Published in final edited form as:

Prostaglandins Leukot Essent Fatty Acids. 2018 December ; 139: 14–19. doi:10.1016/j.plefa.2018.11.001.

Higher baseline expression of the *PTGS2* gene and greater decreases in total colonic fatty acid content predict greater decreases in colonic prostaglandin-E₂ concentrations after dietary supplementation with ω -3 fatty acids

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Abstract

This study evaluated whether mRNA expression of major genes regulating formation of prostaglandin (PG)E₂ in the colon and colonic fatty acid concentrations are associated with the reduction in colonic mucosal PGE₂ after dietary supplementation with omega-3 (ω -3) fatty acids. Supplementation with ω -3 fatty acids was done for 12 weeks using personalized dosing that was expected to reduce colonic PGE₂ by 50%. In stepwise linear regression models, the ω -3 fatty acid dose and subject BMI explained 16.1% of the inter-individual variability in the fold change of colonic PGE₂ post-supplementation. Increases in mRNA gene expression after supplementation were, however, modest and were not associated with changes in PGE₂. When baseline expression of *PTGS1*, *PTGS2* and *HPGD* genes was included in the linear regression model containing dose and BMI, only *PTGS2*, the gene coding for the inducible form cyclooxygenase, was a significant predictor. Higher relative expression of *PTGS2* predicted greater decreases in colonic PGE₂, accounting for an additional 13.6% of the inter-individual variance. In the final step of the regression model, greater decreases in total colonic fatty acid concentrations predicted greater decreases in colonic PGE₂, contributing to an additional 18.7% of the variance. Overall, baseline BMI, baseline expression of *PTGS2* and changes in colonic total fatty acids together accounted for 48% of the inter-individual variability in the change in colonic PGE₂. This is consistent with

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biochemical data showing that fatty acids which are not substrates for cyclooxygenases can activate cyclooxygenase-2 allosterically. Further clinical trials are needed to elucidate the factors that regulate the fatty acid milieu of the human colon and how this interacts with key lipid metabolizing enzymes. Given the central role of PGE₂ in colon carcinogenesis, these pathways may also impact on colon cancer prevention by other dietary and pharmacological approaches.

Keywords

Cyclooxygenase; fatty acids; colon cancer; prostaglandin E₂; fish oils; inflammation

1. INTRODUCTION

Data from both clinical trials and animal studies support the role of increased dietary intakes of ω -3 fatty acids for reducing pro-inflammatory processes in colon tissue and preventing colon cancer [1]. We recently conducted a clinical trial using personalized dosing of ω -3 fatty acids to establish a dose of ω -3 fatty acids that reduces a key pro-inflammatory mediator, prostaglandin (PG)E₂, in colonic mucosa by 50% [2]. This magnitude reduction in colonic PGE₂ was based on preclinical data showing protection from colon carcinogenesis with a 50% or greater reduction in PGE₂ [3–8]. The dosing was initially done using a model that relied on the linear relationship between serum eicosapentaenoic acid (EPA): arachidonic acid (AA) ratios and reduction in colonic PGE₂ in rodents [9, 10]. Hence, a model-determined increase in the serum EPA:AA ratio, which is easily measured in blood samples, was the dosing target. Using a Bayesian design, the dosing model was updated to include human data as it became available during the conduct of the trial [2].

The trial goal was a 50% reduction in PGE₂ the minimal reduction associated with colon cancer prevention [3–8]. Increased PGE₂ production and/or decreased degradation is associated with pro-inflammatory processes and increased carcinogenesis, but a low concentration of PGE₂ is needed to maintain tissue homeostasis in the colon [11]. PGE₂ does have an integral role in maintaining normal colonic processes, such as repair of intestinal injury via its proliferative effects on the epithelium, and in dampening responses of immune cells to allow for the resolution of inflammation [12, 13]. The contrasting roles of PGE₂ in epithelium and immune cells may be one reason why fish oils for treatment of inflammatory bowel diseases have not proven to be beneficial, and pro-inflammatory effects of fish oils have been reported in colitis animal models [14, 15].

