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Effects of a Mediterranean diet intervention on anti- and pro-inflammatory eicosanoids, epithelial proliferation and nuclear morphology in biopsies of normal colon tissue

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Abstract

This randomized trial evaluated the effects of intervention with either a Healthy Eating or a Mediterranean diet on colon biomarkers in 120 healthy individuals at increased colon cancer risk. The hypothesis was that eicosanoids and markers of proliferation would be favorably affected by the Mediterranean diet. Colon epithelial biopsy tissues and blood samples were obtained at baseline and after six months of intervention. Colonic eicosanoid concentrations were evaluated by HPLC-MS-MS, and measures of epithelial proliferation and nuclear morphology were evaluated by image analysis of biopsy sections. There was little change in pro-inflammatory eicosanoids and in plasma cytokine concentrations with either dietary intervention. There was, however, a 50% increase in colonic prostaglandin E₃ (PGE₃), which is formed from eicosapentanoic acid, in the Mediterranean arm. Unlike PGE₂, PGE₃ was not significantly affected by regular use of non-steroidal anti-inflammatory drugs at baseline, and normal weight subjects had significantly higher colon PGE₃ than overweight or obese subjects. Increased proliferation in the colon at baseline, by Ki67 labeling, was associated with morphological features that defined smaller nuclei in the epithelial cells, lower colon leukotriene concentrations and higher plasma cytokine concentrations. Dietary intervention had little effect on measures of epithelial proliferation or of nuclear morphology. The increase in PGE₃ with a Mediterranean diet indicates that in normal colon, diet might affect protective pathways to a greater extent than pro-

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Conflicts of Interest:

None of the authors have conflicts of interest with the research reported within.

inflammatory and proliferative pathways. Hence, biomarkers from cancer models might not be relevant in a true prevention setting.

Keywords

colon cancer prevention; nutrition; diet; eicosanoids; cytokines

INTRODUCTION

Epidemiological evidence has indicated that diet has a sizable role in modulating risk of colon cancer [1]. Although many dietary components have been suggested to have preventive effects, the role of diet in cancer prevention might be maximized when dietary patterns are considered versus any one single component. An overall healthy diet, such as the Mediterranean diet or the diet recommended by the U.S. Department of Agriculture (USDA), was associated with reduced risk of colorectal adenomas in both men and women in a large U.S. cohort [2]. An intervention study that utilized healthy eating goals, the Polyp Prevention Trial, did not show prevention of adenoma recurrence; however, the intervention was preventive in the subset of individuals with excellent compliance [3]. Interventions powered for cancer endpoints have not been done with Mediterranean diets, but extensive epidemiological research has indicated that this type of diet is preventive of many cancers including that of the colon [4–6].

Mediterranean diets are similar to diets recommended by the USDA in that both types of eating patterns involve increased intakes of fruits and vegetables and whole grains relative to a typical Western diet. A Mediterranean diet is, however, unique in its relatively higher content of mono-unsaturated fatty acids (MUFA) and n3 fatty acids, and lower total poly-unsaturated fatty acids (PUFA) [4]. This is relevant to colon cancer prevention since differences in fat intakes may affect the production of pro-inflammatory eicosanoids produced from cyclooxygenases (COX) and lipoxygenases (LOX). Prostaglandins (PG) are produced from metabolism of arachidonic acid, n-6, by constitutive COX-1 in normal tissue and by inducible COX-2 in tumors. Increased COX-2 and PGE₂ is tightly linked with increased colon cancer risk [7]. The LOX products include hydroxyeicosatetraenoic acids (HETEs), and of these, 15-HETE appears to have a preventive role while 5-HETE and 12-HETE have pro-carcinogenic roles [8]. Both 15-HETE and 13-HODE, produced from metabolism of linoleic acid, n-6, by 15-LOX have been associated with prevention, although this may be due more to the tumor-suppressor role that has been proposed for 15-LOX expression [9, 10]. When n-3 fatty acids are available to COX and LOX enzymes, however, this can change the amount and types of eicosanoids that are produced in cells. The products formed from n-3 fatty acids (PGE₃ and leukotriene B₅) appear to inhibit proliferation and have an anti-inflammatory role [11–13].

