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ORIGINAL ARTICLE

Eicosapentaenoic acid (EPA) reduces crypt cell proliferation and increases apoptosis in normal colonic mucosa in subjects with a history of colorectal adenomas

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Abstract
Background and aims Omega-3 fatty acids in fish oil exert a protective effect on the development of colorectal cancer in animal models. Patients with colorectal adenomas have been shown to have increased crypt cell proliferation and decreased apoptosis in macroscopically normal appearing colonic mucosa. We investigated whether dietary supplementation with eicosapentaenoic acid (EPA) could alter crypt cell proliferation and apoptosis in such patients.
Patients/methods Thirty subjects were randomised to either 3 months of highly purified EPA in free fatty acid form (2 g/day) or to no treatment. Colonic biopsies were taken at the initial colonoscopy and repeated 3 months later, and analysed for cell proliferation and apoptosis (immunohistochemistry) and mucosal fatty acid content.
Results/findings Crypt cell proliferation was significantly reduced whilst apoptosis was significantly increased after EPA supplementation. Neither crypt cell proliferation nor apoptosis were altered in the control group. EPA in the mucosa increased significantly after EPA supplementation, whereas there was no significant change in controls.
Conclusions Dietary supplementation with EPA significantly increases levels of this fatty acid in colonic mucosa, associated with significantly reduced proliferation and increased mucosal apoptosis. Further studies are needed to assess the potential efficacy of EPA supplementation in preventing polyps in the chemoprevention of colorectal cancer.

Keywords Apoptosis · Anoikis · Cell proliferation · Eicosapentaenoic acid · Colorectal neoplasms

Abbreviations

EPA	eicosapentaenoic acid
DHA	docosahexaenoic acid
PUFAs	polyunsaturated fatty acids
BMI	body mass index

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Introduction

In the year 2000, there were an estimated 945,000 new cases of cancer of the colon and rectum in the world, accounting for 9.4% of the total number of estimated new cancers in the world that year [1]. There is an up to 25-fold variation between countries with the highest and lowest incidence of colorectal cancer consistent with underlying

environmental influences [1]. It is thought that the causation of approximately 80% of cases of colorectal cancer is related to diet [2].

Western diets are characterised by relatively high amounts of saturated and animal fat, as well as omega-6 polyunsaturated fatty acids (PUFAs) and a relative deficiency in omega-3 PUFAs [3]. In evolutionary terms, these changes in our diet have occurred rapidly, and are thought to be potent promoters of chronic diseases such as atherosclerosis, obesity, diabetes and a number of cancers [3]. Animal models of colorectal cancer have provided convincing evidence that a Western-style diet high in saturated and animal fats and omega-6 PUFAs promotes colorectal carcinogenesis, whereas diets rich in omega-3 PUFAs do not [4]. In an ecological study, Caygill et al. [5] found an inverse correlation between colorectal cancer mortality rates for 24 European countries and the consumption of fish and fish oil when expressed as a proportion of total or animal fat. This correlation was significant whether the intakes were in the period current to the mortality data or 10 or 23 years before cancer mortality [5]. Notably, this apparent protective effect of fish and fish oil consumption was only seen in countries with high (>85 g caput^{-1} day $^{-1}$) animal fat intake [5].

Fish oils are a rich source of the omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and there is an increasing interest in the potential use of omega-3 fatty acids in the chemoprevention of colorectal cancer [6]. Previous studies have shown that fish oil can reduce abnormally high cell proliferation rates in normal mucosa from subjects with colorectal adenomas [7]. More recently, the importance of apoptosis in the development of a number of cancers has been emphasised, and fish oil has been shown to increase apoptosis in animal models of colorectal carcinogenesis. We therefore wanted to investigate the effect of a highly purified preparation of the omega-3 fatty acid, eicosapentaenoic acid on cell proliferation and apoptosis in the normal appearing mucosa from patients with colorectal adenomas.

Materials and methods

Subjects

Thirty patients found to have one or more colorectal adenomas at colonoscopy were recruited for the study, between May 2003 and June 2004. Patients had been deemed to clinically warrant colonoscopy (see Table 1 for indications) and were recruited from outpatient colonoscopy sessions at the endoscopy unit of St George's Hospital, London. Exclusion criteria were: (1) previously resected colorectal cancer; (2) previously diagnosed inflammatory bowel disease or current evidence of active mucosal inflammation; (3) current acute or chronic life-threatening disease; (4) blood coagulation disorders; (5) pregnancy; (6) family history suggestive of hereditary non-polyposis colorectal carcinoma or familial polyposis coli; (7) use of non-steroidal anti-inflammatory drugs on more than an occasional basis (less than three tablets/week); (8) regular use of folic acid supplements; and (9) use of antibiotics in the 6-week period before recruitment. All subjects gave written informed consent before the colonoscopy. The study was reviewed and approved by the St George's Hospital Ethics committee.

Table 1 Comparison of baseline demographics between the EPA and non-treated groups

	EPA group (n=14)	Non-treatment group (n=14)	P value (test)
Age	53.1±14.1	55.4±11.5	0.653 (t test)
Males/females	11/3	7/7	0.115 (χ^2 test)
BMI	26.4±3.0	26.3±2.9	0.995 (t test)
No. of portions fish per week	1.6±1.0	1.3±0.7	0.427 (Mann–Whitney U test)
Mean units alcohol per week	13.0±6.20	10.3±7.9	0.323 (t test)
Smokers/non-smokers	5/9	2/12	0.190 (χ^2 test)
Indications for colonoscopy (more than one factor may apply)			
Rectal bleeding	8/14	8/14	1.000 (χ^2 test)
Change bowel habit	0/14	1/14	0.309 (χ^2 test)
Family history of colorectal cancer	6/14	5/14	0.699 (χ^2 test)
Previous history of colorectal adenomas	3/14	4/14	0.663 (χ^2 test)

Interventions

Patients underwent colonoscopy under intravenous sedation after bowel preparation with two sachets of sodium picosulphate and fluids only 24 h before the procedure. In addition to polypectomy, six mucosal biopsies were taken 30 cm from the anal margin with 3 mm biopsy forceps. Biopsies were always taken from normal appearing mucosa at least 5 cm away from any polyp. Three biopsies were immediately placed in formalin for immunohistochemistry, and the other three biopsies were snap frozen in liquid nitrogen and stored at −70°C until analysis for mucosal fatty acids.

