Highly purified eicosapentaenoic acid as free fatty acids strongly suppresses polyps in Apc\textsuperscript{Min/+} mice

Lucia Fini\textsuperscript{1,2*}, Giulia Piazzi\textsuperscript{1,3*}, Claudio Ceccarelli\textsuperscript{4}, Yahya Daoud\textsuperscript{5}, Andrea Belluzzi\textsuperscript{6}, Alessandra Munarini\textsuperscript{3,7}, Giulia Graziani\textsuperscript{8}, Vincenzo Fogliano\textsuperscript{8}, Michael Selgrad\textsuperscript{1}, Melissa Garcia\textsuperscript{1}, Antonio Gasbarrini\textsuperscript{2}, Robert M. Genta\textsuperscript{9}, C. Richard Boland\textsuperscript{1} and Luigi Ricciardiello\textsuperscript{1}

\textsuperscript{1}Department of Internal Medicine, Baylor Research Institute and Sammons Cancer Center, Baylor University Medical Center, Dallas, TX, USA; \textsuperscript{2}Department of Internal Medicine, Catholic University of Sacred Heart, Rome, Italy; \textsuperscript{3}Center for Applied Biomedical Research (CRBA), S.Orsola-Malpighi Hospital, University of Bologna, Italy; \textsuperscript{4}Clinical Department of Radiological and Histopathological Sciences, S.Orsola-Malpighi Hospital, University of Bologna, Italy; \textsuperscript{5}Institute for Health Care Research and Improvement, Baylor Health Care System, Baylor University Medical Center, Dallas, TX, USA; \textsuperscript{6}Gastroenterology Unit, University of Bologna, Italy; \textsuperscript{7}Division of Endocrinology, Dept. of Clinical Medicine, S.Orsola-Malpighi Hospital, University of Bologna, Italy; \textsuperscript{8}Department of Nutrition Science, Second University of Naples, Naples, Italy; \textsuperscript{9}Department of Pathology, VA Medical Center, Dallas, TX, USA.

Running title: EPA-FFA represses polyps in Apc\textsuperscript{Min/+} mice

Key words: Apc\textsuperscript{Min/+} mice, familial adenomatous polyposis, colon cancer, COX-2, β-catenin

* These authors equally contributed to this work

Corresponding author: Dr Luigi Ricciardiello, Dipartimento di Medicina Clinica, Universita’ degli studi di Bologna, Via Massarenti 9, Bologna, 40138, Italy. E-mail: luigi.ricciardiello@unibo.it

Abbreviations used in this paper: COX-2, Cyclooxygenase-2; ω-3 PUFAs, ω-3 polyunsaturated fatty acids; CRC, colorectal cancer; EPA-FFA, eicosapentaenoic acid as free fatty acids; IHC,
immunohistochemistry; *wt, wild-type; Ctrl, control; AA, arachidonic acid; EPA, eicosapentaenoic acid; ALA, \( \alpha \)-linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; LO, lipoxygenase; QS, quick score; FAP, familial adenomatous polyposis; NSAIDS, non-steroidal anti-inflammatory drugs

**Competing Interest:** This work was supported by SLA Pharma AG, Switzerland. Dr. Ricciardiello received a research grant by SLA Pharma AG, Switzerland. The authors had total control over the data and its interpretation, and SLA Pharma AG had no input in the formulation or writing of the manuscript.

**Role of each author**

**Lucia Fini:** acquisition of the data, analysis and interpretation of the data, drafting of the manuscript

**Giulia Piazzi:** acquisition of the data, analysis and interpretation of the data, drafting of the manuscript

**Claudio Ceccarelli:** analysis and interpretation of the data, technical support

**Yahya Daoud:** statistical analysis, analysis and interpretation of the data

**Andrea Belluzzi:** acquisition of the data, critical revision of the manuscript for important intellectual content

**Alessandra Munarini:** technical and material support

**Giulia Graziani:** analysis and interpretation of the data, technical support

**Vincenzo Fogliano:** analysis and interpretation of the data, technical support

**Michael Selgrad:** analysis and interpretation of the data, technical support

**Melissa Garcia:** technical and material support

**Antonio Gasbarrini:** analysis and interpretation of the data.

**Robert M. Genta:** analysis and interpretation of the data

**C. Richard Boland:** analysis and interpretation of the data, critical revision of the manuscript for important intellectual content, obtained funding
Luigi Ricciardiello: study concept and design, analysis and interpretation of data, drafting of the manuscript, obtained funding, study supervision.