The present study compared the effects of intervention with Healthy Eating or Mediterranean diets on colonic eicosanoids, epithelial proliferation and nuclear morphology. These biomarker endpoints would be one way to evaluate whether a Mediterranean diet, which was more complex and had a larger number of dietary goals than the Healthy Eating

diet, would have relatively more potential for colon cancer prevention. In an intervention study, as compared with observational studies, it is possible to better isolate the specific effects of diet on biomarker endpoints. The hypothesis was that a Mediterranean diet would result in larger changes in eicosanoid profiles than the Healthy Eating diet and that this would affect epithelial proliferation and nuclear morphology. Epithelial proliferation is often utilized as a target in the carcinogenic process, but in normal tissue, more subtle changes in proliferation might be present.

Increased colon crypt height or depth, as measured from the base to the luminal surface, has been associated with preventive approaches such as increased calcium and physical activity [14, 15]. Changes in nuclear morphology also may be important early in the carcinogenic process, and this includes the nuclear features of colonic epithelial cells such as size, variability in size, and circularity that are altered to a large extent in malignancy and dysplasia [16]. Since nuclei become more elliptical as cells stop proliferating and move up the crypt, nuclear features may be related to the proliferative state of cells [17]. Quantitative, computer-assisted, nuclear morphology was used in this study to detect subtle changes in nuclear shape and size that might not be discernable otherwise.

In order to evaluate the effects of diet on all of these biomarkers, colon biopsies of unprepped, normal colon tissue were obtained from a randomized study before and after 6 months of intervention with either Healthy Eating or Mediterranean diets. The effects of the dietary intervention were evaluated on eicosanoid profiles, epithelial proliferation and nuclear morphology. In addition, we evaluated the potential influence of circulating cytokine concentrations on inter-individual differences in colonic proliferation measures. This was done in a prevention setting with a population at increased risk due to family history of colon cancer or to a previous adenoma.

METHODS

Subjects and Dietary Intervention

This study utilized samples and data from a dietary intervention trial, the Healthy Eating Study. The study took place from July 2007 to November 2010 in Ann Arbor, MI, and 120 persons at increased risk of colon cancer were enrolled and 93 finished 6 months of study. The details of recruitment and retention have been published [18]. The primary aim of the study was to evaluate changes in colonic fatty acids [19]. Increased risk was defined as a strong family history of colon cancer or a personal history of adenomas or colon cancer. The recruited subjects were 72% female, 88% Caucasian, mean age 52 (range 22–82) and body mass index 27 kg/m² (range 19–35) [18]. The study was approved by the University of Michigan Institutional Review Board (HUM00007622) and was listed on the Clinical Trials website maintained by the National Institutes of Health (registration number NCT00475722).

Subjects were randomized to follow a standard Healthy Eating diet or a Mediterranean diet for six months. Individualized counseling with a registered dietitian was implemented to help subjects achieve study goals. Both diets had goals for increasing fruit, vegetable and whole grain intakes, but the fat intake goals were different. The Healthy Eating diet had

limits on saturated fat. The Mediterranean diet increased intakes of monounsaturated fats from plant sources and decreased polyunsaturated fat intakes, as previously described [20]. Diet was assessed using two days of written records and two 24-hour recalls at 0 and 6 months. The average of these four days was used to determine nutrient intakes at each time point using the Nutrition Data System for Research software (NDSR), version 2010. Due to 9 instances of missing days, the average number of days of diet analyzed at each visit was 3.93. Compliance to the dietary goals was good, as reported previously [20].

Colon Biopsies

Colon biopsies were obtained by flexible sigmoidoscopy without prior preparation of the bowels to reserve the biological features of the tissue as much as possible. The biopsies were obtained with biopsy forceps circumferentially well beyond the colo-rectal junction (more than 15 cm above the anus) to ensure that colon was sampled. Tissues sampling was done at sites that were visibly free of feces. Eight biopsies were obtained, each weighing about 5 mg and containing the mucosal epithelium and stroma. After removal from the subject, six of the biopsies were immediately placed into liquid nitrogen (6 biopsies), and two biopsies (the third and eighth) were placed in ice-cold, phosphate-buffered saline (pH 7.4). Each of the biopsies in saline were oriented in tissue cassettes on a piece of filter paper and fixed in 10% formalin-90% phosphate buffered saline pH 7.4 within 20 minutes. Biopsies were in formalin for 22–25 hours prior to being transferred to 70% ethanol, and they were stored in 70% ethanol until embedding could take place.