EPA was administered as an enteric coated oral formulation of highly purified (99%) eicosapentaenoic acid in the

free fatty acid form which is released in the proximal small intestine (ALFA™—SLA Pharma UK, Watford, UK). Patients were randomised into two groups: (1) 2 g of ALFA™ per day for 3 months (two 500 mg capsules with breakfast, and two capsules with the evening meal); (2) no treatment for 3 months (avoiding ingestion of any fish oil supplements). Patients in the EPA group were given six containers each containing 60 capsules (360 capsules in total) and were asked to bring the containers with them at the completion visit for capsule counting. Patients were contacted by telephone after 1 and 2 months of intervention to assess compliance and for any side effects. After 3 months, patients attended for the completion visit when a flexible sigmoidoscopy was performed after preparation by phosphate enema. Six mucosal biopsies were repeated 30 cm from the anal verge and processed in the same manner as after colonoscopy. Whilst some studies have reported that the type of bowel preparation itself can have an effect on the rate of mucosal cell proliferation [8, 9], others have not [10]. However, we standardised the bowel preparation used in both treatment groups to avoid any such bias in the comparison of proliferation rates between the two groups.

Objectives and study endpoints

The main objectives of the study were to determine the tolerability of supplementation with highly purified EPA in patients with colorectal adenomas as well as the effect on mucosal cell proliferation and apoptosis. The primary endpoints were cell proliferation as assessed by Ki-67 labelling indices in the colonic crypts, and mucosal apoptosis as assessed by M30 labelling in the colonic mucosa. The secondary endpoints of the study were to determine the degree of incorporation of EPA into the colonic mucosal phospholipids, as well as any changes in the level of mucosal arachidonic acid levels.

Sample size

Previous studies that have found a reduction in colorectal epithelial proliferation indexes with fish oil supplementation have utilised different methods to measure proliferation in the mucosal biopsies. However, depending on the degree of hyper-proliferation in the baseline biopsies of subjects recruited, percentage reductions in proliferation indices in the fish oil treated group have ranged from 30% [7] to as much as 70% [11]. Therefore, assuming we would obtain at least a 35% reduction in crypt proliferation with omega-3 fatty acid treatment, whilst proliferation in the non-treated group would remain unchanged, we calculated that we would require 15 patients in each group assuming a power level of 80% and a 5% level of significance.

Randomisation details and blinding

Blocked randomisation was used with sequentially numbered sealed envelopes to allocate subjects to each treatment group, with the enrolling investigator being unaware of the block size. All samples were coded to blind them during analysis to the treatment group status of each sample.

Immunohistochemistry

Each of the three mucosal biopsies were orientated in the same plane and embedded together in a paraffin block. Sections (3 μm) were cut at a minimum distance of 40 μm apart to ensure the same crypt was not counted twice, placed on charged slides, dewaxed, and endogenous peroxidase activity was blocked by incubation in a 10% hydrogen peroxidase solution. The Ki-67 antigen was retrieved by incubation for 8 min in Tris–EDTA–citrate buffer pH 7.8 (pre-heated for 24 min) in a microwaveable pressure cooker (Biogenex Laboratories-San Ramon, CA 94583 USA). The M30 antigen was retrieved by incubation in Tris–EDTA pH 9.9 heated for 35 min in 1,000 ml of Tris–EDTA pH 9.9 in an un-pressurised container. All slides were stained on the Menirini Optimax™ autostainer, with modifications. Primary antibodies used were monoclonal mouse anti-human Ki-67 antigen (DakoCytomation) diluted 1 in 6,000, and monoclonal mouse M30 Cyto-DEATH (Roche) diluted 1 in 50. The slides were counter-stained with haematoxylin, differentiated in 1% acid alcohol, dehydrated, cleared, and mounted ready for viewing under the microscope.

Scoring and analysis of ki-67 labelling

All slides were coded to blind them as to the group of origin of each biopsy, and counted by the same examiner. Only full length, well-orientated, longitudinal colonic crypts were counted. As it has previously been shown that at least 12 full-length crypts are required to obtain a reliable estimate of cell proliferation [12], only patients with at least this number of crypts before and after treatment were included in the analysis. Each crypt was divided into two hemi-crypts at the base, and each cell position was numbered from the base of each hemi-crypt to the mucosal surface. Then, the status of each cell, i.e. labelled or un-labelled, was noted at each cell position. The overall labelling index (number of labelled cells divided by the total number of cells) was calculated for the total crypt as well as each of five equal crypt compartments and expressed as a percentage. Due to the relatively low frequency of labelling towards the top of the crypt, the most superficial two compartments (compartments 4 and 5) were combined for analysis. In addition, cell proliferation index distribution curves were constructed for each group

before and after treatment, as they represent a more accurate method for analysing and comparing labelled cells in axially sectioned crypts [13, 14]. These were constructed by plotting the pooled overall frequency of cell labelling against crypt cell position for each group pre- and post-treatment. This allows a good visual comparison of any changes in the relative frequency of labelling of actively cycling cells within the crypt.

Analysis of M30 antibody positivity

As previously reported [15], M30 positivity in normal colonic mucosa was found to be low with apoptotic cells being almost exclusively located in the luminal surface epithelium between the crypts. Apoptotic cells were scored by utilising two different methods. Firstly, each slide was graded on a scale of 0 to 3, after agreement between two examiners, using a previously devised scoring system for M30 positivity [16] as follows: 0 = no positive cells; 1 = single positive cells; 2 = small groups of positive cells; 3 = large areas of adjacent positive cells. Scores were then compared before and after treatment in each group. Secondly, the total numbers of apoptotic cells were counted for each slide and the total length of surface epithelium was measured at an objective magnification of ×100 by tracing all the surface epithelium in each biopsy on a digitiser pad, linked to a computer running Osteomeasure software (Osteometrics, Atlanta, GA, USA). Results were expressed as the number of apoptotic cells per 1,000 microns of surface epithelium.