Statement of Translational Relevance

Today, COX-2 inhibitors could be the best chemopreventive agents against colorectal cancer, but their cardiovascular side effects outweigh their benefits. A new formulation of highly purified EPA supplied as free fatty acid (EPA-FFA), was administered to Apc\textsuperscript{Min/+} mice, leading to a dramatic suppression of polyp number and growth. The magnitude of the results is comparable to those obtained with COX-2 inhibitors and better than most nutraceuticals tested in the same animal model. The marked effect of EPA-FFA on polyp development, in the absence of toxicity, makes EPA-FFA an excellent candidate for both CRC chemoprevention and treatment and possibly could be considered for sporadic colorectal prevention/recurrence in humans.
Abstract

PURPOSE: Although COX-2 inhibitors could represent the most effective chemopreventive tool against colorectal cancer (CRC), their use in clinical practice is hampered by cardiovascular side effects. Consumption of ω-3 polyunsaturated fatty acids (ω3-PUFAs) is associated with a reduced risk of CRC. Therefore, in this study, we assessed the efficacy of a novel 99% pure preparation of ω3-PUFA eicosapentaenoic acid as free fatty acids (EPA-FFA) on polyps in Apc\textsuperscript{Min/+} mice. EXPERIMENTAL DESIGN: Apc\textsuperscript{Min/+} and corresponding wild-type mice were fed control diet (Ctrl) or diets containing either EPA-FFA 2.5% or 5%, for 12 weeks while monitoring food intake and body weight. RESULTS: We found that both EPA-FFA diets protected from the cachexia observed among Apc\textsuperscript{Min/+} animals fed Ctrl diet (p<0.0054), without toxic effect, in conjunction with a significant decrease in lipid peroxidation in the treated arms. Moreover, both EPA-FFA diets dramatically suppressed polyp number (by 71.5% and 78.6%, respectively, p<0.0001) and load (by 82.5% and 93.4%, respectively p<0.0001) in both small intestine and colon. In addition, polyps <1mm were predominantly found in the EPA-FFA 5% arm while those 1-3 mm were more frequent in the Ctrl arm (p<0.0001). Interestingly, in the EPA-FFA groups, mucosal arachidonic acid was replaced by EPA (p<0.0001), leading to a significant reduction in COX-2 expression and β-catenin nuclear translocation. Moreover, in the EPA-FFA arms, we found a significant decrease in proliferation throughout the intestine together with an increase in apoptosis. CONCLUSIONS: Our data make 99% pure EPA-FFA an excellent candidate for CRC chemoprevention.
Introduction

Colorectal cancer (CRC) is the fourth commonest cancer in the US and Western countries, with a global incidence of one million cases every year, and mortality of 500,000 (1). An important strategy for the prevention of CRC could involve pharmacological interference with the multi-step carcinogenesis at an initial stage of tumor development (2). Although cyclooxygenase-2 (COX-2) inhibitors still represent an effective chemopreventive tool against CRC, their use in clinical practice is hampered by cardiovascular and gastrointestinal side effects (3-5).

The role of diet in modulating CRC risk is a well-accepted concept, and natural compounds which have been proven safe over time and easily accessible through the diet represent ideal candidates as chemopreventive agents. The consumption of ω-3 polyunsaturated fatty acids (ω-3 PUFAs) has been associated with a reduced risk of CRC (6). One hypothesis is that their anti-neoplastic effects would involve the incorporation of ω-3 PUFAs into cellular membranes replacing the ω-6 PUFA arachidonic acid (AA) with a consequent reduction of pro-inflammatory mediators (7).

In particular, ω-3 PUFAs produce a growth inhibitory effect in colon cancer cells and animal models (8). In clinical studies, dietary supplementation with ω-3 PUFAs significantly reduces crypt proliferation and increases mucosal apoptosis (9). ω-3 PUFAs include the essential fatty acid α-linolenic acid (ALA) and its metabolites eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). There is evidence that these fatty acids have specific effects on disease prevention, but most clinical trials are designed using fish oil-rich meals instead of ω-3 PUFAs supplementation, and have failed to differentiate among them. Moreover, differences in dosage, combination and formulation represent obstacles for reaching a definitive conclusion (10, 11). In addition,
commercially available fish oils are supplied as ethyl esters which are up to five-fold less bioavailable compared to free fatty acids (FFAs) (12, 13). Because the FFAs formulation does not require hydrolysis by pancreatic lipase, it is more efficiently absorbed, and is subsequently reconstituted into triglycerides in enterocytes. In an attempt to clarify the effectiveness of EPA as a single compound in colon cancer prevention, we evaluated the effects of substituting an innovative formulation of 99% (highly pure) EPA as the free fatty acid (EPA-FFA) for other dietary fats on the development of polyps in the gastrointestinal tract of Apc\textsuperscript{Min/+} mice, the most widely used animal model to study the chemopreventive potential of dietary nutrients.