PGE₂ is formed from the action of cyclooxygenase (COX) enzymes that oxygenate the ω -6 fatty acid AA. When dietary ω -3: ω -6 fatty acid ratios are increased, ω -3 fatty acids supplant AA and other fatty acids in phospholipids to reduce AA availability [16]. In the case of COX-1, the binding of eicosapentaenoic acid ω -3 (EPA) inhibits AA oxygenation as a second mechanism to reduce PGE₂ [17]. Other long-chain ω -3 fatty acids are poor COX-1 substrates and very modest COX-1 inhibitors [18]. In the case of COX-2, EPA serves as a substrate for the production of 3-series eicosanoids that have relatively lower pro-inflammatory effects versus 2-series eicosanoids, as well as E-series resolvins that act as anti-inflammatory signals [18, 19]. Fatty acids that are not substrates for COXs, namely

saturated and monounsaturated fatty acids, have been shown to allosterically activate COX-2 oxygenation of AA [20].

The clinical trial did result in a median colonic mucosal PGE₂ concentration that was reduced by almost 50% after supplementation with ω -3 fatty acids, but there were large inter- individual differences between participants in the changes achieved [2]. In the present study, we investigated whether inter-individual variability in mRNA expression of genes coding for key enzymes in the production and degradation of PGE₂, namely COX-1, COX-2 and prostaglandin dehydrogenase (PGDH), contribute to inter-individual differences in the reduction of colonic PGE₂ after ω -3 fatty acid supplementation.

The COX enzymes catalyze the rate-limiting reaction for the conversion of arachidonic acid (AA) to PGE₂. Genes coding for COX-1 and COX-2 isoforms, *PTGS1* and *PTGS2*, are constitutively expressed in the colon, although the expression of *PTGS2* in normal colon is generally low [21, 22]. *PTGS2* appears to have a relatively more important role in tumor biology and is greatly induced during carcinogenesis [21, 22]. For the degradation of PGE₂, the rate-limiting step is catalyzed by 15-hydroxyprostaglandin dehydrogenase (15-PGDH) [23]. Previous studies have demonstrated that 15-PGDH is highly expressed in normal colonic epithelium and that mRNA expression of the gene *HPGD* is ubiquitously lost in colon cancer [23, 24]. It was therefore important to investigate to what extent differences in expression of *PTGS1*, *PTGS2* and *HPGD* together with differences in colonic fatty acid concentrations could have on the efficacy of ω -3 fatty acid supplementation on reducing colonic PGE₂ concentrations.

2. METHODS

2.1. Study participants

This Phase Ib clinical trial was approved by the University of Michigan Internal Review Board (HUM00051786) and registered at www.clinicaltrials.org (NCT# 01860352, Effects of Fish Oil on the Colonic Mucosa). The study was conducted at the University of Michigan in Ann Arbor, MI, and all participants gave signed, informed consent to participate. Participants were recruited through a web-based registry, flyers and word-of-mouth. Inclusion criteria included being generally healthy and having normal complete blood counts, clotting time, and hepatic chemistry tests. Exclusion criteria included pregnant or lactating women, use of anti-coagulation medications, steroids, chronic use of non-steroidal anti-inflammatory drugs, high blood pressure, history of Crohn's disease, ulcerative colitis or other inflammatory diseases, no history of cancer diagnosed within the last five years, and allergies to fish or fish oil. Individuals taking medications or supplements that might affect outcomes, such as aspirin or fish oils, were excluded or given the option of a 3-week wash-out period. A total of 47 eligible participants completed the trial, biopsies for gene expression were available from 45 subjects and mRNA amplification was successful in 44 subjects.

The agent used in the study was a fish oil preparation highly enriched in EPA (EPA-xtra, from Nordic Naturals, Watsonville, CA). Study subjects were asked to provide blood and colon biopsy samples at study entry. Following the baseline sampling, subjects were asked to

consume a low dose of ω -3 fatty acids for two weeks (approximating a dietary EPA: ω -6 fatty acid ratio of 0.1), to provide another blood sample, and then to take a high dose of ω -3 fatty acids for another two weeks (approximating a dietary EPA: ω -6 fatty acid ratio of 0.3), followed by another blood sample. Each individual's own serum EPA:AA fatty acid ratios as a function of dose were used to calculate a personalized dose needed to achieve the target EPA:AA ratio that would be expected to reduce colonic PGE₂ by 50%. The target dose was provided for 12 weeks and this was followed by another colon biopsy and blood sampling. The average target dose was 5.5 g of EPA-xtra per day. Details of the dosing and eicosanoid changes have been published [2].

