explored. Multiple imputations (M=20) were performed under the *Missing At Random* assumption [21] for all primary outcomes and analysed using linear mixed models.

Results from analyses are presented as mean differences (MD) with 95% confidence intervals (CI). NFκB muscle protein is presented as the natural logarithmic transformation due to non-normality. All statistical analyses were conducted using STATA (StataCorp, Texas USA) by a Derby Clinical Trials Unit statistician and a member of the research team (BD).

Results

Recruitment

One hundred and twenty patients were assessed for eligibility and from these 64 were recruited and randomised to the study; 32 to EPA and 32 to placebo, with baseline data collected for 32 EPA and 29 placebo patients (Table 1). By cessation of the study, 31 EPA patients and 25 placebo patients had fully completed the study with both muscle biopsies (Figure 1). An intention to treat analysis was performed on all patients achieving baseline data for primary outcomes, with missing data imputed on sensitivity analysis.

Baseline data

There were no baseline differences in age, height, gender, body weight, or LMM between the groups. Similarly, both groups were equally matched for operation performed and cancer staging (Table 1).

Compliance

Intervention compliance was almost identical between the two groups (EPA: 136±6.33 (87%) vs. placebo: 137±7.57 (88%) capsules consumed, p=0.68).

Primary outcomes

Muscle protein expression of NF-κB (arbitrary units) showed no difference between EPA and placebo groups when adjusting for baseline values (MD -0.002; 95% CI -0.19 to 0.19); p=0.98).

There were no differences between the EPA and placebo group in whole-body LMM following CRC resection when adjusting for baseline values (MD 704.77g; 95% CI -1045.6g to 2455.13g; p=0.42) (Figure 2). There was no difference between the groups in hand grip strength at follow up (MD 0.1; 95% CI -1.88 to 2.08; p=0.81). Furthermore, there was no difference between the groups for AT when adjusting for baseline values (MD 1.11 mls/kg/min; 95% CI -0.52 mls/kg/min to 2.74 mls/kg/min; p=0.18).

Secondary outcomes

There was no significant differences between the groups in postoperative temperature (p=0.67) (Figure 3A). In addition, there was no significant difference in white cell count between the groups (p=0.83) (Figure 3B). LoS did not significantly differ between the groups (EPA: 7.5 days (6-13.5) vs. placebo: 3.82 days (5-10), p=0.41). There was no difference between the groups in complications (p=0.62).

Sensitivity analysis

When re-analysing primary outcomes using multiple imputation, similar to the main analysis, there was no significant differences for NF-κB (p=0.82), LMM (p=0.74), HG (p=0.53) and AT (p=0.84).

Discussion

This study has shown that perioperative EPA is safe and tolerable for CRC patients. However, it did not show perioperative EPA to benefit patients having resection of CRC.

Surgery is known to elicit a marked physiological stress response, and in this study patients in both EPA and placebo groups displayed increases in white cell count after operation which returned to normal by five days post-surgery. In tandem with this HGS was reduced in the immediate post-operative period, with these reductions extending up to four weeks after operation. Similarly, whole-body LMM was also reduced over the same time-frame, although there was no difference between the groups.

Despite these reductions in muscle mass and muscle function, we failed to detect a statistically significant or clinically meaningful reduction in cardiorespiratory fitness, a known risk factor for poor post-operative outcomes. In addition, and at odds with our previous observations, we also found no change in NFκB around the time of CRC resection [22].

Furthermore, addressing our primary study aim, nutritional supplementation with EPA conferred no advantage in terms of body composition, physical performance or clinical outcomes (LoS and postoperative complications); although it must be acknowledged that this study was not statistically powered for clinical variables.

These findings are in contrast to those of previous studies which have suggested potential beneficial effects of EPA in the both the pre and post-operative period [10,

23-25]. However, not all EPA trials have reported positive findings. A recent randomised control trial of standard diet versus standard diet supplemented with ProSure® (compromising supplementation with 2.2g EPA per day) found no difference in bodyweight at 1 or 3 months post-operatively between the treatment and control groups [23]. Similarly, in a separate study, EPA supplementation in CRC, providing 2g of EPA daily, did not show any benefit of EPA on clinical outcomes of complications, length of stay or hospital re-admissions [25].