Methods

Animal treatments and tissue harvesting

This study was approved by the Institutional Animal Care and Use Committee at the Baylor Research Institute of Dallas, USA. Five weeks old male Apc\textsuperscript{Min/+} mice on a C57BL/6J background and corresponding wild-type mice (n=48) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and housed in a temperature and humidity controlled animal facility with a 12h light/dark cycle. To avoid gender related differences during the study protocol, only male animals were used in this study. After one week, mice were evenly randomized in six groups to be fed the control diet (Ctrl) or diets enriched with EPA-FFA 2.5% or EPA-FFA 5%. The Ctrl diet was based on a modified AIN-93G diet (Research Diets, New Brunswick, NJ) where corn oil, which is ω-6 PUFAs predominant, was substituted for soybean oil that contains both ω-3 and ω-6 PUFAs (Supplementary Table 1). EPA-FFA diets were obtained from the Ctrl diet substituting highly purified (99%) EPA-FFA (ALFA\textsuperscript{™}, SLA Pharma AG, Liestal, Switzerland) for corn oil. The three
diets were isocaloric. In order to preserve EPA-FFA stability, the food was enriched with antioxidant vitamins, sealed and nitrogen flushed in foil bags, stored at 4°C and used within 3 days after opening. Based on animal strain requirements and previous experience, a fixed equal amount of food was provided daily. Mice were allowed to drink tap water ad libitum. Body weight was monitored weekly. After 12 weeks, mice were sacrificed by cervical dislocation under anesthesia with isofluorane (IsoFlo, Burns Vet Supply, Saint Paul, MN) and blood was collected by intracardiac puncture and immediately stored at -80°C. The entire gastrointestinal tract was immediately removed, washed with phosphate buffered saline (PBS), and divided into five segments [I-IV from proximal small intestine (I) to distal small intestine (IV), and colon]. Each segment was cut longitudinally and rinsed with PBS. Fresh tissue samples were collected and stored at -80°C. Segments were then flattened on filter paper and fixed overnight in 10% buffered formalin. The following day, segments were washed with PBS, and stained with 0.2% methylene blue (Sigma Aldrich, St. Louis, MO) in PBS. The number, location, and size of visible tumors were determined at 10X magnification using an Olympus SZX-ILLB100 microscope (Center Valley, PA) by two independent and blinded investigators. Polyp size was determined by caliper measurement of the largest (Dim1) and the perpendicular diameter (Dim2). Based on Dim1, polyps throughout the intestinal tract were classified into 3 categories (<1mm, 1-3 mm, >3mm). Polyp area (A) was calculated according to the equation \( A = \pi \times \frac{\text{Dim1}}{2} \times \frac{\text{Dim2}}{2} \). Polyp load was expressed as the sum of all the polyp areas.

**Histological and immunohistochemical analysis**

Small intestine and colon were Swiss-rolled, formalin-fixed and paraffin-embedded. One slide for each specimen was stained with hematoxylin and eosin and evaluated independently by two blinded pathologists for histological characterization of the polyps (low grade/high grade dysplasia). For
immunohistochemistry (IHC), slides were dewaxed, rehydrated and subjected to peroxidase inhibition and antigen retrieval with citrate buffer pH 6.0 at 98°C for 40 min. Before incubation with mouse monoclonal antibodies, samples were layered for 1h with goat anti-mouse IgG (Vector Laboratories, Burlingame, CA; dilution 1:50). Slides were incubated with rabbit monoclonal anti-COX-2 (clone SP21, Thermo Scientific, Fremont, CA; dilution 1:150), mouse monoclonal anti-Ki-67 (clone MM1, Leica Biosystems Newcastle Ltd, Newcastle, UK; dilution 1:1400) and mouse monoclonal anti-β-catenin (clone 14, BD Transduction, Franklin Lakes, NJ; dilution 1:2500) and processed using the non-biotin amplified complex (NovoLink Polymer Detection System, Leica Biosystems, Newcastle, UK) according to the manufacturer's procedure.

COX-2 was quantified according to positive tumor cell percentage and staining intensity (Quick Score, QS) (14). The Ki-67 proliferation index was expressed as the ratio between positive nuclei and total number of nuclei per crypt analyzed on 8 and 15 full length, well orientated, longitudinal crypts from the small intestine and colon, respectively.

**Apoptosis analysis**

Apoptosis was evaluated with the DeadEnd Fluorimetric TUNEL System (Promega, Madison, WI), according to the manufacturer’s recommendations.

**Determination of lipid peroxidation**

Lipid peroxidation was evaluated on blood samples by malondialdehyde (MDA) (15). The concentrations were expressed as nmol MDA per mL of serum.

**Mucosal fatty acid analysis**
Normal tissue samples were collected from the small intestine (I segment). Each mucosal fatty acid content was determined as described (16). Fatty acid levels are expressed as relative percentages of total fatty acids. Heptadecanoic acid (17:0) was used as internal standard.

**Statistical analysis**

Sample sizes were based on the average number of polyps and variances in the Apc$^{Min/+}$ strain. We established that eight mice in each group would be sufficient to detect a difference in polyp means among the three groups with a power of 85% and a minimal reduction of 15% (one-way ANOVA test). Relative weight gain was defined as: (weight at time t – weight at time t$_0$) / weight at time t$_0$.

A generalized linear mixed model was used to evaluate relative weight gain changes over time and among the three groups. ANOVA test was used to analyze continuous variables while Chi square and Fisher exact tests were applied for categorical variables. Correlation analysis was used to evaluate the relationship between variables. JMP version 8.02 (Cary, NC) and SAS version 9.2 (Cary, NC) were used for the statistical analysis. Significance was assigned at p<0.05.
Results

EPA-FFA diets abrogate the effect of genotype on body weight and protect from lipid peroxidation

The relative weight gain profiles are shown in Supplementary Figure 1 and Supplementary Table 2. Wild-type and Apc^{Min/+} mice displayed increasing profiles of relative weight gain when fed either EPA-FFA diets while the Apc^{Min/+} Ctrl arm exhibited a significant fall after 9 weeks (p<0.0054), corresponding to the period when polyp appearance had significant health impact on the mutant mice. Moreover, we tested whether EPA-FFA supplementation, by altering the pool of total fats, would affect lipid peroxidation. As shown in Figure 1, as expected the highest level of MDA was registered among Apc^{Min/+} animals fed Ctrl diet. We found that EPA-FFA 5% significantly reduced lipid peroxidation compared to Ctrl diet in both wild-type and Apc^{Min/+} mice (p<0.0001). A reduction was also observed for the EPA-FFA 2.5% arms, although it reached statistical significance only in the Apc^{Min/+} group (p<0.0015).

EPA-FFA diets markedly suppress polyp number

We evaluated the effects of EPA-FFA on the development of polyps in the gastrointestinal tract of Apc^{Min/+} mice, which spontaneously develop multiple intestinal polyps within several weeks of birth (17-19). Our results showed that both EPA-FFA diets markedly suppressed polyp number (Table 1). Similar to previous reports (17, 18, 20), the Apc^{Min/+} Ctrl group displayed 38.63±7.44 polyps per animal. Both the treated arms showed significantly fewer polyps per animal with a mean of 11.00±2.14 and 8.25±2.55 for the EPA-FFA 2.5% and EPA-FFA 5% respectively, corresponding to reductions of 71.5% (Ctrl vs. EPA-FFA 2.5%, p<0.0001) and 78.6% (Ctrl vs. EPA-FFA 5%, p<0.0001). Both EPA-FFA diets were equally effective in reducing the number of polyps (EPA-
FFA 2.5% vs. EPA-FFA 5%, p=n.s.). A significant reduction in number of polyps was observed in each segment of the small intestine and colon in the animals fed either EPA-FFA 2.5% or EPA-FFA 5% (p<0.0009). The overall distribution of polyps throughout the intestinal tract after EPA-FFA administration was proportional to the distribution in the Apc<sup>Min/+</sup> mice receiving Ctrl diet (Table 1). All polyps found in the small intestines and colons were characterized by low grade dysplasia apart from the colons of two mice fed the Ctrl diet that developed cancers.

**EPA-FFA significantly affects polyp load and size**

We also examined the effect of EPA-FFA treatment on polyp load and size. Polyp load (mm<sup>2</sup>) was 74.2±29.3, 13.0±2.7 and 4.9±2.0 in the Ctrl, EPA-FFA 2.5% and EPA-FFA 5% arms, respectively (Ctrl vs. EPA-FFA 2.5%, p<0.0001; Ctrl vs. EPA-FFA 5%, p<0.0001; EPA-FFA 2.5% vs. EPA-FFA 5%, p=n.s.) corresponding to a reduction by 82.5% and 93.4% for the EPA-FFA 2.5% and EPA-FFA 5% compared to the Ctrl diet, respectively. As shown in Figure 2, polyps measuring <1mm were significantly more common in the EPA-FFA 5% arm compared to the other groups, whereas polyps 1-3 mm in size were predominantly found in the Ctrl group. No polyps greater than 3 mm were found in the EPA-FFA 5% diet group.