Mucosal fatty acid analysis

The biopsies were weighed and rinsed in 500 μl of ice cold PBS and then homogenised. The homogenate was transferred into a suitable glass tube and 750 μl of chloroform to methanol (1:2) was added and mixed at room temperature for 30 min. The mixture was then centrifuged at 2,500 rpm and the supernatant placed into a new tube. A further 600 μl of chloroform to methanol (1:2) was then added to the pellet and left to stand for 20 min. This was then centrifuged at 2,500 rpm and combined with the first supernatant. To the supernatants was then added 450 μl of chloroform and 450 μl of water, which were mixed and centrifuged at 2,000 rpm for 10 min as to separate the phases. The extraction was repeated three times for each sample. The extract was evaporated to dryness under nitrogen and rinsed in 400 μl of KOH/methanol and heated at 80°C for 10 min. Then 600 μl of BF_3/methanol was added, the mixture covered and heated for a further 10 min at 80°C. When cooled, three extractions were performed with 1 ml of *n*-hexane. The extract methyl esters were evaporated to dryness and rinsed in 5 μl of *n*-hexane, and then stored at −20°C until analysis.

The fatty acid methyl esters were analysed using a Varian 3400 Gas chromatograph (Varian, Palo Alto, CA, USA) equipped with a Supelco® (Bellefonte, PA, USA) SP-2330 fused capillary silica column (length 30 m, 0.25 mm internal diameter, 0.20 μm film thickness) with a flame ionization detector. Gas flow through the column was 20 ml/min of helium, with a sample injection volume of 1 μl. The column temperature was initially 185°C for 10 min, then increased by 2°C/min to 205°C then held at 205°C for 10 min, with the injection temperature being 220°C. Samples were then detected at 250°C. The areas under the peaks were expressed as percentages of the total areas of all major fatty acid peaks.

Dietary fat intake

To estimate each individual's usual level of fat intake, a previously devised and validated 13-item screening questionnaire for fat intake was administered to each participant, and scored accordingly [17]. This questionnaire has been shown to provide essentially the same amount of information on total fat and saturated, oleic, and linoleic fatty acids as three 4-day food records [17], and shows good correlation with estimates of fat intake derived from a more detailed and established 100-item food frequency questionnaire [18].

Statistical methods

Mean labelling indices for the total and individual five crypt compartments showed a normal distribution and were therefore compared using paired *t* tests. Apoptosis scores and mucosal fatty acid composition were not normally distributed and were therefore analysed using the Wilcoxon signed rank test and the Mann–Whitney *U* test. Statistical analysis was performed using SPSS v 12.0 (SPSS, Chicago, IL, USA).

Results

Compliance and side effects

Of the 30 patients who were recruited, 28 completed the study, whilst one patient from each group withdrew. Of the 14 patients randomised to take the EPA capsules and completing the study, nine patients took 2 g per day for 3 months without any side effects, with pill counts showing over 95% of the prescribed number of capsules having been used. Three patients experienced diarrhoea whilst taking 2 g per day. The diarrhoea subsided when the dosage was reduced in one patient to 1.5 g per day and 1 g in another two. Two patients experienced abdominal pain after using

the capsules and stopped taking the capsules 10 and 14 days before the re-biopsy date. As these patients had taken 2 g per day for over 10 weeks, they were re-biopsied as planned.

Group baseline demographics and clinical characteristics

There were no statistically significant differences between the two groups in the mean age, BMI, number of weekly portions of fish consumed or alcohol and tobacco use or clinical factors leading to colonoscopy (Table 1). There were also no significant differences between the two groups in estimated total fat, saturated fat, oleic and linoleic fatty acid intake (data not shown).

Crypt cell proliferation (ki-67 labelling)

Of the 14 patients recruited to each group, 13 patients in the EPA treated group, and 10 patients in the non-treatment group had at least 12 or more, full-length, well-orientated colonic crypts before and after treatment for analysis of crypt cell proliferation. There were no significant differences in the mean labelling indices of either the whole crypt, or each of the crypt compartments, at the start of the study between the two groups. A summary of the Ki-67 crypt cell proliferation indices before and after treatment in the EPA and non-treatment groups is shown in Table 2. The paired labelling indices for the whole crypts and the upper 40% of the crypts are shown in Fig. 1, respectively.

The labelling index curves from our subjects are very similar in shape to those seen in other published studies of human colonic mucosa [19, 20]. Comparison of the labelling index curves showed a significant downwards shift in the combined frequency of labelling at each crypt cell position after EPA treatment ($p<0.001$, Wilcoxon signed ranks test), whereas the frequency of labelling increased significantly in the non-treatment group ($p<0.001$). Proliferation index labelling curves before and after treatment for the EPA supplemented and no treatments groups are shown in Fig. 2.

Mucosal apoptosis (M30 antibody labelling)

Apoptotic cells were always seen in the luminal epithelium between the crypts. Both the apoptosis grade and the number of apoptotic cells increased significantly after EPA supplementation, and a summary of the changes in apoptosis grade and scores in each group is shown in Table 3. Examples of apoptosis grades 1 to 3 are shown in Fig. 3.

Mucosal fatty acid content

Due to technical reasons, results of mucosal fatty acid content were available only for 10 patients in the EPA treated group and 13 patients in the control group. As expected, mucosal EPA content increased significantly in the EPA-treated group whereas the percentage contribution of EPA to mucosal fatty acids remained largely unchanged in the non-treatment group (Table 4). Whilst the decrease in percentage contribution of arachidonic acid to mucosal fatty acids in the EPA-treated group was not statistically significant ($p=0.093$, Wilcoxon signed ranks test), the mean overall change in arachidonic acid content between the two groups was significantly different ($p=0.021$, Mann–Whitney U test). The paired changes in the percentage contribution of EPA to total mucosal fatty acids in each group are shown in Fig. 4.