We have previously shown elevated expression of NFκB, a key modulator of inflammation, in the skeletal muscle of CRC patients while the cancer is *in situ*, with a return to normality following curative CRC resection [22]. This finding combined with pre-clinical studies showing attenuated NFκB activity by EPA [12], is what led us to this study. However, in this present study we did not show this reduction in muscle NFκB after surgery and this may be explained by a number of factors. Firstly, almost half of the patients in this study had a documented post-operative complication compared to none in our previous work; this higher complication rate is likely explained by the inclusion of anterior resections. Anterior resection is a more physiologically challenging operation, evidenced by the prolonged LoS in this subgroup following surgery. This may, in turn, have led to continued inflammatory and stress responses to surgery in these patients, resulting in maintained heightened NFκB within the muscle. In addition, in contrast to our earlier work, in this study muscle biopsies were taken at five weeks post-surgery (as opposed to six weeks previously). At this time-point any inflammation associated with surgery and/or the primary tumour may be more pronounced, leading to a limited reduction in NFκB.

Our finding of no effect of EPA supplementation on inflammatory status is in contrast to previous reports which have demonstrated that EPA supplementation decreases the expression of, and modulates the action of key pro-inflammatory cytokines (namely TNF-α and IL-6) in human preclinical *in vitro* models and healthy human volunteers [26]. Furthermore, EPA supplementation has been reported to attenuate inflammation in patients with disseminated cancer [27], with several studies reporting reductions in blood borne pro-inflammatory cytokines one week post-operatively following EPA supplementation after major gastrointestinal surgery [24, 28, 29]. Similarly, reductions in LoS of have also been reported with EPA supplementation following major gastrointestinal surgery [29-31]

Given our previous experience and that of others, failing to observe an effect of EPA on expression of NFκB expression is surprising. Previous studies have however looked at blood borne markers of inflammation, raising the question of EPA penetrance and its action at the level of skeletal muscle. Normalisation of inflammatory markers within the muscle will likely follow on from, rather than precede inflammatory changes within the plasma. This temporal factor may account for the absence of an observed reduction in tissue inflammation five weeks post-surgery. Indeed, within this time-frame muscle mass and physical performance were still diminished compared to pre-operative measures, suggesting that skeletal muscle function, structure and possibly metabolism [32-33] , were not normalised by this point.

We accept that there are number of limitations to this study. The study was powered for changes in muscle NFκB expression and therefore likely underpowered for detection of body composition, functional and clinical differences within and between

groups. However, such measures were not the primary aim of the current study, with previous research documenting significant reductions in many of these variables after major abdominal surgery. Moreover, the sample size required to investigate differences in LoS and post-operative morbidity would not have been achievable in a single centre within a meaningful time-frame. Additionally, as the primary focus of the study was to explore changes in muscle mass and function we did not explore for serum markers of inflammation, or activation of muscle NFκB, other than white cell count. Furthermore, despite recruiting sufficient patients for a baseline visit, attrition throughout the study resulted in failure to retain sufficient participants to achieve the *a priori* calculated sample size (in the placebo group only). Despite this, given the clear lack of significance observed in both primary and secondary outcomes we do not believe this compromises our findings. Finally, patients were only studied on two acute study days, with dates chosen to coincide with standard clinical care visits. An increased number of study days, particularly extending later into the post-operative period, may have provided more insight into the longer-term effects of EPA supplementation given around the time of surgical resection.

In conclusion, despite observing significant increases in serum white cell count and reductions in LMM and physical function, we found no clinical or functional benefit of perioperative EPA nutritional supplementation in individuals undergoing curative CRC resection in the period up to five weeks post-operation. Future studies should explore higher doses of EPA administered over a prolonged time-frame with assessment visits planned to give better temporal resolution.

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Conflict of Interest

No author has any conflict of interest to declare.

Author Contributions

TH, BEP, JNL and JPW designed the research; TH conducted the research; TH, BEP, BD and JPW analysed the data; TH, BD, BEP, JNL and JPW wrote the manuscript; JPW has primary responsibility for the final content of the paper.

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TABLES

Table 1. Patient baseline data

[f]=Fisher's exact test [t]=Student's t-test [m]=Mann-Whitney-u test [c]= Chi-squared test

Categorical variables are presented as frequencies with continuous variables as mean (SD), or median (inter-quartile range).