**Mucosal EPA replaces arachidonic acid after EPA-FFA treatment**

The fatty acid composition of non-polypoid small intestinal tissue was evaluated in Apc<sup>Min/+</sup> mice (Table 2). The most representative fatty acids were analyzed, from 16:0 to 22:6 (palmitic acid to docosahexaenoic acid). Mucosal EPA content increased significantly in the EPA-FFA 2.5% and EPA-FFA 5% arms (p<0.0001), in which the amount of EPA was approximately one-fourth to one-fifth of the total mucosal fatty acid content. Interestingly, the highest EPA incorporation was detected in the EPA-FFA 2.5% group, reaching statistical significance compared to EPA-FFA 5%
Contrariwise, the percentage of AA present in the cellular membranes was dramatically reduced from 11.7±2.6 in the Ctrl arm to 1.38±0.23 and 1.62±0.24 in the EPA-FFA 2.5% and EPA-FFA 5% groups, respectively (Ctrl vs. EPA-FFA 2.5%, p<0.0001; Ctrl vs. EPA-FFA 5%, p<0.0001). A significant negative correlation was found between AA or its precursor (linoleic acid) and EPA or its metabolites (DPA and DHA) suggesting that the pro-inflammatory ω-6 PUFAs were displaced by EPA-FFA (Supplementary Table 3).

**EPA-FFA decreases COX-2 expression and β-catenin nuclear translocation**

Based on the widely accepted concept that ω-3 PUFAs can modulate COX-2 expression, we evaluated the impact of EPA-FFA supplementation on COX-2 and β-catenin expression in the Apc\(^{Min/+}\) mice using IHC (Figures 3 and Supplementary Figure 2). Compared to the Ctrl diet, in both the small intestine and colon, the COX-2 QS was markedly reduced by both EPA-FFA 2.5% and EPA-FFA 5%, with no differences between EPA-FFA diets in the small intestine, and a greater effect in the EPA-FFA 5% arm in the colon (EPA-FFA 2.5% vs. EPA-FFA 5%, p=0.04). Interestingly, in each arm there were no differences in COX-2 QS values between colon and small intestine suggesting that COX-2 inhibition occurs equally in both small and large intestine. Furthermore, nuclear β-catenin translocation was frequently found in the dysplastic areas of the small intestine and colon of the Apc\(^{Min/+}\) animals fed the Ctrl diet, with a reduction of positive nuclei in both EPA-FFA treated mice (Supplementary Figure 2). Interestingly, no positive nuclei were found in the colons of any treated animals.

**EPA-FFA inhibits cellular proliferation and increases apoptosis**

We evaluated cellular proliferation in both the small intestines and colons of Apc\(^{Min/+}\) mice (Figure 4). In the small intestine, we found a decrease in the Ki-67 proliferation index in both EPA-FFA
treated arms, reaching statistical significance only with EPA-FFA supplementation at the highest concentration (Ctrl vs. EPA-FFA 5%, \(p=0.038\)). In the colon, both EPA-FFA diets significantly inhibited cellular proliferation (Ctrl vs. EPA-FFA 2.5%, \(p=0.0001\); Ctrl vs. EPA-FFA 5%, \(p=0.0003\)). Finally, an increase in apoptosis was observed among the EPA-FFA treated animals in both the small intestines and colons of Apc\(^{Min/+}\) mice (Supplementary Figure 3).

**Discussion**

In this study, we tested the efficacy of an innovative formulation of eicosapentaenoic acid - 99% (highly) purified and supplied as the free fatty acid (EPA-FFA) - and showed that it strongly suppressed polyp number (by 71.5% and 78.6% in EPA-FFA 2.5% and 5% groups compared to the Ctrl) and polyp load (by 82.5% and 93.4% in EPA-FFA 2.5% and 5% groups compared to the Ctrl) in Apc\(^{Min/+}\) mice. In addition, EPA-FFA significantly affected polyp size in a concentration-dependent manner. This was also reflected by protecting against weight loss associated with the mutant genotype. These effects were associated with a suppression of COX-2 expression and reduction of \(\beta\)-catenin nuclear translocation and cellular proliferation, together with an increase in apoptosis. Importantly, the decreased proliferation and enhanced apoptosis found in both small intestines and colons of Apc\(^{Min/+}\) mice fed EPA-FFA 5% could explain why smaller polyps were present in this group. *In vitro* studies have identified many ‘ideal’ agents for CRC chemoprevention, which then failed when tested in more complex systems (2, 21, 22). The Apc\(^{Min/+}\) mouse, a model of FAP, is widely used to evaluate the chemopreventive potential of dietary nutrients and chemotherapeutic agents before being tested in clinical settings. Many compounds are effective in
reducing polyps in this model (17-19, 23). In particular, non-steroidal anti-inflammatory drugs (NSAIDs) have significant tumor suppressive activity in animal models, with reductions ranging from 64-90% with sulindac and celecoxib (21). In clinical settings, anti-COX-2 drugs, extensively tested in secondary prevention studies, are highly effective, although their long-term administration is associated with a significant increased risk of cardiovascular toxicity (3-5, 24). Many micronutrients such as polyphenols or vitamins, have been identified as possible chemopreventive agents, although the magnitude of the effects is smaller when compared with synthetic compounds, and the results in the clinical trials have been controversial (21). Importantly, caloric intake, as well as the quality and the relative percentage of macronutrients, are involved in the pathogenesis of CRC. Specifically, the quantity (total fats), quality (animal or vegetable) and type (saturated, mono-saturated or poly-unsaturated) of fats have been shown to influence polyp formation in Apc\textsuperscript{Min/+} mice (25).

In the present study, using eicosapentaenoic acid as the free fatty acid (which is completely absorbable) at the highest purity ever tested, we observed a dramatic suppression of polyp number and polyp burden. Moreover, the EPA-FFA preparation significantly affected polyp size in a concentration-dependent manner. The magnitude of these results suggests that EPA-FFA are as effective as NSAIDs as preventive agents, and may be more potent than other nutraceuticals. However, based on the design of the study, with the drug administered starting from week 6, we cannot distinguish whether the effects were on suppression of formation or regression of existing polyps.

In the last few decades, there has been ongoing interest in the role of \( \omega-3 \) PUFAs in CRC prevention. The anti-neoplastic mechanism of \( \omega-3 \) PUFAs, including EPA, is not fully understood (7). One hypothesis is that EPA incorporates into the cellular membranes competing with the \( \omega-6 \) PUFA
AA as the substrate for COX and lipoxygenases (LO). AA and ω-3 PUFAs are metabolized by COX into prostanoids and by LO to leukotrienes respectively, resulting in opposite effects on tumor growth, apoptosis, angiogenesis and inflammation (26-28). In addition, ω-3 PUFAs can also be converted into potent anti-inflammatory mediators such as resolvins and protectins (29, 30). It is reasonable to speculate that the dietary balance between ω-3 and ω-6 PUFAs would impact the carcinogenic process.

In the diets of the present study, EPA-FFA (ω-3 PUFA) was substituted for corn oil (ω-6 PUFAs) in the Ctrl diet. Dietary modification in the ω-3/ω-6 PUFAs ratio was reflected by changes in the phospholipidic composition of the intestinal mucosa. Mucosal AA was replaced by EPA in the ApcMin/+ mice, demonstrating that the EPA-FFA preparation was efficiently incorporated into cellular membranes. To the best of our knowledge, the levels of EPA incorporation measured in the present study are the highest ever reported in animals or humans. Unexpectedly, the EPA-FFA 2.5% group showed a slightly higher EPA incorporation than the EPA-FFA 5% group. Since our data suggest that the biological findings are dose-dependent, we speculate that the EPA-FFA 2.5% diet may have reached a steady-state level of incorporation in this specific system, but that the 5% diet accumulation might continue in other tissues in which cell turnover is slower (e.g. red blood cells, adipocytes etc). In CRC, over-expression of COX-2 results in increased production of PGE2, affecting cell proliferation, apoptosis, migration, and other signaling pathways, including NF-kB (31) and Wnt. In particular, Castellone and colleagues identified a pivotal role for cross-talk between the COX-2 and β-catenin pathways (32). In our study we found AA replacement by EPA in the cellular membranes, and, as a possible consequence, we observed a strong decrease in COX-2 expression accompanied by a decrease in nuclear β-catenin translocation. In addition, a significant inhibition of cellular proliferation and an increase in apoptosis were found. Although this study
lacks functional data on COX-2 enzyme activity, our results suggest that the macroscopic effects of EPA-FFA on Apc<sup>Min</sup/><sup>+</sup> tumorigenesis could be explained by modulation of the two major pathways involved in CRC development: COX-2 and β-catenin/Wnt signalling.

In the present study, mutant and wild-type treated mice displayed comparable weight gain profiles, while mice receiving the Ctrl diet showed cachexia or excess in weight gain, respectively. Our data show that EPA-FFA treatment efficiently prevents weight loss in Apc<sup>Min</sup/><sup>+</sup> mice, abrogating the effect of the genotype. On the contrary, in the wild-type arms, EPA-FFA treatment contributed to weight gain control. In fact, it has been reported that ω-3 PUFA treatment can modulate adipose tissue function, preventing hyperplasia and hypertrophy, and controlling adipose tissue inflammation (33).

ω-3 PUFAs consumption is safe and well tolerated (34). However, in long-term treatment, some concerns have been raised that higher doses of ω-3 PUFAs would increase lipid peroxidation, which could be controlled by concomitant antioxidant supplementation (7). Importantly, we found significantly decreased lipid peroxidation in treated animals in a dose-dependent manner, which was also found in wt animals. Furthermore, in order to balance for possible differences in oxidative stress, diets also included an equal amount of vitamin mix (10 gm of mix/kg of diet) containing substantial amounts of antioxidants vitamins, including vitamin E (75 IU).

Previous studies have demonstrated a protective role for ω-3 PUFAs as ethyl esters in Apc<sup>Min</sup/><sup>+</sup> mice, and reported a 40-50% reduction in tumor load (35, 36). A comparable amount of EPA-FFA produces a stronger effect, which makes the highly purified EPA-FFA preparation similar in potency to the most powerful pharmacological agents. It is likely that the impact of these lipids is
influenced by the type (EPA vs. DHA), form (free fatty acid vs. ethyl ester) and purity of the ω-3 PUFAs used.

There is evidence that individual ω-3 PUFAs may have specific and independent effects (10). In Apc\(^{Min/+}\) mice, Petrik et al. reported tumor suppression of 48% in EPA-fed mice, whereas weaker effects were obtained with DHA (36). Moreover, better absorption was reported for EPA over DHA (13). Overall, most clinical trials report a strong and consistent association between EPA intake and reduced risk for colorectal neoplasia, whereas the evidence related to DHA alone has been unconvincing (11).

Commercially available fish oils are mainly supplied as ethyl esters because is less expensive, more malleable and conveniently processed for distribution. Conflicting reports have been published on fatty acid ethyl esters bioavailability. Some reports suggest that EPA supplied as ethyl ester is ineffective compared to free acid or trygliceride (37, 38), while others found that ethyl ester and triglyceride are equipotent in humans (13, 39-42) In our study, fish oil supplementation was given as FFAs, which are efficiently absorbed and reconstituted into triglycerides by enterocytes, and do not require hydrolysis by pancreatic lipase. The magnitude of this effect is considerably greater than previous reports using ω-3 PUFA supplementation. We believe that this difference could be explained by the characteristics of the FFA formulation. Although this is the first study using EPA-FFA in animal models, this particular formulation was recently tested in patients who underwent colectomy and ileorectal anastomosis for FAP, and a significant decrease in polyp number and size in the rectum was observed among those who received EPA-FFA for 6 months compared to matched controls (16). Finally, the same formulation has been tested in a clinical trial reporting a greater reduction in cellular proliferation and increase in apoptosis in the normal mucosa of patients with colorectal adenomas than had been seen in an equivalent trial in which ω-3 PUFAs were
administered as ethyl esters (9, 43). In conclusion, EPA-FFA is an attractive candidate to reduce cancer risk in an effective, safe and inexpensive way. More importantly, this intervention has efficacy that resembles what has been obtained with NSAIDs and coxibs, but without the attendant toxicity. The important findings of both the clinical trial and the present study strongly suggest that EPA-FFA could be a successful candidate as chemopreventive agent in FAP mutation carriers. Also, it is reasonable to speculate that this approach could be considered in relation to sporadic colorectal prevention/recurrence in humans.

Acknowledgements

We thank Prof. Mark A. Hull for the critical review of the manuscript.
References


10. Anderson BM, Ma DW. Are all n-3 polyunsaturated fatty acids created equal? Lipids Health Dis 2009;8:33.


36. Petrik MB, McEntee MF, Johnson BT, Obukowicz MG, Whelan J. Highly unsaturated (n-3) fatty acids, but not alpha-linolenic, conjugated linoleic or gamma-linolenic acids, reduce tumorigenesis in Apc(Min/+) mice. J Nutr 2000;130:2434-43.


38. Hudson EA, Tisdale MJ. Comparison of the effectiveness of eicosapentaenoic acid administered as either the free acid or ethyl ester as an anticachectic and antitumour agent. Prostaglandins Leukot Essent Fatty Acids 1994;51:141-5.


Tables

Table 1. Effect of EPA-FFA dietary supplementation at different concentrations on polyp number in Apc\textsuperscript{Min/+} mice (mean±SD)

<table>
<thead>
<tr>
<th></th>
<th>Ctrl (n=8)</th>
<th>EPA-FFA 2.5% (n=8)</th>
<th>EPA-FFA 5% (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Small Intestine</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I Segment</td>
<td>3.50±0.53</td>
<td>1.38±0.74</td>
<td>1.75±1.28</td>
</tr>
<tr>
<td>II Segment</td>
<td>5.0±1.2</td>
<td>2.13±0.99</td>
<td>2.00±0.76</td>
</tr>
<tr>
<td>III Segment</td>
<td>11.88±4.19</td>
<td>3.25±1.04</td>
<td>1.88±1.13</td>
</tr>
<tr>
<td>IV Segment</td>
<td>17.00±3.25</td>
<td>4.00±1.51</td>
<td>2.00±0.93</td>
</tr>
<tr>
<td><strong>Total Small Intestine</strong></td>
<td>37.38±7.07</td>
<td>10.75±2.43</td>
<td>7.63±2.13</td>
</tr>
<tr>
<td><strong>Colon</strong></td>
<td>1.25±1.49</td>
<td>0.25±0.46</td>
<td>0.63±0.74</td>
</tr>
<tr>
<td><strong>Small Intestine+ Colon</strong></td>
<td>38.63±7.44</td>
<td>11.00±2.14</td>
<td>8.25±2.55</td>
</tr>
<tr>
<td><strong>% Reduction</strong></td>
<td></td>
<td>71.5%</td>
<td>78.6%</td>
</tr>
</tbody>
</table>
Table 2. Effect of EPA-FFA dietary supplementation at different concentrations on mucosal fatty acid content (mean±SD)