Table 2 Summary of Ki-67 cell proliferation in the EPA and non-treatment groups

	EPA group			Non-treatment group		
	Pre	Post	Change	Pre	Post	Change
No. of columns analysed	368	366	–	274	276	–
Mean no. of columns per patient (SD)	28.3 (4.8)	27.5 (4.7)	–	27.4 (6.2)	27.6 (6.4)	
Range	24 – 42	24 –38		24 – 44	24 – 44	
Mean no. of cells per column (SD)	67.6 (7.3)	66.1 (6.31)	–	69.9 (7.3)	68.8 (5.91)	–
Range	58.7 to 80.1	56.9–77.6		59.6–79.3	61.8–76.6	
Mean total crypt LI (SD)	28.3 (7.9)	22.42 (5.5)*	−5.86 (8.8)**	26.1 (6.7)	29.7 (8.1)	3.62 (8.0)
Mean compartment 1 LI (SD)	45.8 (9.8)	46.1 (6.7)	0.23 (11.6)	44.9 (7.2)	49.3 (10.5)	4.3 (11.9)
Mean compartment 2 LI (SD)	49.8 (11.9)	40.15 (11.1)*	−9.62 (15.0)****	45.1 (6.9)	50.5 (13.3)	5.37 (8.99)
Mean compartment 3 LI (SD)	30.9 (11.6)	18.61 (9.8)***	−12.30 (14.0)****	23.7 (10.8)	29.92 (12.66)	6.20 (10.7)
Mean compartment 4 and 5 LI (SD)	7.39 (5.5)	3.62 (3.0)*	−3.76 (5.54)**	8.22 (8.7)	9.49 (6.38)	1.27 (8.40)

Change represents mean change in pre- and post-values (SD).
*$p<0.05$ vs pre-EPA value (paired t test)
**$p<0.05$ vs change in non-treatment group (Mann–Whitney U test)
***$p<0.01$ vs pre-EPA value (paired t test)
****$p<0.01$ vs change in non-treatment group (Mann–Whitney U test)

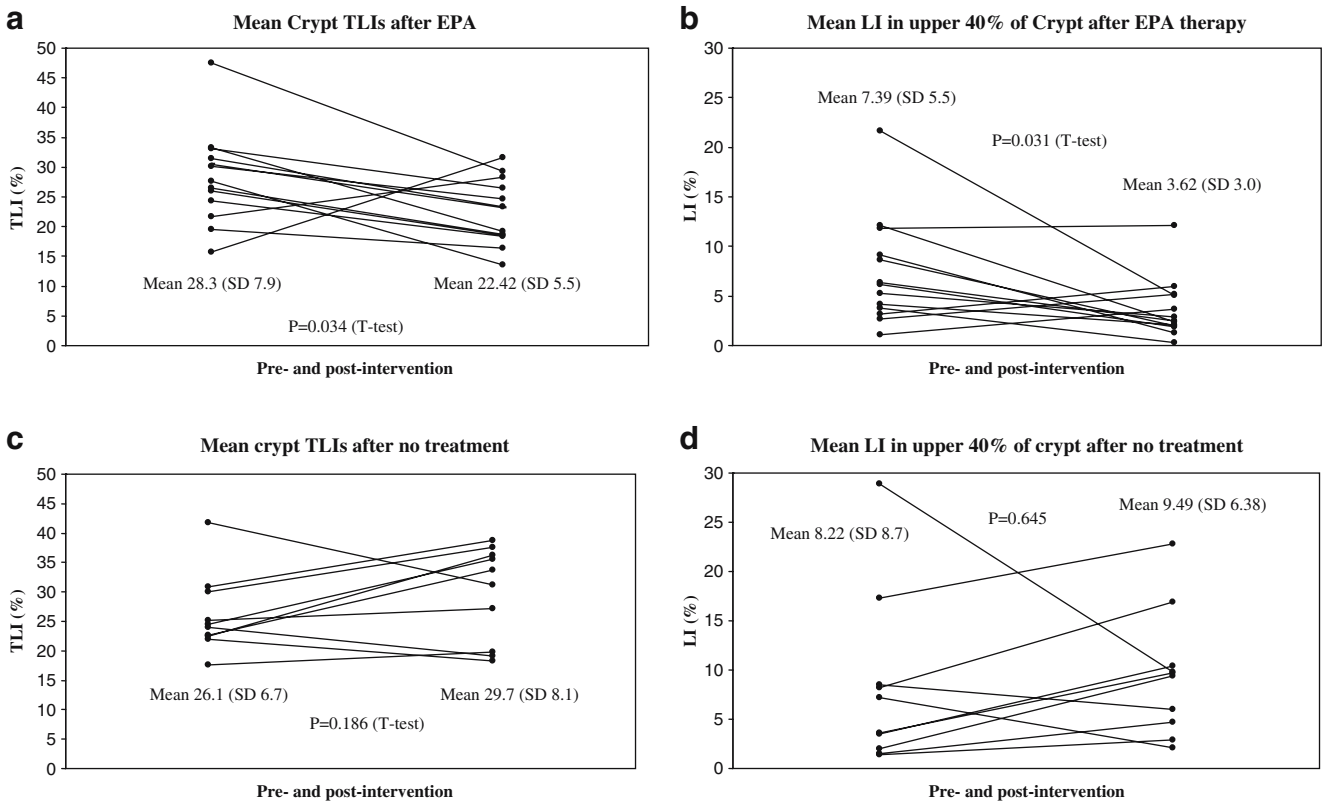

Fig. 1 Paired mean labelling indices (LIs) in the total crypts (TLIs) and upper 40% of the crypts in each subject before and after 3 months of EPA (**a** and **b**), respectively, and no treatment (**c** and **d**)

Correlation between changes in mucosal cell proliferation and apoptosis and levels of mucosal EPA

A summary of the correlations between changes in cell proliferation and apoptosis with change in the mucosal levels of EPA is shown in Table 5. There was a weak but significant inverse correlation between increasing mucosal levels of EPA and the number of labelled cells in the total crypt compartments (TLI) as well as in crypt compartments 2, 3 and combination of 4 and 5. There was no significant correlation between the change in mucosal EPA content and either the number or grade of apoptotic cells.

Discussion

Animal models have consistently shown a protective effect of omega-3 fatty acids on the development of colorectal cancer when compared with animals fed on high fat diets typical of a western diet [4]. Normal homeostasis of colonic mucosa relies on a balance between proliferation at the base of the crypt, and apoptosis at the surface epithelium. Apoptosis along the surface epithelium is therefore the result of physiological turnover of senescent cells, whereas apoptosis occurring in the lower proliferative zones of the crypt is an occasional phenomenon and thought to be in response to genetic damage [21, 22]. Supplementation with low doses of EPA has been found to be associated with both reduced crypt cell proliferation and increased apoptosis in normal rat mucosa [23] and the protective effect of fish oil on colorectal carcinogenesis in carcinogen-induced colorectal cancer in rats is associated with increased mucosal apoptosis [24, 25]. Recently it has been shown that the distribution of apoptotic cells within rat colonic crypts associated with fish oil supplementation depends on the mechanism of DNA damage utilised. Apoptotic cells increase in the base of the crypt to remove methylation-induced (alkylating) DNA damage, whereas apoptosis increases in the top of the crypt to remove oxidation induced DNA damage [26].

Two recent human studies have described a generalised decreased level of mucosal apoptosis throughout the colon in subjects with colorectal adenomas [27, 28], which may predispose these subjects to the development of polyps. Cheng et al. [29] found that low dose omega-3 fatty acid supplementation (100 mg of EPA and 400 mg of DHA per day) significantly increased rates of apoptosis in the normal colonic mucosa of patients with colorectal adenomas after

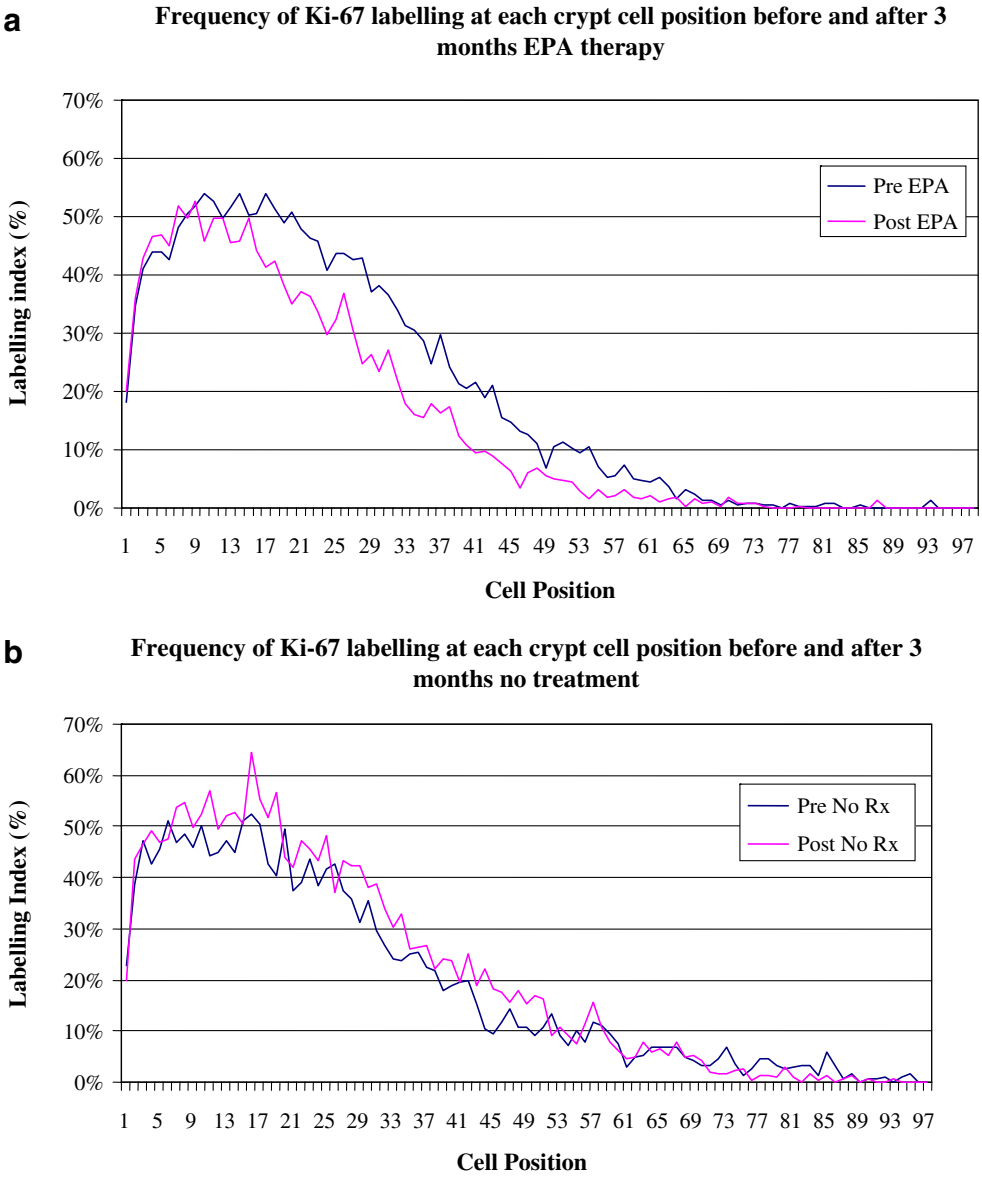

Fig. 2 Proliferation index labelling curves before and after treatment for **a** EPA group and **b** no treatment group

2 years of supplementation. No significant changes in rates of apoptosis between the control and supplementation groups were seen after 12 months, and no effect on crypt proliferation as measured by Ki-67 labelling occurred after 12 or 24 months of supplementation. Levels of omega-3 fatty acids in the colonic mucosa were significantly increased after 12 months of therapy [29]. Why are these results different from ours? Firstly, a higher dose of EPA was given to subjects in our study and therefore there may be a threshold dosage of EPA supplementation at which crypt cell proliferation becomes significantly reduced. This hypothesis is supported by the fact that whereas a significant increase in apoptosis was only seen after 24 months of supplementation using a relatively low dose of omega-3 fatty acids, the higher dosage of EPA used in our study not only resulted in significantly reduced proliferation, but also significantly higher rates of mucosal apoptosis after only 3 months of therapy. Secondly, the free fatty acid form of EPA has been shown to be more efficiently absorbed [30–32], and may be more biologically active than the more commonly used ethyl ester or triglyceride preparations of EPA. Whilst there are no human studies comparing the biological activity of different EPA formulations, a study in colonic adenocarcinoma bearing mice found that whilst the free acid fatty acid of EPA was effective in reversing host body weight loss and inhibiting tumour growth, the ethyl ester form of EPA was ineffective in either respect at the same dose level, even when administered with a high fat diet [33].

The increase in apoptosis seen with EPA supplementation in our study was evident from both the significant increase in the number of M30-labelled cells, as well as the

Table 3 Summary of the apoptosis grade scores and number of cells before and after treatment in each group

	EPA group			Non-treatment group		
	Pre	Post	Change	Pre	Post	Change
Mean apoptosis grade (SD)	0.36 (0.50)	1.50*** (1.02)	1.14** (0.86)	0.57 (0.51)	0.86 (0.36)	0.29 (0.61)
Range (0–3)	0–1	0–3	−1–+2	0–1	0–1	−1–+1
Mean no. apoptotic cells/1,000 microns surface epithelium (SD)	0.10 (0.20)	7.57**** (14.33)	7.47* (14.33)	0.29 (0.41)	1.01 (1.63)	0.72 (1.77)
Range	0–0.70	0–54.4	−0.20–+54.4	0–1.20	0–6.10	−1.20–+6.10

*p=0.008 vs change in non-treatment group (Mann–Whitney U test)
**p=0.005 vs change in non-treatment group (Mann–Whitney U test)
***p=0.003 vs pre-EPA mean score (Wilcoxon signed ranks test)
****p=0.001 vs pre-EPA mean value (Wilcoxon signed ranks test)

significant increase in the apoptosis score. Whereas only single positive cells (scoring 1) were seen in both groups before treatment, and also in the non-treatment group after 3 months, post-treatment slides from six patients in the EPA-treated group exhibited obvious groups of apoptotic cells in the luminal epithelium, accounting for the very significant increase in the mean apoptosis score for the EPA group. Apoptotic cells in our study demonstrated using M30 immunoreactivity were seen in the luminal surface epithelium. This was also the case in the study by Cheng et al. [29], despite using a different staining technique. Koornstra et al. [15], using M30 immunoreactivity, found that whilst no apoptotic cells were seen in the lower half of the crypt in genuinely normal mucosa, they were present in this location in over half of the cases of normal mucosa taken from the surgical resection margins of colonic adenocarcinomas. Apoptosis in the lower half of the colonic crypts has also been reported in other pathological conditions such as intestinal acute graft vs host disease associated with allogenic bone marrow transplantation, and in response to drug induced colitis attributable to NSAIDs [34]. Therefore, it may be expected that any physiological increase in apoptosis in normal colonic mucosa due to dietary intervention with omega-3 fatty acids, would occur in the luminal epithelium, as was seen in our study and that of Cheng et al. [29]. Colonic crypt cells are carpeted by a

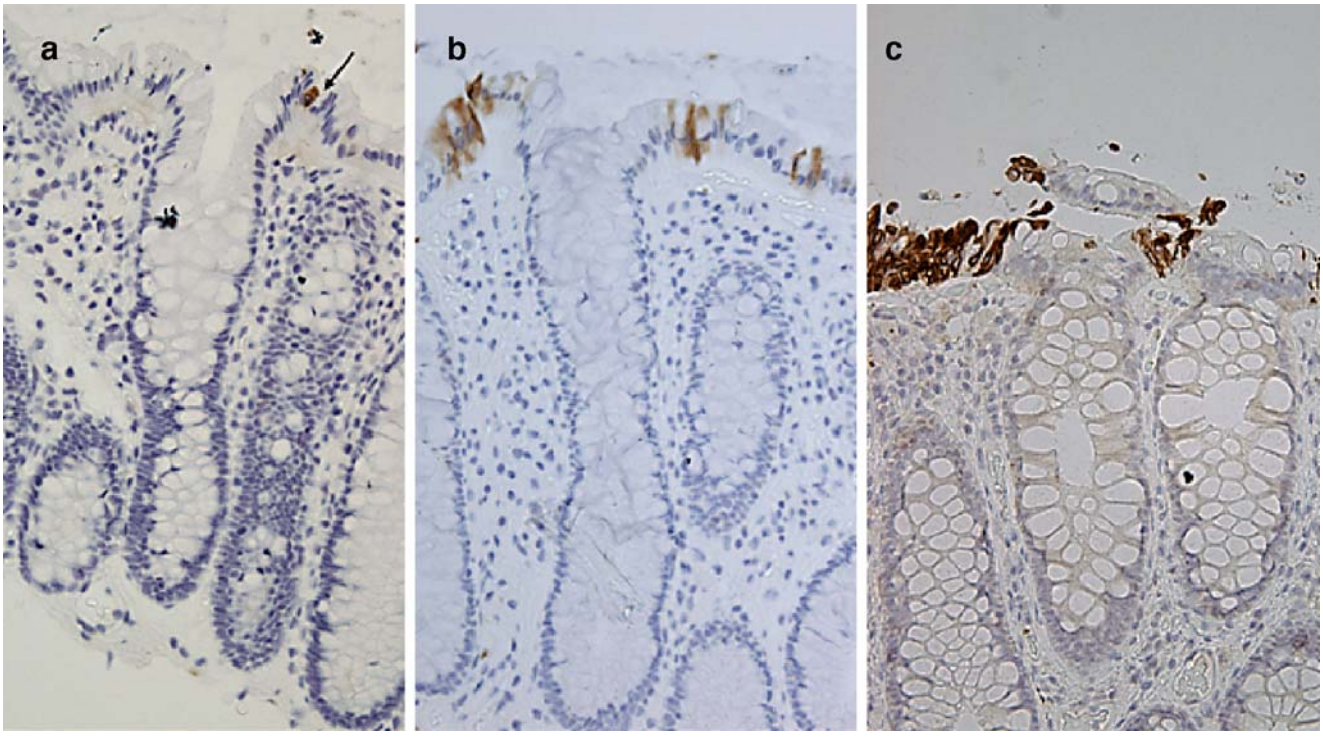

Fig. 3 Colon mucosa (×200) stained with M30 antibody to demonstrate apoptotic cells: **a** grade 1 apoptosis score with a single apoptotic cell (*arrowed*); **b** grade 2 apoptosis (*small groups of positive cells*); **c** grade 3 apoptosis (*large areas of adjacent positive cells*). Apoptosis scores of grades 2 and 3 were only seen in subjects after EPA supplementation

Table 4 Percentage contribution to total mucosal fatty acids of the main mucosal fatty acids in the EPA and no treatment group

	EPA group			Control group		
	Pre	Post	Change	Pre	Post	Change
Palmitic acid (C16:0)	22.63 (1.96)	23.62 (3.04)	0.99 (4.08)	22.71 (2.04)	22.52 (1.73)	−0.19 (1.84)
Stearic acid (C18:0)	12.60 (3.30)	15.92 (7.75)	3.33 (7.59)	12.66 (3.03)	14.10 (3.68)	1.44 (3.12)
Oleic acid (C18:01ω9)	35.16 (6.77)	31.62 (7.17)	−3.53 (7.38)	37.35 (4.70)	35.12 (4.22)	−2.23 (4.08)
Linoleic acid (C18:2ω6)	15.30 (2.35)	14.02 (3.26)	−1.28 (2.38)	15.47 (1.03)	15.40 (1.97)	−0.07 (1.42)
Arachidonic acid (C20:4ω6)	9.44 (3.02)	7.99 (2.46)	−1.45 (2.67)*	8.35 (2.83)	9.10 (1.86)	0.75 (1.43)
Eicosapentaenoic acid (C20:5ω3)	1.15 (0.62)	2.65 (1.60)**	1.51 (1.17)***	0.80 (0.46)	0.79 (0.53)	−0.009 (0.15)
DPA (C22:5ω3)	1.36 (0.58)	1.72 (1.06)	0.35 (1.25)	0.92 (0.36)	1.06 (0.38)	0.14 (0.26)
DHA (C22:6ω3)	2.38 (1.06)	2.46 (1.37)	0.08 (0.94)	1.73 (0.83)	1.92 (0.82)	0.18 (0.36)

*p=0.021, EPA supplementation group vs control group (Mann–Whitney U test)
**p=0.005 post- vs pre-value, EPA group (Wilcoxon signed ranks test)
***p<0.0001, EPA supplementation group vs control group (Mann–Whitney U test)

basement membrane with which they interact during their upward migration towards the surface epithelium, and it may be detachment from the underlying basement membrane that triggers apoptosis, a process known as *anoikis* [35]. Whilst this is difficult to study in vivo, incubation of the human adenocarcinoma cell line HT29 with EPA has been shown to lead to detachment of the cells from the substratum, which is followed by apoptosis [36, 37]. It has been suggested that anoikis functions in vivo as a surveillance mechanism to prevent dysplasia and preserve normal tissue architecture by destroying cells that attempt to deviate from their normally operative spatial constraints [38]. Prostaglandin E_2 has been shown to inhibit anoikis in intestinal epithelial cells [39] and therefore EPA may increase anoikis by reducing the production of PGE_2 from arachidonic acid by cyclooxygenases, in particular, COX-2. Levels of PGE_2 are significantly increased in surgically resected human colonic cancers compared with paired samples of normal colonic tissue [40], and PGE_2 increases the growth and motility of colorectal carcinoma cells [41], and promotes tumour survival by the inhibition of apoptosis, stimulation of cell proliferation and promotion of tumour angiogenesis [42]. Furthermore, metabolism of EPA via the cyclooxygenase pathway leads to the production of the 3-series prostaglandins such as PGE_3, which has weak, if any effect on cell proliferation [43]. Therefore, inhibition of COX-2 activity together with a reduction in

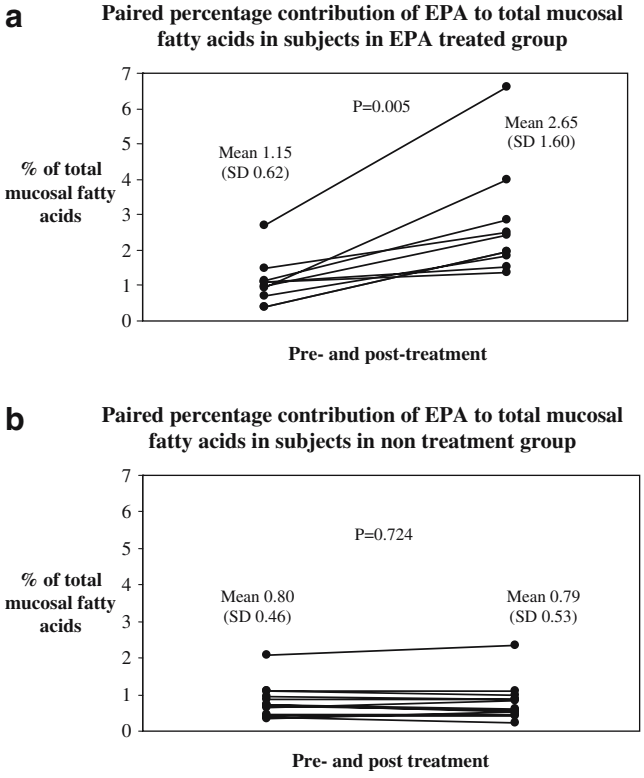

Fig. 4 Paired changes in the percentage contribution of eicosapentaenoic acid (EPA) to total mucosal fatty acids in subjects from the **a** EPA-treated group and **b** non-treated group