Table 2. Post-operative complications per Clavien-Dindo classification of surgical complications

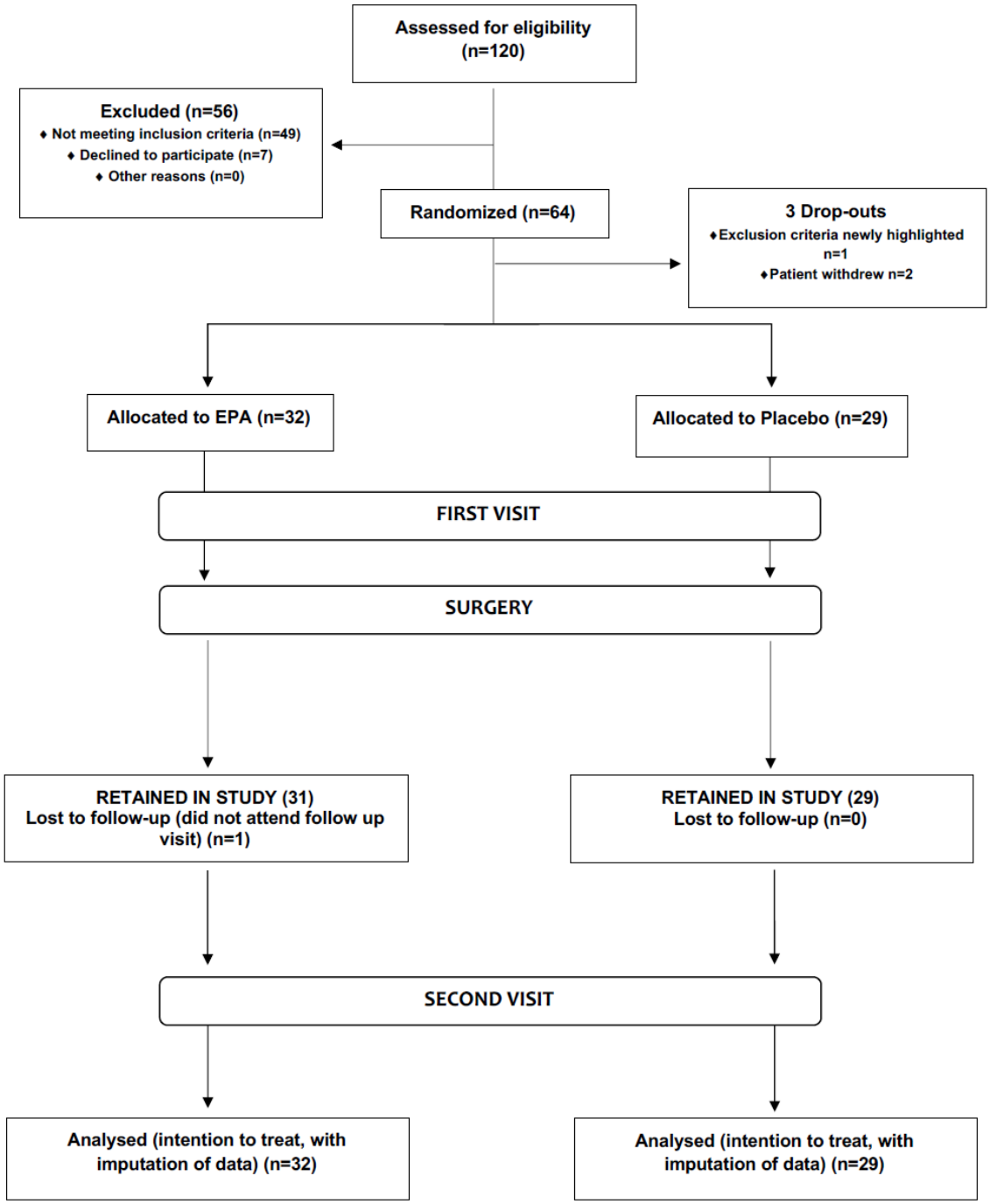
Figure Legends

Figure 1. Study CONSORT diagram

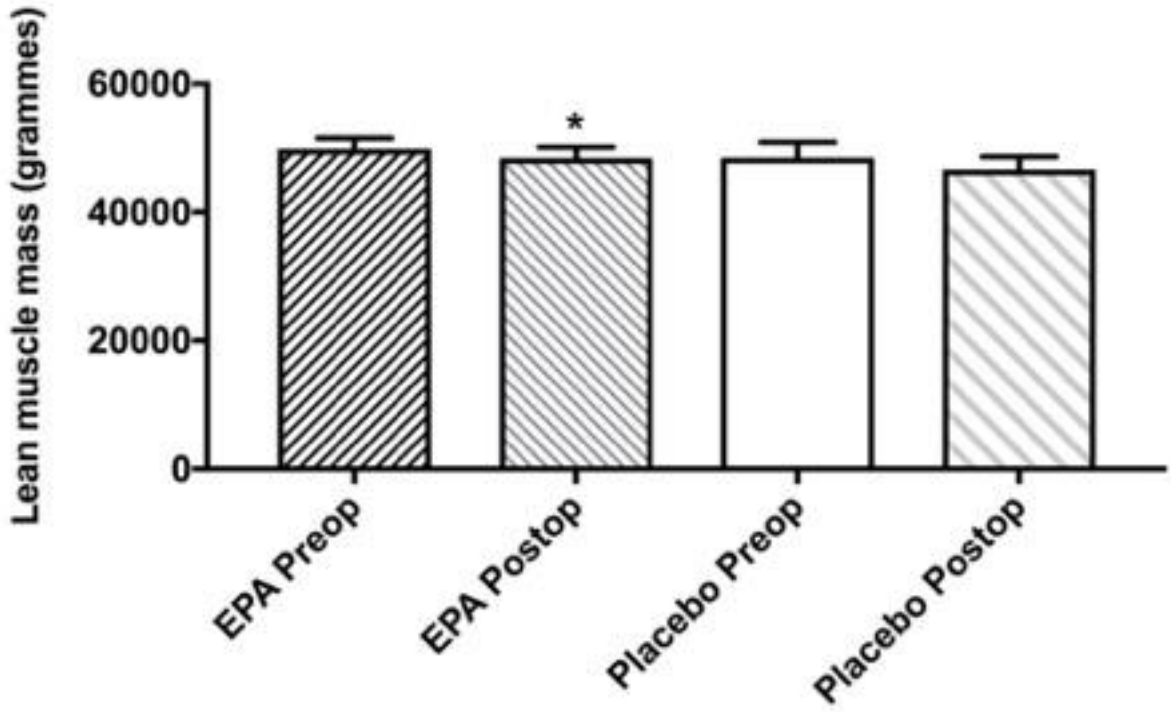
Figure 2. Whole-body lean muscle mass (LMM) before (Preop) and 4-weeks after (Postop) colorectal cancer resection surgery with (EPA; N=32) or without (placebo; N=29) perioperative eicosapentaenoic acid supplementation. Statistical analysis via Student's t-tests.

Figure 3. Clinical outcomes (**A.** maximum temperature, **B.** white cell count) for 5 postoperative days (POD) after colorectal cancer resection surgery with (EPA; N=32) or without (placebo; N=29) perioperative eicosapentaenoic acid supplementation. Analysis via Mann-U-Whitney statistical testing.