2.2. Colon mucosal biopsies and serum

Participants underwent flexible sigmoidoscopy at study entry and 20–28 hours after the last ω -3 fatty acid target dose. The participants did not prepare their bowels before the procedure. Participants were placed in a left lateral decubitus position and a flexible sigmoidoscope was passed to 20 to 25 cm from the anal sphincter. Tissue samples were taken using Radial Jaw 4 Jumbo forceps (Marlborough, MA) by opening and pressing the forceps perpendicular to the mucosal surface with mild pressure. Biopsies for analysis of gene expression, eicosanoids or fatty acids were frozen in liquid nitrogen within 60 seconds and frozen at -80°C until analysis. Assays for fatty acids and eicosanoids were done on a homogenate prepared from four biopsies, and gene expression was analyzed in a separate biopsy due to the different processing methods required. Serum samples were prepared after allowing whole blood to coagulate for 30 minutes followed by centrifugation at 1500 g for 30 minutes. Aliquots were frozen at -80°C until analysis.

2.3. Eicosanoid and fatty acid analyses

The eicosanoid and fatty acid analyses were performed on homogenates prepared from four colon biopsies. The biopsies (about 20 mg tissue each) were pulverized and homogenized with 800 μL of ice-cold phosphate-buffered saline containing 1 mM EDTA and 0.1 mM indomethacin using an Ultrasonic processor in an ice-cold water bath. Protein was determined in 20 μL of the homogenate using the Bradford assay (BioRad Protein Assay Reagent, Hercules, California).

Eicosanoids were extracted from 500 μL of the homogenate after adding 20 μL of a buffer (0.75M citric acid, 0.25 M ammonium acetate, pH 2.7) and 20 μL of deuterated internal standards. Two extractions were done using 2 mL of hexane: ethyl acetate (1:1, v/v, 0.1% BHT) each time. The evaporated extract was reconstituted in 80 μL methanol and subjected to HPLC-MS-MS analysis using a chiral column as described previously [25].

Concentrations of eicosanoids were calculated versus standard curves constructed for each analyte and expressed as ng eicosanoid per mg protein.

Fatty acids were determined using a 150 μL aliquot of the homogenate or 100 μL of serum. The internal standard, 17:0, was added and two extractions were performed using chloroform:methanol 2:1 (on a volume basis). Dried lipid extracts or standards were then derivatized to form fatty acid methyl esters by adding 90 μL of chloroform:hexane (1:1) and 10 μL Meth-Prep II (Alltech Inc., Deerfield, IL) at room temperature prior to GC-MS.

analysis, as described previously [26]. Fatty acid concentrations were calculated in $\mu\text{g/ml}$ using standard curves for each fatty acid. The total content of fatty acids in the biopsies was determined by summing the 13 fatty acids analyzed and calculating μg of total fatty acids per mg protein, to normalize against variations in biopsy size. To calculate EPA:AA ratios, the mole percent of EPA and of AA, as a percent of total fatty acids, was used.

2.4. RNA extraction and reverse transcription

One frozen biopsy of approximately 5–10 mg tissue from each participant at each time point was used for RNA extraction. RNA was extracted using 1 ml TRIzol Reagent following the manufacturers' protocol (Invitrogen, Waltham, MA). RNA was purified using a PureLink RNA Mini Kit (Invitrogen 12183–018A) and reverse transcription was done using an Applied Biosciences High Capacity Reverse Transcriptase Kit (Invitrogen 4368813). The cDNA samples were amplified in a thermal cycler for 10 minutes at 25°C, 120 minutes at 37°C, then for 5 minutes at 85°C.