For each formalin-fixed biopsy, two adjacent slides were made at eight levels through the biopsy using a 4 μ m section thickness. One slide from each level was stained with hematoxylin and eosin (H&E) to identify the levels at which the largest number of full-length crypts were located. Slides made from those levels were then used for immunohistochemistry (IHC) for Ki67 staining. One subject refused the ending biopsy making 212 biopsies available for eicosanoid analysis (of 213 subject visits). An additional 4 subject visits did not yield adequate tissue sections with full-length crypts for IHC analysis, yielding a total of 208 biopsies available for Ki67 labeling and nuclear morphology. Nine of the sections were not stained adequately for morphology. The numbers of samples analyzed are shown in Table 1.

Plasma samples

Fasting blood samples were obtained at baseline and 6 months after an overnight fast. Plasma was prepared from EDTA tubes within 30 minutes of drawing the blood and aliquots were stored at -80°C until analysis. Cytokines were measured in the Immunology Core facility that utilized ELISA DuoSets from R&D Systems (Minneapolis, MN) using the manufacturer's suggested protocol. Inter-batch differences in this core facility generally have been less than 5%. The cytokine assayed were those representing a T-helper cell type 1 response (IL1 β , IL6, IL8, interferon γ , TNF α) and those representing a T-helper cell type 2 or more immune-modulatory response, (IL4, IL10, and IL13).

Eicosanoid quantitation

Reverse-phase liquid chromatography with tandem mass spectral detection (LC-MS-MS) was used to profile eicosanoids (PGE₂, PGE₃, LTB₄, LTB₅, 5-HETE, 12-HETE, 15-HETE, 13-HODE) using methods previously developed for rodent tissues [21]. Briefly, homogenates of four frozen colon biopsies, each about 5 mg tissue, were prepared. One fourth was used for fatty acid analysis, one fourth for carotenoid analysis and one half for eicosanoid analysis. Extraction of eicosanoids for LC-MS-MS analysis was with ether. The analysis was carried out with a Luna Phenyl-Hexyl analytical column (2 × 150 mm, 3 μm particle size, Phenomenex, Torrance, CA) and deuterated internal standards (Cayman Chemical, Ann Arbor, MI). Since deuterated compounds were not available for PGE₃ and LTB₅, PGE₂-d₄ was used for quantifying both PGE₂ and PGE₃, and LTB₄-d₄ was used for quantifying LTB₄ and LTB₅. A small portion of the homogenate was also used for analysis of total protein by the Bradford assay.

Ki67 proliferation marker

Immunohistochemical staining for Ki67 positive cells was done using an automated immunohistochemical stainer with Ki67 antibodies from Novocastra Laboratories Ltd (1:120). Positive nuclei were visualized with horseradish peroxidase-conjugated streptavidin and 3, 3'-diaminobenzidine substrate. A light hematoxylin counterstain was used to help visualize negative nuclei. Bright field microscopy was performed using an Olympus BX51 microscope with attached Olympus DP-70 high resolution digital camera. For each slide, only full-length crypts were enumerated for Ki67 labeling. Two slides from two different biopsies were averaged for each subject at each time point, and the number of crypts analyzed was an average of 7.3 (SD 2.4, range 1–12) for 208 subject-visits. The remaining seven subject-visits had less than 3 full length crypts but were included in the analyses to minimize the number of missing data points. The number of crypts analyzed was a covariate in the analyses.

Image analysis was performed using MCID Elite 7.0 software (Linton, Cambridge, England) using the Grain Counting algorithm to enumerate all nuclei and positively stained nuclei. Full-length crypts were located and each hemicrypt, or one side of the crypt stemming from the base to the top, was selected by an outline tool. An intensity threshold was set to differentiate Ki67-positive and negative cells: a lower threshold was used to detect Ki67 positive cells which were darker than the negative nuclei detected with a light H&E counterstain. Three values were derived for each hemicrypt: 1) height of crypt given as number of total cells (Ki67-positive and negative), 2) labeling index (LI) as the number of Ki67-positive cells divided by the total number of cells per hemicrypt, and 3) extent of the proliferative zone (EPZ) as the number of cells from the bottom of the crypt to the highest proliferating cell along each side of the crypt. The coefficient of variation between three observers for 15 slides was 1.15% for cell height, 4.80% for LI and 0.71% for EPZ.

Nuclear morphology

Slides for analysis of nuclear morphology were stained with Harris' hematoxylin and eosin (H&E) to maximize the staining of nuclei. Nuclei for morphological analysis were selected in the middle third of all available full length crypts to avoid the immature cells at the crypt

base and the senescent cells at the crypt surface. The Grain Counting algorithm of MCID Elite 7.0 software was used to collect the nuclear area, perimeter, maximal chord, diameter, form factor (a measure of circularity with a perfect circle having a maximal value of 1), major axis length, minor axis length, fiber length, number of end nodes, and branch nodes. The mean and SD was calculated for each subject visit. The number of nuclei imaged per subject-visit was an average of 133 (SD 67, range 45–432) and depended on the number of full-length crypts present in the sections. The coefficient of variation between three observers for these measures was 9.57% for area, 5.14% for diameter and 1.69% for form factor.

Statistical analyses

All data was kept in an SPSS file and analyzed using IBM SPSS Statistics version 20.0.0 (IBM Corporation, Armonk, New York). Variables were transformed if the distribution was not normal before analyses. Natural log transformation was used for eicosanoids except for PGE2 which was a square root transformation. For morphometric measurements, the mean and SD was calculated for each subject at each time point. Natural logarithm was used to transform the mean and standard deviation of nuclear area, and square root was for the standard deviation of crypt height. The other variables did not need transformation. Since inter-individual variability in cytokine concentrations was large, Z-scores were calculated for each cytokine. These were evaluated individually and as a sum since the Spearman correlation between individual cytokines was greater than 0.75 in each case, regardless if it was a T-helper cell type 1 or 2 cytokine (the range for the correlation coefficient was 0.751–0.955). The effects of demographic factors such as gender, obesity, and use of non-steroidal anti-inflammatory factors on each measure at baseline were evaluated using two-sample t-tests.

The change across time in levels of eicosanoids, nuclear features and crypt features were evaluated using a linear mixed model including a random intercept for subjects (Table 1). Time, diet group, and the group-by-time interaction were the primary predictors in the mixed models controlling for select baseline variables (age, regular use of non-steroidal anti-inflammatory drugs or NSAIDs, BMI) and percent of dietary goals met. Age was included as a covariate since mean age at baseline was higher in the Mediterranean group versus the Healthy Eating group (55 years versus 50 years, $p = 0.03$). To control for any batch to batch variation in analytical methods, analysis batch was also included as a categorical covariate.

To facilitate evaluation of the relationships between nuclear factors, eicosanoids and colonic proliferation measures at baseline, principal component analysis with a varimax rotation was performed to identify factors present in the morphology and eicosanoid data (Tables 2–3). This method identifies individual variables that are associated with each other and can be used to simplify subsequent analyses. The factors were selected based on the percent of the variance that could be accounted for in the original variables with maximal divergence in factor loading between identified factors. Correlations were then evaluated between the derived morphometry and eicosanoid factors, colon crypt proliferative characteristics (crypt height, labeling index and extent of the proliferative zone), and plasma cytokine z-scores.

RESULTS

Effects of Dietary Intervention on Colon Biomarkers

The dietary interventions resulted in changes in self-reported diet as well as in serum carotenoids and fatty acids [19, 20]. Briefly, in the Healthy Eating arm, intake of saturated fat decreased 40%, monounsaturated fat decreased 25% and servings of fruit and vegetables increased 67%. In the Mediterranean arm, intake of saturated fat decreased 27%, monounsaturated fat increased 30% and servings of fruit and vegetables increased 83%. Participants met 88% of five dietary goals in the Healthy Eating arm and 82% of ten goals in the Mediterranean arm at 6 months [20]. Serum markers changed more modestly. Carotenoids increased about 20% in both arms, and in the Mediterranean arm serum n-3 fatty acids and monounsaturated fatty acids both increased by 13% [19].

In the present study, each of the biomarker endpoints in colon was evaluated for changes after six months of dietary intervention. The only biomarker to change significantly over time was PGE₃ which increased in the Mediterranean diet arm from baseline to 6 months (Table 1). Changes in other eicosanoids, nuclear morphology, epithelial proliferation were not statistically significant. Changes in cytokine z-scores also were not significant, except for IL10 which increased slightly in the Healthy Eating arm at 6 months ($p = 0.042$, not shown). The covariates in the models included age, gender, baseline BMI, percent of dietary goals met at 6 months and regular use of NSAIDs. Of these, the factors with the most interesting effects on biomarker values at baseline were NSAID use, BMI and gender. NSAID use had a significant fixed effect in the mixed linear models for PGE₂, 13-HODE and 12-HETE only.

Percent of dietary goals met at 6 months, as evaluated by the study dietitian, was not a significant fixed factor in any of the analyses. Use of cholesterol medications such as statins, or blood pressure medications also were not significantly correlated with any of the outcome variables, and these were therefore not used as covariates in further analyses. Results in Table 1 were similar when covariates were restricted to age, gender and NSAID use. When the models were restricted to non-users of NSAIDs, the increase in PGE₃ in the Mediterranean diet arm remained significant and the other changes remained non-significant. Nearly identical results were obtained if use of any anti-inflammatory medications in the previous 2 weeks was used as a covariate instead of regular NSAID use, but regular NSAID use had stronger effects.

Effects of Demographic Factors on Colon Biomarkers

There were 25 subjects who reported regular use of NSAIDs and this was associated with significantly lower mean baseline PGE₂, 10.3 (SD 8.7) vs. 19.6 (SD 14.0), $p < 0.001$, and lower mean 13-HODE, 49.6 (SD 37.8) v. 77.9 (SD 59.3), $p = 0.025$ based on two-sample t-tests for users ($n = 25$) versus non-users ($n = 95$) of NSAIDs, respectively. PGE₃ was slightly and not significantly lower in NSAID users (1.29, SD 1.08) versus nonusers (1.08, SD 1.36), $p = 0.417$. Each cytokine z-score, and the composite score, was significantly decreased by NSAID use with the exception of IL8 for which $p = 0.066$. The composite z-score was -2.01 (SD 0.57) in NSAID users and 0.36 (SD 8.30 in NSAID non-users ($p = 0.008$).

The only variable to differ significantly by gender at baseline was LTB₄. LTB₄ was higher in women (0.36, SD 0.24, n=86) than men (0.24, 0.17, n=34) with $p=0.001$. Crypt height was 53.5 cells (SD 10.9, N=33) in men versus 49.4 cells (SD 9.7, N=85) in women with $p = 0.052$. Crypt height was higher in obese versus normal weight subjects at baseline but this was not significant $p = 0.115$. The only two variables to differ significantly by normal weight versus overweight or obese status were PGE₃ and the standard deviation of the extent of the proliferative zone (EPZ). PGE₃ was higher in the 40 normal weight subjects versus the 80 overweight obese subjects (1.78, SD 1.31, vs. 0.98, SD 0.94, $p < 0.001$). Conversely, the SD of EPZ was higher in overweight/obese versus normal weight subjects (0.131, SD 0.058 vs. 0.108, SD 0.055, $p = 0.037$) indicating more variability for EPZ in the obese subjects.

Relationships between measures of colonic proliferation and other biomarkers

There were three measures of colonic proliferation assessed by image analysis: crypt height (the number of cells from the base to the apex of the crypt), labeling index (percent of cells in each crypt positively stained with Ki67), and extent of the proliferative zone (height of the highest Ki67 positive cells along each crypt side divided by total height of the crypt, EPZ). The three measures of crypt proliferation were significantly associated with each other, in a manner that might be expected. The EPZ was positively correlated with the overall Ki67 labeling index ($r=0.660$, $p < 0.001$) but not with crypt height ($r = -0.030$, $p=0.746$). Increased crypt height was associated negatively with the labeling index ($r = -0.194$, $p = 0.036$). Since labeling index is the number of Ki67 positive cells divided by the total number of cells per hemi-crypt, taller crypts did not appear to contain a great number of Ki67-positive cells, resulting in a lower labeling index.

To better understand the relationships between measures of epithelial proliferation with the other colon biomarkers measured, correlations were conducted at baseline with data from all 120 subjects. For nuclear morphology and eicosanoid concentrations, this analysis was done using the factors generated from principal component analysis to reduce the number of variables. This was important given the modest sample size. The principal component analysis identified factors that segregated the epithelial nuclear morphology measures into factors associated with either nuclear size (area, diameter, etc.) or circularity (branch nodes, form factor, etc.), as shown in Table 2. This two-factor solution accounted for 89% of the variance in nuclear morphology features.

The colonic eicosanoid measures segregated into two factors by principal component analysis as shown in Table 3. Factor 1 was positively associated mainly with PGE₂, 13-HODE and the HETEs. Factor 2 was associated mainly with the leukotrienes, although 13-HODE had a sizable contribution as well. PGE₃ was the only variable to load on a 3rd factor and was kept separate. The three-factor solution of the two eicosanoid factors and PGE₃ accounted for 69% of the variance in these measures.