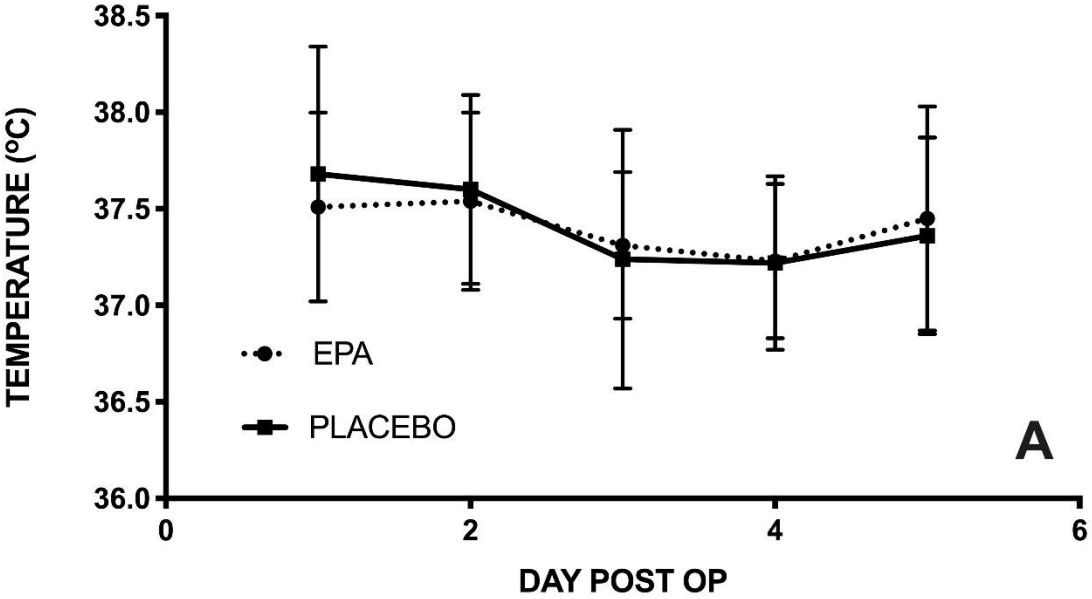
Figure 1

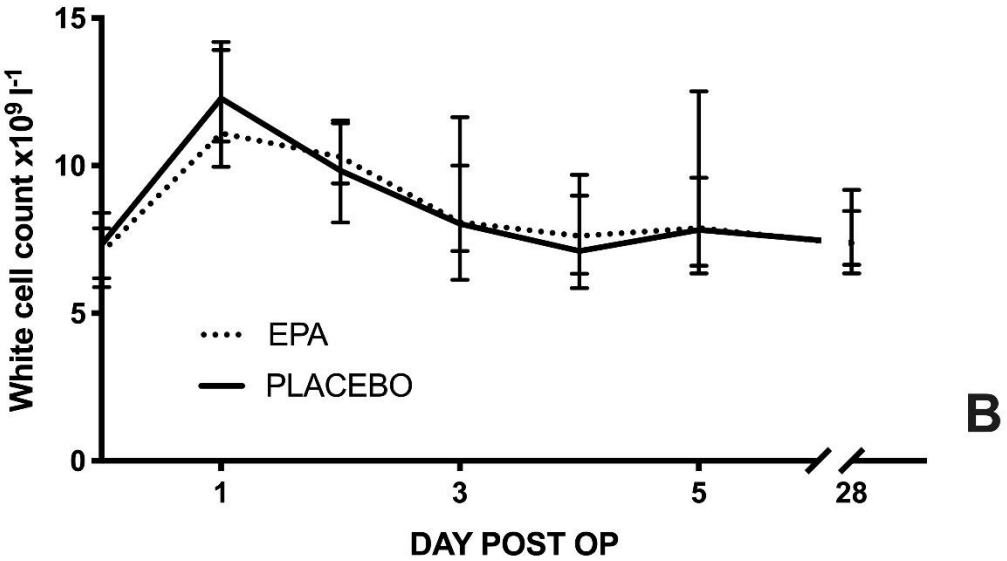

Assessed for eligibility (n=120)
Excluded (n=56)
♦ Not meeting inclusion criteria (n=49)
♦ Declined to participate (n=7)
♦ Other reasons (n=0)
Randomized (n=64)
3 Drop-outs
♦Exclusion criteria newly highlighted n=1
♦Patient withdrew n=2
Allocated to EPA (n=32)
Allocated to Placebo (n=29)
FIRST VISIT
SURGERY
RETAINED IN STUDY (31)
Lost to follow-up (did not attend follow up visit) (n=1)
RETAINED IN STUDY (29)
Lost to follow-up (n=0)
SECOND VISIT
Analysed (intention to treat, with imputation of data) (n=32)
Analysed (intention to treat, with imputation of data) (n=29)

504


Lean muscle mass (grammes)
60000
40000
20000
0
*
EPA Preop
EPA Postop
Placebo Preop
Placebo Postop

505


TEMPERATURE (ºC)
38.5
38.0
37.5
37.0
36.5
36.0
EPA
PLACEBO
A
0
2
4
6
DAY POST OP


White cell count x10⁹ l⁻¹
15
10
5
0
EPA
PLACEBO
B
1
3
5
28
DAY POST OP

506

507

Table 1. Patient baseline data

	EPA	Placebo	*P*
Patient Number	32	29	
Gender Ratio (M:F)	24:8	18:11	0.41[f]
Age (years)	69.03 (7.95)	67.42 (6.92)	0.41[t]
Height (cm)	171 (166.5–175)	168 (162-174)	0.16[m]
Weight (kg)	80.78 (16.44)	86.10 (18.17)	0.23[t]
Muscle mass (g)	49805±1787	48475±2441	0.66[t]
AT (ml/kg/min)	16.7±0.7	15.1±0.8	0.14[t]
Handgrip strength (kg)	34.46±1.91	31.85±2.11	0.36[t]
Duke Staging: *A* *B* *C*	 12 10 10	 10 7 12	 0.96[c]

508

509

Table 2. Post-operative complications per Clavien-Dindo classification of surgical complications

Morbidity	EPA	Placebo	*Total*
Grade I	6	4	10
Grade II	5	8	13
Grade IIIb	3	1	4
Grade IVa	1	0	1
NIL	17	16	33
Total	32	29	61
P = 0.617 (Fisher's exact test)			

510