Table 5 Correlation between changes in cell proliferation and apoptosis with change in the mucosal levels of EPA

Factor	Spearman's correlation coefficient	P value (two-tailed)
Proliferation		
Change LI1 and change EPA	+0.102	0.679
Change LI2 and change EPA	−0.461	0.047
Change LI3 and change EPA	−0.600	0.007
Change LI4 and 5 and change EPA	−0.491	0.033
Change TLI and change EPA	−0.481	0.037
Apoptosis		
Change no. of apoptotic cells and change EPA	+0.367	0.085
Change apoptosis grade and change EPA	+0.385	0.070

the mucosal levels of arachidonic acid may be one of the potential mechanisms responsible for the change in crypt proliferation and apoptosis seen in our study. Whilst the reduction in the mean levels of mucosal arachidonic acid in the EPA supplemented group did not reach statistical significance, the mean overall change between each group was significantly different (Table 4). As significantly lower levels of arachidonic acid were only seen after 6 months of fish oil supplementation in the study by Anti et al. [7], it may be that the duration of supplementation in our study was not of sufficient duration to reduce arachidonic acid levels significantly, but enough to produce a therapeutic effect.

Whilst we have found a significant effect of EPA on proliferation and apoptosis within the colon, there are several limitations in the design of our study that warrant further discussion. Firstly, as we only measured apoptosis by M30 immunoreactivity in this study, we cannot provide any further insight as to the mechanisms by which omega-3 fatty acids such as EPA increase mucosal apoptosis in the colon. Cheng et al. [29] found that the increase in mucosal apoptosis associated with EPA and DHA supplementation was associated with a significant increase in the production of the apoptosis-enhancing protein Bax, whereas no significant effect on p53 expression or the apoptosis-suppressing protein BCl-2 was demonstrated. This would imply that elevated production of Bax protein within normal mucosa is one mechanism by which EPA increases mucosal apoptosis in the colon [29]. Further studies would be useful to not only validate our results, but to also measure changes in the expression of other proteins known to regulate apoptosis, and to also correlate any such changes with changes in mucosal prostaglandin production, particularly PGE_2. Secondly, in this trial, a placebo was not used, which has the disadvantage of subjects knowing their treatment group status, and potentially altering their diet or lifestyle accordingly during the study period. Many clinical studies investigating the effect of omega-3 fatty acids have used placebos containing omega-6 fatty acids, such as corn oil, linoleic acid, or safflower seed oil. It is questionable as to whether using such fatty acids are appropriate as a placebo, as they compete with omega-3 fatty acids for metabolism and give rise to pro-inflammatory eicosanoids such as prostaglandin E_2, which can stimulate cell proliferation and inhibit apoptosis. Therefore, we opted not to use a placebo and randomise subjects to EPA or no treatment. To ensure good scientific technique to the study, all samples were coded to ensure that the treatment status of a patient was not known when the results were analysed. Furthermore, in accordance with the published guidelines from the International Conference on Harmonisation (ICS) of technical requirements for registration of pharmaceuticals for human use (July 2000), it is acceptable to use a no-treatment concurrent control group when there is reasonable confidence that the study endpoints are objective. As the endpoints in this study are all histological and biochemical measurements of cellular processes, and there are no subjective assessments in our study, we felt that not using a placebo would not affect the scientific validity of the study. As there were no significant changes in the EPA content of the colonic mucosa after 3 months in the non-treatment group, we can be reasonably confident that subjects in the control group did not increase their oily fish or fish oil intake during the study period.

In the non-treatment group, there was a non-significant trend towards an increase in both colonic cell proliferation and apoptosis after 3 months despite little change in mucosal fatty acids. This difference could have exaggerated the relative changes in the EPA-treated group. One possible explanation for this could be the differing effects of the two different bowel preparation regimes on colonic mucosal proliferation and apoptosis. On the first visit, the colon was prepared with sodium picosulphate, whereas on the second visit, a single phosphate enema was given. Ideally, we should have used the same bowel preparation on each visit, but we were aware that sodium picosulphate can frequently cause abdominal cramps and diarrhoea, and as a full examination of the colon was not necessary when obtaining the post-treatment biopsies, it was felt compliance would be better using just a phosphate enema on the second visit. It would be interesting in further studies to investigate the effects of different bowel preparation regimes on colonic mucosal apoptosis, as previous studies until now have only focused on proliferation [8–10].

Despite the changes in mucosal proliferation and apoptosis over the 3-month period in this study, we cannot infer that long-term supplementation with EPA would reduce the risk of colorectal neoplasia because these measures have not been validated as biomarkers in the development of colorectal neoplasia in humans. In fact, proliferation indices failed to predict future colorectal adenoma occurrence in a randomised trial of calcium supplementation over a 3-year period [44]. Colonic epithelial apoptosis was found to have a greater prognostic value to detect dietary effects on tumour incidence than cell proliferation in the promotive phase of carcinogenesis in azoxymethane treated rats [45]. Furthermore, in a trial showing that celecoxib causes regression of colorectal adenomas in patients with FAP, a trend was seen between changes in the number of apoptotic cells in the superficial and luminal regions of normal colonic mucosa and polyp regression ($r=0.33$, $p=0.053$) [46]. Therefore, further studies are needed in humans to assess the validity of using apoptosis in normal mucosa as a predictor of future colorectal neoplasia in dietary intervention studies in the chemoprevention of colorectal cancer.

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