2.5. Quantitative real-time PCR (RT-qPCR)

Real-Time PCR was performed by using TaqMan® Environmental Master Mix 2.0 (Applied Biosystems). The primers and probes used for real time q-PCR were purchased from Applied Biosystems (Foster City, CA, USA). The primers used were: *PTGS1* (COX-1, Hs00377726_m1), *PTGS2* (COX-2, Hs00153133_m1), *HPGD* (prostaglandin dehydrogenase, Hs00960586_g1). Gene expression results were normalized to expression of *ACTB* (Actin, Hs01060665_g1) and *KRT20* (cytokeratin, Hs00300643_m1).

For quantification, the standard curve method was used. A 10 μl aliquot of cDNA was taken from each sample, and sequential 5-fold dilutions were prepared from the pooled cDNA to create standard curves (Figure 1). All samples and standard curves were run in duplicate on the same plate for the real-time PCR reactions. The relative amount of each target mRNA expression was established from the standard curve and was subsequently normalized by *ACTB* or *KRT20* expression. The results are therefore expression of any given sample relative to the sample pool. This assay method was previously validated against immuno-histochemical protein expression in human colonic biopsies by Sidahmed et al. [27].

2.6. Statistical analysis

Statistical analyses were conducted using IBM SPSS software version 24 (PASW Statistics, IBM Corporation, Armonk, New York) To evaluate factors that affect change in colonic PGE_2 concentrations after supplementation with ω -3 fatty acids, subjects were classified as responders if they achieved a 50% decrease in colonic PGE_2 concentrations since a 50% decrease was the target of the phase Ib clinical trial. Differences between responders and non-responders were evaluated by two-sided, independent-samples t-tests or Pearson Chi-Square tests (two-sided) with the exception of family history and race for which we conducted Fisher's Exact Test due to the small numbers of subjects (Table 1).

To evaluate differences in colonic gene expression in subgroups defined by demographic factors or by being above or below the median value for variables such as dietary intakes or colonic measures, independent samples t-tests were done. These subgroup analyses were not

done when group size was small: there were only five current users of tobacco products and two non-Caucasian subjects. Spearman correlations were used to evaluate the correlations of colonic PGE₂ concentrations with gene expression at baseline.

Changes in gene expression post supplementation were evaluated by one-sample tests of fold change (post/pre), as determined by a fold change that is significantly different from 1 (Table 2). Expression of the genes of interest at baseline and change in expression post/pre were investigated for association with changes in colonic PGE₂ concentrations in linear regression models. In these models, natural log transformation was used to normalize fold change in colonic PGE₂, the outcome of interest. R² values were used as a measure of incremental contribution of groups of prognostic factors in explaining variability in the outcome (Table 3). In the first two steps of the linear regression, dose and demographic variables were considered for inclusion based on differences between responders and non-responders. The variables with the largest differences between groups in the bivariate analysis, namely BMI (with p=0.05) and dose per kg body weight (p=0.01), were therefore entered. In the third step, stepwise selection was used to enter relative expression of *PTGS1*, *PTGS2* and *HPGD* at baseline, and the stepwise selection method retains only the significant predictors in the final model. In an alternate model, changes in expression of these three genes were evaluated in the third step. In the fourth step, stepwise selection was used to enter changes in colonic total fatty acid variables to determine which fatty acid variables are significant predictors of the reduction in colonic PGE₂.

3.0 RESULTS

3.1. Colonic gene expression and associations with colonic PGE₂ concentrations and demographic factors at baseline

Of the 90 available biopsies, all were successfully amplified but two had values more than 150% of the interquartile range and were excluded due to the possibility of sample handling artefacts. This left 44 pairs of samples for the analyses. The mean efficiency for the PCR standard curve for each primer was between 100% and 105%. Expression in replicate samples differed by an average of 1.6% for *ACTB*, for 1.3% *KRT20*, 1.2% for *PTGS1*, 1.4% for *HPGD*, and 1.3% for *PTGS2*. Samples with greater than 7% difference between duplicates were repeated (four samples for *PTGS2*, two for *HPGD* and one for *PTGS1*). Expression of genes in the normal colonic mucosal biopsy samples was highest for *ACTB*, followed by *KRT20*, *HPGD*, *PTGS1* and *PTGS2* (Figure 1).

The data in Table 1 show the baseline characteristics of study subjects by “responder status”, as defined by whether or not a 50% reduction in colonic PGE₂ was achieved. The intent of these analyses was to identify potential predictor variables for inclusion in the linear regression analyses, and p-values from the independent t-tests therefore were not adjusted for multiple comparisons. These results showed that mean baseline BMI was borderline higher in non-responders (p=0.053), and mean baseline colonic EPA:AA ratio also was higher in non-responders versus responders (p=0.043). The ω -3 fatty acid dose, expressed per kg body weight, baseline PGE₂, and baseline gene expression of *PTGS2* conversely were all significantly lower in non-responders versus responders. The expression of *HPGD*, or

PTGS1 normalized to either actin (Table 1) or to cytokeratin (not shown) did not differ by responder status.

In additional analyses using independent samples t-tests, gene expression did not differ between men and women, or among subjects with values above or below the median for age, BMI, selected dietary intakes (fiber, fat, ω -3 fats), colonic fat content (total fat content, EPA:AA ratio, ω -3 fatty acid content) and colonic concentrations of PGE₂ and PGE₃ (not shown). Spearman correlations also were used to evaluate the correlations of colonic PGE₂ concentrations with gene expression at baseline. The correlations with *PTGS1* ($\rho=0.06$) and with *HPGD* ($\rho=0.08$) had coefficients near zero. The correlation with *PTGS2* ($\rho=0.27$) was better but not significant with $p=0.08$.

3.2. Effects of ω -3 fatty acid supplementation on colonic gene expression and total fatty acid content

There were several statistically significant effects of supplementation on colonic expression of *PTGS1*, *PTGS2*, and *HPGD*, although the nature of the significant differences varied across responder status (Table 2). The mean expression of all three genes increased 20–30%. The variability in individual responses was, however, large with coefficients of variation ranging 34 – 103%. The increases in *PTGS1* and *HPGD* expression post ω -3 fatty acid supplementation were statistically significant in the complete group of 44 subjects, and the increase in *HPGD* also was significant in the responder subgroup.

In the 44 subjects combined, change in mean total colonic fatty acid content was small (fold change of 1.13, SD 0.43), but the variability was substantial. In the 21 subjects who displayed a decrease in total colonic fatty acid content (in $\mu\text{g}/\text{mg}$ protein), mean BMI was the same (27 in either case), but colonic PGE₂ was decreased by 56%. The decrease in colonic PGE₂ for the 23 subjects who did not display a decrease total colonic fatty acid content was only 4% ($p<0.001$ from independent-samples t-tests). In the groups with and without a decrease in total colonic fatty acids, changes in serum EPA:AA ratios (6.7 and 7.2-fold, respectively, $p=0.797$) and change in colonic EPA:AA ratios (5.0- and 3.5-fold, respectively, $p=0.089$) were not significantly different.

3.3. Predictors of reduction in colonic PGE₂ after supplementation

Linear regression models were created to evaluate predictors of the fold-change in colonic PGE₂. In the first two steps of the regression model, we sought to control for dose and demographic factors before evaluating the potential contribution of colonic fatty acids and gene expression in steps 3 and 4. Since the dosing was personalized based on blood EPA:AA responses, the dose provided to each subject varied and was important to include in the model. As could be expected, the beta coefficient for dose of ω -3 fatty acids per kg body weight was negative indicating that a higher dose was associated with a greater reduction in colonic PGE₂, but this was not statistically significant with $p = 0.082$ (Table 3). Of the demographic variables, BMI exhibited the largest differences by responder status and is known to be associated with inflammatory processes. Adding baseline BMI in the model resulted in more than doubling the percent of variation explained, and higher BMI was associated with larger fold change in PGE₂, ie. a smaller decrease ($p = 0.030$). Dose and

BMI together accounted for 16.1% of the variance in colonic PGE₂. Adding age and gender did not improve the model substantively (not shown). There was very little change in body weight during the study (mean 0.9 pounds, SD 5.0, in non-responders; mean -2.2 pounds in responders, SD 4.2, $p = 0.321$ from independent t-tests), and this also was not a significant predictor the linear regression model.

Since there was very little change in gene expression by ω -3 fatty acid supplementation, we then entered relative expression of all three genes at baseline using stepwise selection in the third step of the model. Of the three genes, only baseline *PTGS2* expression was statistically significantly associated with fold change in colonic PGE₂ (Table 3). Higher baseline *PTGS2* expression predicted a greater reduction in PGE₂ and accounted for an additional 13.6% of the variance in change in colonic PGE₂ ($p = 0.003$ for the F-change). When fold change in *PTGS1*, *PTGS2* and *HPGD* gene expression (post/pre) were entered in the third step instead, none of these were significant predictors (not shown). Finally, we entered fold change in total colonic fatty acids and fold change in the EPA:AA ratio (fold change post/pre supplementation) as predictors using stepwise selection. The fold increase in the colonic EPA:AA ratio varied from 1.4–13.7 except for one sample that was an outlier with a 64-fold increase. After excluding the outlier, fold change in EPA:AA ratio was not a significant predictor, and only change in total colonic fatty acid concentrations was retained as a significant predictor in the model, accounting for an additional 18.7% of the variance of change in colonic PGE₂ (Table 3). The positive beta coefficient indicates that a larger decrease in total colonic fatty acids predicted a larger decrease in colonic PGE₂. The overall model accounted for 48.4% of the inter-individual variability in change in colonic PGE₂ after ω -3 fatty acid supplementation ($p < 0.001$ for the overall model). When fold increase in EPA:AA was used without excluding the outlying value, the fold increase in the EPA:AA ratio became a significant predictor that was retained in the model but it only improved the predictive value modestly with a final R^2 of 51.7% (not shown).

4. DISCUSSION

This study investigated the impact of the colonic fatty acid milieu and the relative expression of key genes on the production of PGE₂ in the human colon after ω -3 fatty acid supplementation. Our results show that personalized ω -3 fatty acid supplementation that targeted EPA:AA ratios in serum did not result in large changes in the expression of *PTGS1*, *PTGS2*, or *HPGD* genes in the colon of healthy volunteers, with increases of <30% post-supplementation. However, higher baseline *PTGS2* expression was a significant predictor of decreased colonic PGE₂ post supplementation, as shown in Table 3. This was unexpected since *PTGS2* mRNA is expressed at low levels in the normal human colon. In this generally healthy study population, only one subject had a strong family history of colorectal cancer and none had a personal history of colorectal cancer. Other work also has shown that preventive agents exert relatively greater effects on reducing PGE₂ when baseline PGE₂ concentrations are elevated, or when either protein expression of COX-2 is elevated or expression of 15-PGDH is low [28–31]. Our study was small, but others have shown that inter-individual variability in *PTGS2* expression is modified by both genetic polymorphism and by environmental exposures [32, 33]. *PTGS2* expression therefore might be an important prevention target even in persons at normal risk for colon cancer, although we

cannot rule out a contribution from changes in *HPGD* and *PTGS1* mRNA expression which might become significant in a larger data set.

The other important finding was that change in total fatty acid concentration in the colon post-supplementation was a stronger predictor of change in PGE₂ concentration than change in colonic EPA:AA fatty acid ratios. Biochemical data show that EPA is an excellent inhibitor of COX-1 via binding to the active site of the enzyme [17]. In the case of COX-2, however, fatty acids that are not substrates affect cyclooxygenase activity via their binding to the allosteric monomer [18, 34]. The major fatty acids in human tissues, SFA and MUFA, are not substrates for COX-1 nor COX-2, but these fatty acids activate COX-2 [18]. Other mechanisms by which SFA and MUFA could augment the pro-inflammatory state of the colon is through the activation of inflammatory pathways via toll-like receptors [35]. Evidence is now growing that there is an important interplay between signaling through toll-like receptors and cyclooxygenase pathways in regulating the inflammatory environment in the GI tract [36].

It was unexpected that the effects of dietary supplementation with ω -3 fatty acids would be modulated by fatty acid concentrations in the colonic mucosa since in our study there were no significant changes in body weight. It is, however, well-known that ω -3 fatty acids can reduce serum triglycerides via stimulation of extracellular lipolysis [37]. