

Highly Purified Eicosapentaenoic Acid as Free Fatty Acids Strongly Suppresses Polyps in Apc^{Min/+} Mice

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Abstract

Purpose: Although cyclooxygenase (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 (ω -3-PUFAs) is associated with a reduced risk of CRC. Therefore, in this study, we assessed the efficacy of a novel 99% pure preparation of ω -3-PUFA eicosapentaenoic acid as free fatty acids (EPA-FFA) on polyps in Apc^{Min/+} mice.

Experimental design: Apc^{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^{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 less than 1 mm in size were predominantly found in the EPA-FFA 5% arm whereas those 1 to 3 mm in size 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.

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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Colorectal cancer (CRC) is the fourth commonest cancer in the United States and Western countries, with a global incidence of 1 million cases every year, and mortality of 500,000 (1). An important strategy for the prevention of CRC could involve pharmacologic interference with the multistep 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 antineoplastic 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

Translational Relevance

Today, cyclooxygenase (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*^{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.

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 5-fold less bioavailable compared to free fatty acids (FFA; refs. 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*^{Min/+} mice, the most widely used animal model to study the chemopreventive potential of dietary nutrients.

Material and 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. Five-week-old male *Apc*^{Min/+} mice on a C57BL/6J background and corresponding *wild-type* (*wt*) mice ($n = 48$) were obtained from The Jackson Laboratory and housed in a temperature and humidity controlled animal facility with a 12-hour light/dark cycle. To avoid gender-related differences during the study protocol, only male animals were used in this study. After 1 week, mice were evenly randomized in 6 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,) 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, SLA Pharma AG) for corn oil. The 3 diets were isocaloric. 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. On the basis of 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 isoflurane (IsoFlo, Burns Vet Supply) 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 5 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) in PBS. The number, location, and size of visible tumors were determined at 10 \times magnification using an Olympus SZX-ILLB100 microscope by 2 independent and blinded investigators. Polyp size was determined by caliper measurement of the largest (Dim_1) and the perpendicular diameter (Dim_2). On the basis of Dim_1 , polyps throughout the intestinal tract were classified into 3 categories (<1 mm, 1–3 mm, >3 mm). Polyp area (A) was calculated according to the equation $A = \pi (Dim_1/2) (Dim_2/2)$. Polyp load was expressed as the sum of all the polyp areas.

Histologic 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 2 blinded pathologists for histologic 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 minutes. Before incubation with mouse monoclonal antibodies, samples were layered for 1 hour with goat anti-mouse IgG (Vector Laboratories; dilution 1:50). Slides were incubated with rabbit monoclonal anti-COX-2 (clone SP21, Thermo Scientific; dilution 1:150), mouse monoclonal anti-Ki-67 (clone MM1, Leica Biosystems Newcastle Ltd; dilution 1:1,400) and mouse monoclonal anti- β -catenin (clone 14, BD Transduction; dilution 1:2,500) and processed using the non-biotin-amplified complex (NovoLink Polymer Detection System, Leica Biosystems) according to the manufacturer's procedure.

COX-2 was quantified according to positive tumor cell percentage and staining intensity (quick score, QS; ref. 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), according to the manufacturer's recommendations.

Determination of lipid peroxidation

Lipid peroxidation was evaluated on blood samples by malondialdehyde (MDA; ref. 15). The concentrations were expressed as nanomoles of MDA per milliliter 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. Hep-tadecanoic 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 8 mice in each group would be sufficient to detect a difference in polyp means among the 3 groups with a power of 85% and a minimal reduction of 15% (1-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 3 groups. ANOVA test was used to analyze continuous variables whereas 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 and SAS version 9.2 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 Fig. 1 and Supplementary Table 2. *Wild-type* and

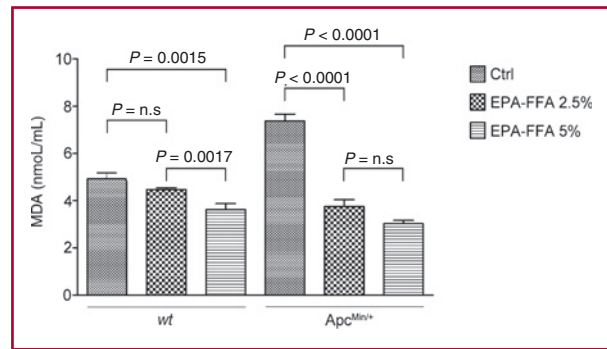


Fig. 1. Effect of EPA-FFA dietary supplementation on lipid peroxidation. Significant protection from lipid peroxidation was observed among EPA-FFA 5% treated groups. Data are presented as mean \pm SD.

$Apc^{Min/+}$ mice displayed increasing profiles of relative weight gain when fed either EPA-FFA diets whereas 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 *wt* 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

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

	Ctrl (n = 8)	EPA-FFA 2.5% (n = 8)	EPA-FFA 5% (n = 8)
Small intestine			
I Segment	3.50 \pm 0.53	1.38 \pm 0.74	1.75 \pm 1.28
II Segment	5.0 \pm 1.2	2.13 \pm 0.99	2.00 \pm 0.76
III Segment	11.88 \pm 4.19	3.25 \pm 1.04	1.88 \pm 1.13
IV Segment	17.00 \pm 3.25	4.00 \pm 1.51	2.00 \pm 0.93
Total small intestine	37.38 \pm 7.07	10.75 \pm 2.43	7.63 \pm 2.13
Colon	1.25 \pm 1.49	0.25 \pm 0.46	0.63 \pm 0.74
Small intestine + colon	38.63 \pm 7.44	11.00 \pm 2.14	8.25 \pm 2.55
% Reduction		71.5	78.6

Table 2. Effect of EPA-FFA dietary supplementation at different concentrations on mucosal fatty acid content (mean \pm SD)

Arm	Fatty acid				
	% Palmitic	% Stearic	% Oleic	% Linoleic	% Linolenic
Ctrl ($n = 8$)	21.9 \pm 2.6	24.1 \pm 3.2	14.6 \pm 4.1	24.9 \pm 2.1	0.14 \pm 0.13
EPA-FFA 2.5% ($n = 8$)	19.7 \pm 4.4	22.39 \pm 2.95	9.19 \pm 5.80	17.4 \pm 1.7	0.16 \pm 0.07
EPA-FFA 5% ($n = 8$)	19.2 \pm 3.1	17.2 \pm 2.9	12.6 \pm 2.8	24.4 \pm 2.5	0.14 \pm 0.13

(Continued on the following page)

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%, $P = \text{n.s.}$). 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 $Apc^{\text{Min/+}}$ 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 2 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^2) 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%, $P = \text{n.s.}$) 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 less than 1 mm were significantly more common in the EPA-FFA 5% arm compared to the other groups, whereas polyps 1 to 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 nonpolypoid small intestinal tissue was evaluated in $Apc^{\text{Min/+}}$ mice (Table 2). The most representative fatty acids were analyzed, from 16:0 to 22:6 (palmitic acid to DHA). 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% ($P = 0.045$). 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, docosapentaenoic acid, 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

On the basis of 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 $Apc^{\text{Min/+}}$ mice using IHC (Figs. 3 and Supplementary Fig. 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%

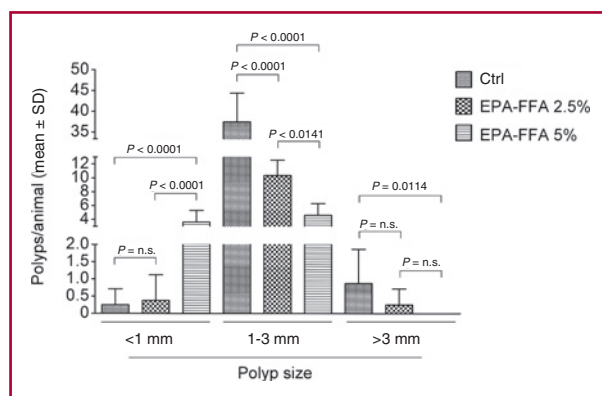


Fig. 2. Effect of EPA-FFA dietary supplementation on polyp size in the intestine of $Apc^{\text{Min/+}}$ mice. The EPA-FFA 5% arm displayed more polyps measuring ≤ 1 mm compared to the other groups. Data are presented as mean \pm SD.

Table 2. Effect of EPA-FFA dietary supplementation at different concentrations on mucosal fatty acid content (mean \pm SD) (Cont'd)

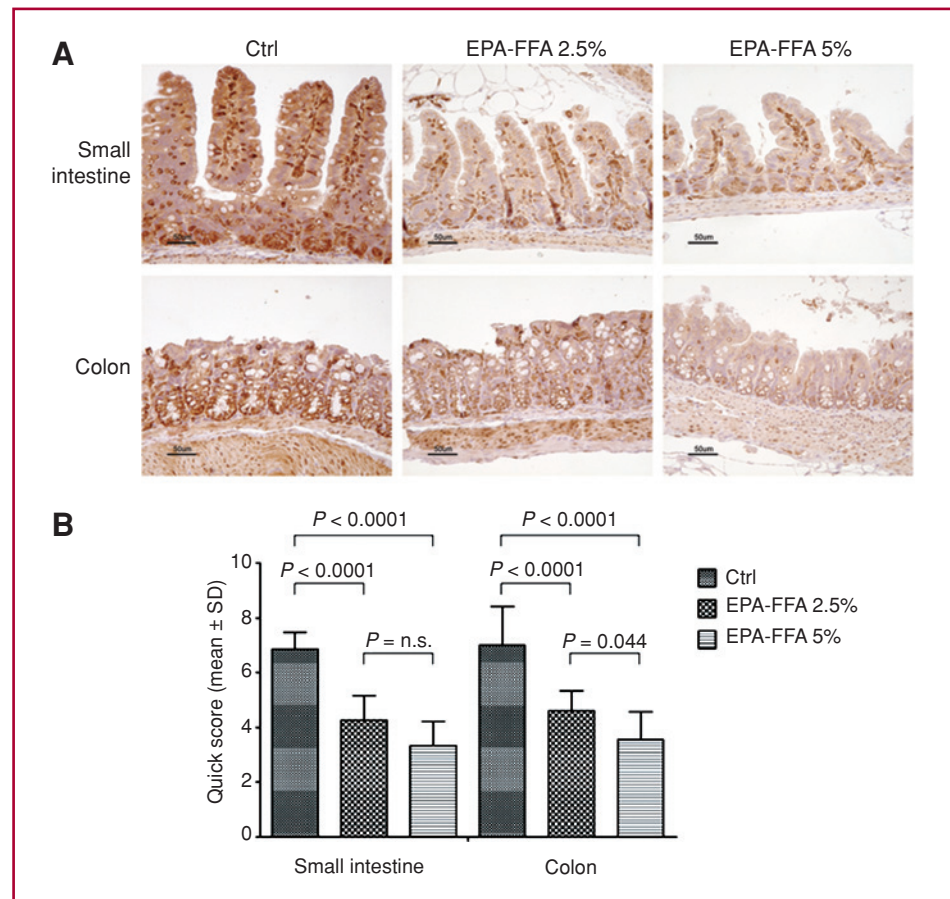
Arm	Fatty acid			
	% AA	% EPA	% DPA	% DHA
Ctrl (<i>n</i> = 8)	11.7 \pm 2.6	0.42 \pm 0.32	0.11 \pm 0.06	1.72 \pm 0.30
EPA-FFA 2.5% (<i>n</i> = 8)	1.38 \pm 0.23	24.2 \pm 4.9	2.40 \pm 0.29	2.70 \pm 0.49
EPA-FFA 5% (<i>n</i> = 8)	1.62 \pm 0.24	20.5 \pm 3.4	1.95 \pm 0.35	2.02 \pm 0.25

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 Fig. 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 (Fig. 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,

Fig. 3. EPA-FFA reduces COX-2 expression. A, representative COX-2 IHC in tissues from the small intestine and colon of *Apc*^{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 \pm SD.



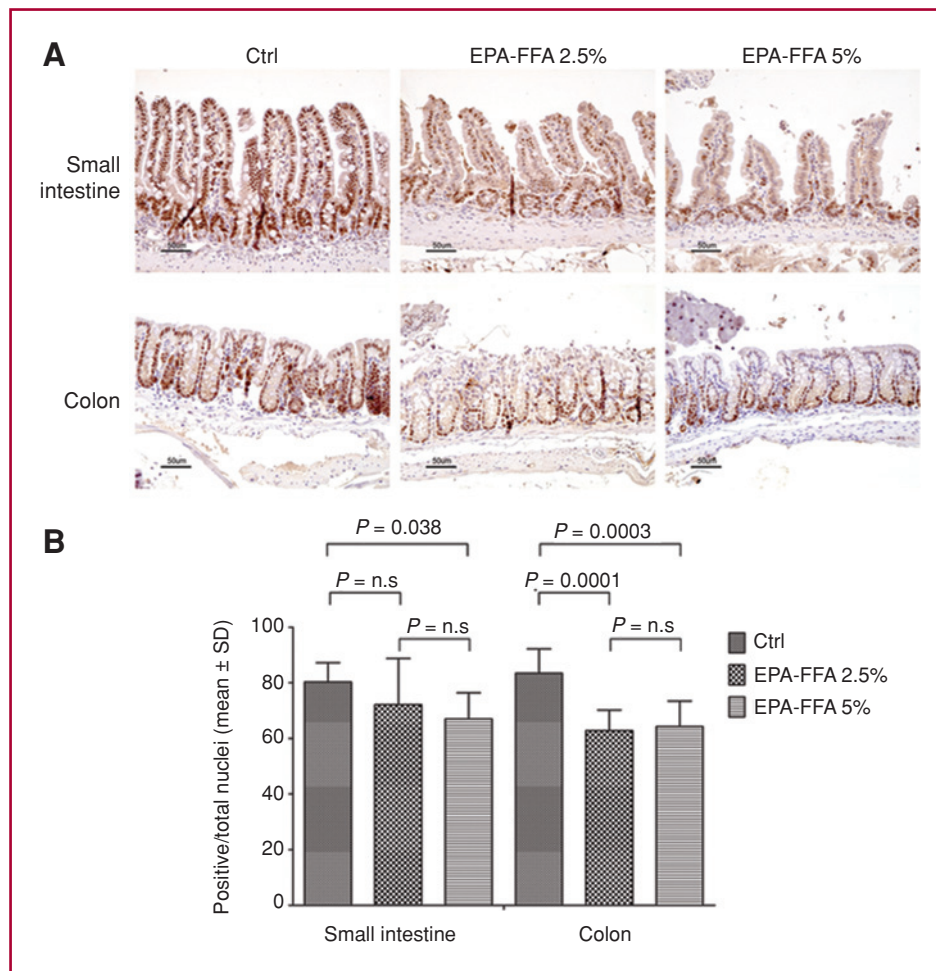


Fig. 4. EPA-FFA inhibits cellular proliferation. A, representative Ki-67 IHC in tissues from small intestines and colons of $Apc^{Min/+}$ mice. B, quantification of Ki-67 staining. While a significant reduction of proliferation was observed in the small intestine and colon of animals fed EPA-FFA 5%, the effect of EPA-FFA 2.5% was restricted to the colon. Data are presented as mean \pm SD.

an increase in apoptosis was observed among the EPA-FFA-treated animals in both the small intestines and colons of $Apc^{Min/+}$ mice (Supplementary Fig. 3).

Discussion

In this study, we tested the efficacy of an innovative formulation of EPA—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 β -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, nonsteroidal anti-inflammatory drugs (NSAIDs) have significant tumor suppressive activity in animal models, with reductions ranging from 64% to 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,

monosaturated or polyunsaturated) of fats have been shown to influence polyp formation in $Apc^{Min/+}$ mice (25).

In the present study, using EPA as the FFA (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 on the basis of 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 polyyps.

In the last few decades, there has been an ongoing interest in the role of ω -3-PUFAs in CRC prevention. The antineoplastic mechanism of ω -3-PUFAs, including EPA, is not fully understood (7). One hypothesis is that EPA incorporates into the cellular membranes competing with the ω -6-PUFA AA as the substrate for COX and lipoxygenases (LO). AA and ω -3-PUFAs are metabolized by COX into prostanooids 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 $Apc^{Min/+}$ 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. As 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, overexpression of COX-2 results in an increased production of PGE₂, affecting cell proliferation, apoptosis, migration, and other signaling pathways including NF- κ B (31) and Wnt. In particular, Castellone and colleagues identified a pivotal role for crosstalk 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 $Apc^{Min/+}$ tumorigenesis could be explained by modulation of the 2 major pathways involved in CRC development: COX-2 and β -catenin/Wnt signaling.

In the present study, mutant and *wt* treated mice displayed comparable weight gain profiles, whereas 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 $Apc^{Min/+}$ mice, abrogating the effect of the genotype. On the contrary, in the *wt* 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, to balance for possible differences in oxidative stress, diets also included an equal amount of vitamin mix (10 gm of mix per 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 $Apc^{Min/+}$ mice, and reported a 40% to 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 pharmacologic agents. It is likely that the impact of these lipids is influenced by the type (EPA vs. DHA), form (FFA 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 they are less expensive, more malleable, and conveniently processed for distribution. Conflicting reports have been published on bioavailability of fatty acid ethyl esters. Some reports suggest that EPA supplied as ethyl ester is ineffective compared to free acid or triglyceride (37, 38), whereas 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 a 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.

Disclosure of Potential Conflicts of Interest

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Supplementary Figures and tables

Supplementary Table 1: Diet characteristics

Diets	AIN-93G		Ctrl Diet		EPA-FFA 2.5%		EPA-FFA 5%	
	gm%	kcal%	gm%	kcal%	gm%	kcal%	gm%	kcal%
Protein	20	20	20	20	20	20	20	20
Carbohydrate	64	64	64	64	64	64	64	64
Fat	7	16	7	16	7	16	7	16
Total		100		100		100		100
Kcal/gm	4.0		4.0		4.0		4.0	
Ingredient	gm	kcal	gm	kcal	gm	kcal	gm	kcal
Casein	200	800	200	800	200	800	200	800
L-Cystine	3	12	3	12	3	12	3	12
Corn Starch	397.486	1589.944	397.486	1589.944	397.486	1589.944	397.486	1589.944
Maltodextrin 10	132	528	132	528	132	528	132	528
Sucrose	100	400	100	400	100	400	100	400
Cellulose, BW200	50	0	50	0	50	0	50	0
<i>Soybean Oil</i>	70	630	0	0	0	0	0	0
<i>Corn Oil</i>	0	0	70	630	45	405	20	180
<i>EPA</i>	0	0	0	0	25	225	50	450
t-Buthylhydroquinone	0.014	0	0.014	0	0.014	0	0.014	0
Mineral Mix S10022G	35	0	35	0	35	0	35	0
Vitamin Mix V10037	10	40	10	40	10	40	10	40
Choline Bitartrate	2.5	0	2.5	0	2.5	0	2.5	0
FD&C Yellow Dye #5	0	0	0	0	0	0	0	0
FD&C Red Dye #40	0	0	0.025	0	0	0	0.05	0
FD&C blue Dye #1	0	0	0.025	0	0.05	0	0	0
Total	1000	4000	1000.05	4000	1000.05	4000	1000.05	4000

Vitamin Mix V10037 (used at 10 gm/kg diet). Amount in 10 gm: Vitamin A Palmitate 4,000 IU; Vitamin D3 1,000 IU; Vitamin E Acetate 75 IU; Phylloquinone 0.75 mg; Biotin 0.2 mg; Cyancocobalamin 25 ug; Folic Acid 2 mg; Nicotinic Acid 30 mg; Calcium Pantothenate 16 mg; Pyridoxine-HCl 7 mg; Riboflavin 6 mg; Thiamin HCl 6 mg; Sucrose 9.7192

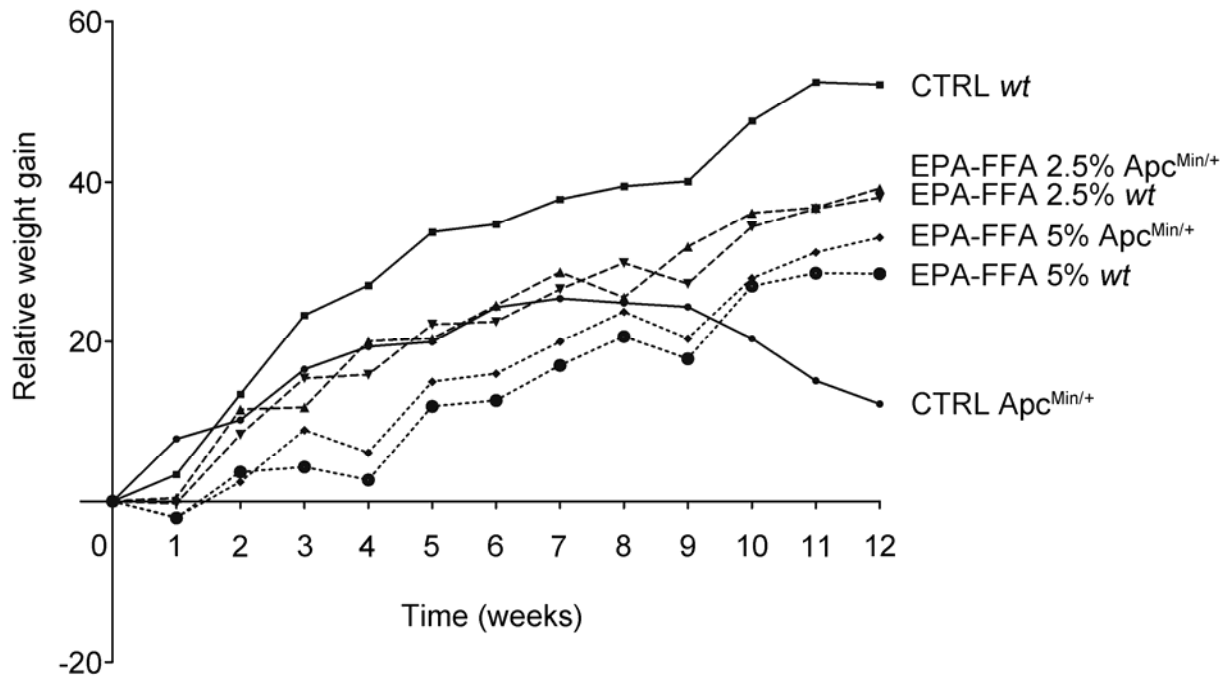
Mineral Mix S10022G (used at 35 gm/kg diet). Amount per 1000 gm of mix: Calcium Carbonate (40% Ca) 357 gm; Potassium Phosphate Monobasic (28.7% K, 22.8% P) 196 gm; Potassium Citrate(36.2% K) 70.78 gm; Potassium Sulfate (44.9% K, 18.4% S) 46.6 gm; Magnesium Oxide (60.3% Mg) 24 mg; Sodium Chloride (39.3% Na, 60.7% Cl) 74 gm; Cupric Carbonate (57.5% Cu) 0.3 gm; Potassium Iodate (59.3% I) 0.01gm; Ferric Citrate (21.2% Fe) 6.06 gm; Manganous Carbonate (47.8% Mn) 0.63gm; Sodium Selenate (41.8% Se) 0.01025 gm; Zinc Carbonate (52.1% Zn) 1.65 gm; Chromium K Sulfate 0.275 gm; Ammonium Molybdate 0.00795 gm; Sodium Silicate 1.45 gm; Lithium Chloride 0.0174 gm; Boric Acid 0.0815 gm; Sodium Fluoride 0.0635 gm; Nickel Carbonate 0.0318 gm; Ammonium Vanadate 0.0066 gm; Sucrose 221.026 gm.

Supplementary Table 2. Relative weight gain (in grams) over 12 weeks (T-01 to T-12, weeks 1-12) (mean±SD)

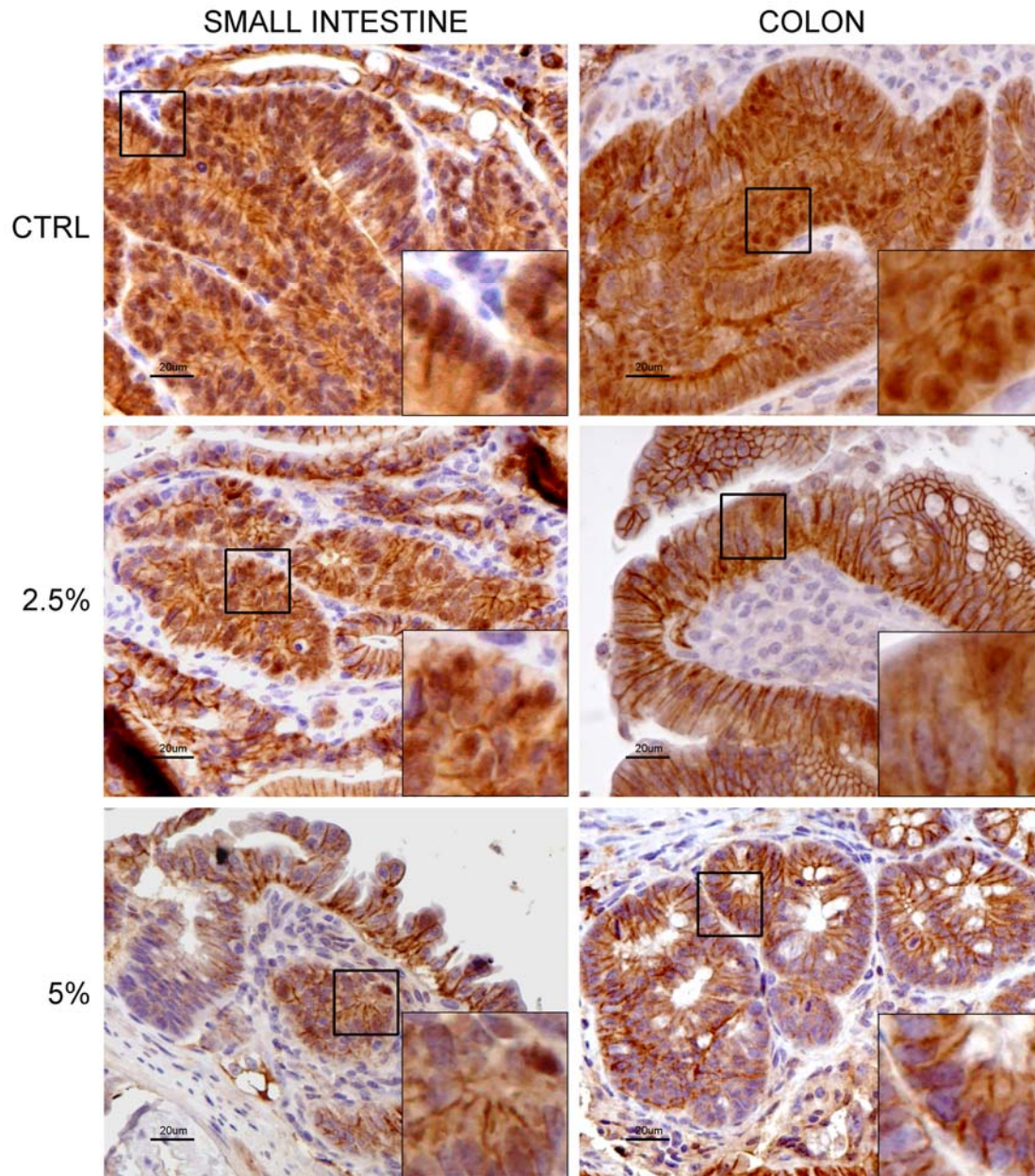
Time period	Apc ^{Min/+} mice			Wild- type mice		
	Ctrl	EPA-FFA 2.5%	EPA-FFA 5%	Ctrl	EPA-FFA 2.5%	EPA-FFA 5%
T-01	7.85± 4.09	0.44± 3.04	-2.00±1.70	3.38±3.45	-0.27±2.74	-2.07±3.89
T-02	10.20 ±4.63	11.51± 1.18	2.44±3.68	13.41± 6.84	8.36±5.73	3.68±5.61
T-03	16.52± 5.60	11.78 ±2.73	8.96±3.58	23.29± 10.57	15.40±6.67	4.31±5.36
T-04	19.34 ±5.19	20.03 2.66	6.12±3.65	27.05± 13.49	15.84±6.29	2.68±3.51
T-05	19.96± 7.32	20.35 ±2.72	14.97±5.13	33.77± 14.61	22.11±7.80	11.91±6.69
T-06	24.28± 10.59	24.50± 4.57	15.97±4.73	34.73± 15.83	22.44±8.37	12.62±6.46
T-07	25.39± 9.84	28.69± 4.16	19.95±6.08	37.83± 16.40	26.54±10.44	16.98±8.72
T-08	24.83± 11.13	25.51 ±5.32	23.71±7.62	39.47± 14.31	29.82±9.16	20.60±6.78
T-09	24.34± 14.06	31.87 ±5.67	20.34±7.44	40.10± 14.10	27.24±9.90	17.83±8.59
T-10	20.36± 15.86	36.10± 3.83	27.91±8.00	47.61± 13.26	34.39±9.25	26.94±9.06
T-11	15.10± 13.47	36.78 ±3.89	31.16±10.89	52.45± 13.04	36.63±8.23	28.57±8.50
T-12	12.20± 13.91	39.19± 4.87	33.02±11.59	52.15± 12.33	38.03±8.16	28.48±8.34

Supplementary table 3. Pairwise correlation analysis of mucosal fatty acid content

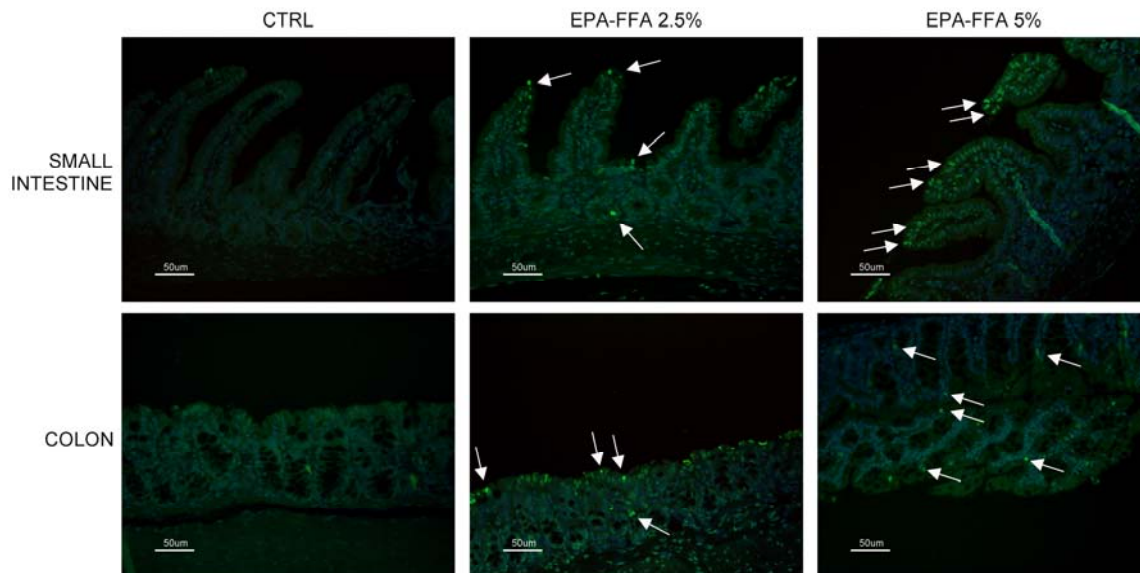
Variable	Vs. variable	Correlation	Lower 95%	Upper 95%	<i>p</i>
DPA	AA	-0.9060	-0.9589	-0.7920	<.0001
EPA	AA	-0.9053	-0.9586	-0.7907	<.0001
DHA	Linoleic	-0.5939	-0.8045	-0.2505	0.0022
DPA	Linoleic	-0.5915	-0.8033	-0.2471	0.0023
DHA	AA	-0.5861	-0.8003	-0.2393	0.0026
EPA	Linoleic	-0.4730	-0.7360	-0.0860	0.0196
AA	Linoleic	0.4577	0.0667	0.7269	0.0245
DPA	EPA	0.9395	0.8633	0.9738	<.0001
DHA	DPA	0.7168	0.4408	0.8689	<.0001
DHA	EPA	0.7100	0.4296	0.8655	0.0001



Supplementary Figure 1. Effect of EPA-FFA dietary supplementation on body weight in $Apc^{Min/+}$ and corresponding *wild-type* mice. EPA-FFA diets protected from the weight loss observed in $Apc^{Min/+}$ animals fed Ctrl diet.



Supplementary Figure 2. Representative β -catenin IHC in dysplastic tissues from the small intestines and colons of $Apc^{Min/+}$ mice. In the EPA-FFA treated groups, reduced β -catenin nuclear translocation was found in the small intestine, while the absence of nuclear staining was observed in the colon.



Supplementary Figure 3. EPA-FFA enhances apoptosis. Representative TUNEL Assays in tissues from small intestine and colon of $Apc^{Min/+}$ mice. An increase in apoptosis was observed in the small intestine and colon of animals fed EPA-FFA 2.5% and 5%. Arrows indicate TUNEL positive cells.