<table>
<thead>
<tr>
<th>Fatty acid Arm</th>
<th>%Palmitic</th>
<th>%Stearic</th>
<th>%Oleic</th>
<th>%Linoleic</th>
<th>%Linolenic</th>
<th>%AA</th>
<th>%EPA</th>
<th>%DPA</th>
<th>%DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl (n=8)</td>
<td>21.9±2.6</td>
<td>24.1±3.2</td>
<td>14.6±4.1</td>
<td>24.9±2.1</td>
<td>0.14±0.13</td>
<td>11.7±2.6</td>
<td>0.42±0.32</td>
<td>0.11±0.06</td>
<td>1.72±0.30</td>
</tr>
<tr>
<td>EPA-FFA 2.5%</td>
<td>19.7±4.4</td>
<td>22.39±2.95</td>
<td>9.19±5.80</td>
<td>17.4±1.7</td>
<td>0.16±0.07</td>
<td>1.38±0.23</td>
<td>24.2±4.9</td>
<td>2.40±0.29</td>
<td>2.70±0.49</td>
</tr>
<tr>
<td>(n=8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EPA-FFA 5%</td>
<td>19.2±3.1</td>
<td>17.2±2.9</td>
<td>12.6±2.8</td>
<td>24.4±2.5</td>
<td>0.14±0.13</td>
<td>1.62±0.24</td>
<td>20.5±3.4</td>
<td>1.95±0.35</td>
<td>2.02±0.25</td>
</tr>
<tr>
<td>(n=8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure Legends

Figure 1. Effect of EPA-FFA dietary supplementation on lipid peroxidation. Significant protection from lipid peroxidation was observed among EPA-FFA 5% treated groups. Data are presented as mean±SD.

Figure 2. Effect of EPA-FFA dietary supplementation on polyp size in the intestine of Apc\textsuperscript{Min/+} mice. The EPA-FFA 5% arm displayed more polyps measuring ≤ 1 mm compared to the other groups. Data are presented as mean±SD.

Figure 3. EPA-FFA reduces COX-2 expression. (A) Representative COX-2 IHC in tissues from the small intestine and colon of Apc\textsuperscript{Min/+} mice. (B) Quantification of COX-2 staining. A significant reduction in COX-2 expression was observed in the small intestine and colon of EPA-FFA treated animals. Data are presented as mean±SD.

Figure 4. EPA-FFA inhibits cellular proliferation. (A) Representative Ki-67 IHC in tissues from small intestines and colons of Apc\textsuperscript{Min/+} mice. (B) Quantification of Ki-67 staining. While a significant reduction of proliferation was observed in the small intestine and colon of EPA-FFA treated animals. Data are presented as mean±SD.
animals fed EPA-FFA 5%, the effect of EPA-FFA 2.5% was restricted to the colon. Data are presented as mean±SD.
A

**SMALL INTESTINE**

CTRL  | EPA-FFA 2.5%  | EPA-FFA 5%

**COLON**

CTRL  | EPA-FFA 2.5%  | EPA-FFA 5%

B

![Bar chart](https://clincancerres.aacrjournals.org/)

**Quick Score (Mean±SD)**

- **SMALL INTESTINE**
  - CTRL: p<0.0001
  - EPA-FFA 2.5%: p<0.0001
  - EPA-FFA 5%: p=n.s.

- **COLON**
  - CTRL: p<0.0001
  - EPA-FFA 2.5%: p=0.044
  - EPA-FFA 5%: p<0.0001
A

SMALL INTESTINE

CTRL

EPA-FFA 2.5%

EPA-FFA 5%

COLON

B

p=0.038

p=n.s.

p=0.0003

p=0.0001

Positive/total nuclei (Mean±SD)

CTRL

EPA-FFA 2.5%

EPA-FFA 5%

SMALL INTESTINE

COLON
Highly purified eicosapentaenoic acid as free fatty acids strongly suppresses polyps in Apc^{Min/+} mice

Lucia Fini, Giulia Piazzi, Claudio Ceccarelli, et al.

Clin Cancer Res  Published OnlineFirst October 28, 2010.

Updated version  Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-10-1990

Supplementary Material  Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2010/12/08/1078-0432.CCR-10-1990.DC1

Author Manuscript  Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.