Increased colon crypt height at baseline was positively associated with the two nuclear morphology factors from principal component analysis that defined larger and more circular nuclei. Thus, crypts with greater height had larger and more circular nuclei, consistent with delayed differentiation to elongated colon epithelial cells (Table 4). The only significant

correlation of nuclear size with other colonic measures, other than those shown in Table 4, were for the nuclear size factor with PGE₃ (Spearman $r = -0.225$, $p = 0.015$) and with 12-HETE ($r = 0.333$, $p < 0.001$). This indicates that higher PGE₃ at baseline was associated with smaller nuclear size and the opposite was true for 12-HETE.

PGE₃ was not associated with the measures of epithelial proliferation. Eicosanoid factor 2, which was loaded mainly by LTB₄, LTB₅ and 13-HODE, was negatively associated with Ki67 labeling index and with the extent of the proliferative zone (Table 4). LTB₄, LTB₅, 13-HODE were also negatively correlated with labeling index ($p < 0.04$ in each case, not shown). To evaluate if systemic inflammation could also be related to colonic proliferation, we evaluated the relationships between plasma cytokine concentrations and colonic proliferation. This was done using a cytokine z-score that was the sum of the z-scores for each cytokine. The individual cytokine concentrations were highly correlated with each other, and this allowed for generation of a meaningful composite z-score. This cytokine z-score was positively and significantly associated with the Ki67 labeling index (Table 4).

DISCUSSION

The association between PGE₂ and colon carcinogenesis is well known, but there have been relatively few studies that have examined the effects of diet on reducing PGE₂ in the normal, but at-risk, colon. For primary cancer prevention, changes in normal epithelium are of interest. In this study, biopsies of apparently normal colon tissue were obtained by flexible sigmoidoscopy before and after six months of dietary intervention. Subjects were randomized to either a Healthy Eating or Mediterranean diet, and compliance to dietary goals was very good, as reported previously [20]. The dietary interventions had little effect on concentrations of PGE₂ and other eicosanoids in the colon tissue, with the notable exception of an increase in PGE₃ with the Mediterranean intervention (Table 1). Interestingly, the data indicated that PGE₃ and PGE₂ concentrations were dependent on different factors. Unlike PGE₂, PGE₃ concentrations at baseline were not significantly different in users versus non-users of NSAIDs, and PGE₃ concentrations were significantly higher in normal weight versus overweight or obese subjects.

The significance of increased PGE₃ in the colon after following a Mediterranean diet is likely a beneficial change. PGE₃ is formed when 20:5 (eicosapentanoic acid, n-3) is the substrate for COX-2 [22, 23]. PGE₃ appears to have lower pro-inflammatory potential than PGE₂, inhibit colon stem cell expansion, and has been shown to inhibit platelet aggregation in vitro [12, 13, 24]. The analogous product from n-3 fatty acid metabolism by 5-LOX is LTB₅. In vitro, LTB₅ did not stimulate proliferation of colon cancer cells, unlike the LTB₄ that is formed from n-6 fatty acid metabolism [11]. Levels of leukotrienes in this study were low in the colon biopsies, and this may be why a significant effect of diet intervention was not detected (Table 1).

The lack of intervention effect on PGE₂ concentrations in this study was not expected since the Mediterranean diet did involve a number of dietary factors that should work together to reduce PGE₂. Phytochemicals from plant based foods and olive oil both have been shown to decrease the activity of COX and LOX [25]. High amounts of dietary n-3 fatty acids

decreased PGE₂ concentrations in the mouse colon [21]. Oleic acid (n-9) from olive oil does not appear to be a substrate for COX, but it may have allosteric effects on inhibiting COX activity [26, 27]. The effects of both n-3 and n-9 fatty acid intakes on decreasing eicosanoid levels appear to be maximal when n-6 fatty acid intake is low, and n-6 intakes were decreased with the Mediterranean intervention [20]. In humans, however, it may be difficult to attain large changes in colon fatty acids such as that achieved in animal models. Indeed, changes in colonic fatty acids with the Mediterranean intervention were modest relative to that attained in the blood [19]. This indicates the importance of metabolic factors, and possibly genetic factors, on fatty acid concentrations in colon tissue.

There have been very few studies that have examined the impact of diet on nuclear morphology. One intervention using low-fat dairy foods did show a transient decrease nuclear size in normal human mucosa [28]. Dietary fatty acids can affect the composition of cell membranes, which in turn affects fluidity, and dietary antioxidants from fruits and vegetables can prevent lipid peroxidation. This makes it reasonable to expect that the dietary interventions employed might have affected nuclear features via membrane effects, but there were no effects of diet on nuclear features in this present study as shown in Table 1.

The effects of diet on colon crypt height in humans have been studied to a greater extent than the effects of diet on nuclear morphology and eicosanoids. Increased colon crypt height alone, without an increase in number of proliferating cells, is not likely to increase risk. In fact, increased crypt height has been associated with preventive approaches, including physical activity and dietary fiber and/or short-chain fatty acids [14, 15, 29–31].

Unlike crypt height, increased epithelial proliferative activity and upward expansion of the proliferating cells towards the apex of the crypt is considered to be an early biological change in the carcinogenic process [32]. Epithelial proliferation in the colon was shown to be increased in persons who are at risk for colon cancer as compared to those at normal risk [33]. The proliferative index in human colorectal tissue has been reported to be decreased by dietary intakes of calcium, lower total fat, and higher fiber, fish, fruits and vegetables in some, but not all, studies [34–37]. This difference across studies could be due to the complex nature of diet and interactions among nutrients. For example, a fish oil intervention reduced rectal proliferation only when overall fat intake was low [38, 39]. Measurement of epithelial proliferation in the colon also is technically challenging. The present study used standardized, timed, procedures for biopsy processing and computer-assisted image analysis for quantifying proliferation, but we did not detect a change in proliferation after intervention, as detected by expression of Ki67. Other markers of proliferation, such as hTERT, which captures longer-term proliferation, were not investigated in this study nor were markers of apoptosis [40].

In order to evaluate whether inflammation-related measures were associated with epithelial proliferation, we conducted additional analyses with the baseline measures. We hypothesized that increased epithelial proliferation might be associated with increased eicosanoids, plasma cytokines and adverse features of nuclear morphology (Table 4). Increased plasma cytokines were associated with a higher Ki67 labeling index, indicating that factors contributing to an increased systemic pro-inflammatory state might increase

colon cancer risk. Plasma cytokines in healthy individuals display very large inter-individual differences, the complex etiology and full significance of which is still elusive [41]. In addition, it was interesting that both labeling index and extent of the proliferative zone were negatively associated with eicosanoid factor 2 that loaded mainly on leukotrienes. The apparently protective effect of leukotrienes on epithelial proliferation in the colon was interesting. There is a LTB_4 receptor that is expressed in colon, BLT2, that differs from BLT1 expressed in phagocytes, and BLT2 has been found to have protective effects on colon mucosal integrity [42]. We hypothesize BLT2 might be relatively more important in normal, vs. inflamed, human colon tissue. Finally, increased colon crypt height was associated with more circular and larger nuclei (Table 4). This could be due to the observation that nuclei have been noted to become more elliptical when cells stop proliferating as they move up the colon crypt from the base to the apex [17].

Overall, the present results indicate that dietary intervention based on either healthy eating or Mediterranean goals had little effect on colonic proliferation or morphology, but colonic PGE_3 was significantly increased by the Mediterranean intervention. In normal tissue, PGE_2 may already be low in most individuals, making it difficult to detect reductions in PGE_2 . In addition, we found lower Ki-67 labeling index to be associated with morphological features that defined smaller nuclei in the epithelial cells, higher colonic leukotrienes and lower plasma cytokines. Limitations of the study include that it was conducted over only six months and that the samples size was not large. Dietary and/or lifestyle changes that more strongly affect markers of pro-inflammatory states may be of interest for future studies. It is possible that calorie intakes and/or energy balance may have stronger effects on inflammatory changes than changes in diet quality. In this regard, it remains to be determined if aspects of obesity and/or weight change might have stronger effects than diet quality on pro-inflammatory biomarkers in normal human colon tissue.

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TABEL 1

Eicosanoid levels in colonic mucosa over time in the two diet groups. Covariates in the linear mixed models were baseline age, gender, BMI, percent of dietary goals met at 6 months and regular NSAID use.

Analyte	Healthy Eating Baseline	6 Months	Mediterranean Baseline	6 Months
Eicosanoids (pg/μg protein)	N=61	N=46	N=59	N=46
PGE ₂	17 (14)	17 (14)	18 (13)	19 (16)
PGE ₃	1.3 (1.2)	1.5 (1.7)	1.2 (1.1)	1.8 (1.8)^a
15-HETE	8.9 (12)	8.0 (7.4)	7.0 (7.0)	6.6 (7.4)
13-HODE	68 (43)	68 (52)	76 (68)	67 (48)
12-HETE	26 (44)	25 (42)	27 (72)	26 (42)
5-HETE	2.8 (2.6)	3.6 (5.1)	3.5 (5.5)	2.8 (2.4)
LTB ₄	0.33 (0.29)	0.32 (0.19)	0.32 (0.26)	0.33 (0.19)
LTB ₅	0.41 (0.44)	0.33 (0.23)	0.36 (0.37)	0.30 (0.20)
Ki 67 Proliferation Measures	N=60	N=45	N=58	N=45
Labeling Index	0.21 (0.06)	0.21 (0.04)	0.21 (0.06)	0.22 (0.06)
Extent Proliferative Zone	0.60 (0.09)	0.61 (0.07)	0.59 (0.08)	0.61 (0.07)
Crypt Height (cells/crypt)	50 (9)	51 (11)	51 (11)	50 (11)
SD Labeling index	0.07 (0.04)	0.07 (0.03)	0.07 (0.02)	0.07 (0.04)
SD Proliferative Zone	0.13 (0.06)	0.13 (0.05)	0.12 (0.05)	0.13 (0.06)
SD Crypt Height	8.2 (4.7)	8.4 (3.9)	8.4 (3.8)	7.4 (3.3)
Morphology Measures	N=59	N=41	N=58	N=41
Nuclear Circularity	0.70 (0.05)	0.71 (0.05)	0.70 (0.05)	0.69 (0.05)
Nuclear area	197 (37)	216 (66)	199 (27)	204 (45)
Nuclear diameter	9.4 (0.94)	9.9 (1.3)	9.4 (0.9)	9.4 (1.0)
SD Circularity	0.13 (0.02)	0.12 (0.02)	0.13 (0.02)	0.13 (0.02)
SD Nuclear area	59 (13)	63 (19)	60 (11)	59 (13)
SD Nuclear diameter	2.3 (0.3)	2.4 (0.4)	2.3 (0.3)	2.3 (0.3)

^a Significantly different than baseline for that diet group, $p < 0.05$ from mixed linear models after transformation of select variables to achieve normality. Transformations used were square root for PGE₂ and 13-HODE, natural log for the other eicosanoids and square root for the SD of crypt height. Data shown are untransformed means and SD of all available data at each time point for ease of interpretation.

TABLE 2

Principle component analysis of nuclear morphometric features in epithelial cells in the middle third of colonic crypts. This 2-factor solution accounted for 89% of the variance in nuclear morphology features. Features with highest loading for each factor (> 0.6) are bolded for ease of interpretation.

Nuclear feature	Factor 1 (size)	Factor 2 (circularity)
Area	0.956	0.163
Perimeter	0.783	0.589
Maximal chord	0.818	0.425
Diameter	0.873	−0.358
Maximal diameter	0.942	−0.051
Form Factor	0.161	−0.937
Major axis length	0.872	0.220
Minor axis length	0.935	−0.161
Fiber length	0.450	0.889
Number of end nodes	0.010	0.910
Number of branch nodes	0.019	0.911

TABLE 3

Principle component analysis of colonic eicosanoids. Prostaglandin E₃ (PGE₃) was the only variable to load on a 3rd factor and was kept separate. Bolded values show major contributors to each factor.

Eicosanoid	Factor 1 (HETEs, PGE ₂)	Factor 2 (Leukotrienes)
LTB ₄	0.294	0.647
LTB ₅	– 0.143	0.906
PGE ₂	0.759	0.004
13-HODE	0.741	0.450
15-HETE	0.792	0.050
12-HETE	0.865	0.200
5-HETE	0.568	0.005

TABLE 4

Correlation between colon crypt characteristics (height, labeling index, proliferation) and serum cytokines, factors generated from principal component analysis of nuclear morphology and colon eicosanoids in colon biopsy tissues at baseline. Table entries are Spearman correlation coefficient followed by the p-value. Eicosanoid Factor 1 and PGE₃ were not significantly associated with any of the crypt proliferation measures and are not shown.

Factors	Crypt height	Labeling Index	Extent of the Proliferative Zone
Eicosanoid Factor 2 (leukotrienes)	0.024, 0.797	-0.268, 0.003	-0.197, 0.033
Plasma cytokine z-score	-0.109, 0.241	0.207, 0.025	0.093, 0.316
Nuclear factor 1 (size)	0.389, <0.001	-0.185, 0.048	-0.097, 0.302
Nuclear factor 2 (circularity)	0.229, 0.014	-0.077, 0.411	-0.033, 0.726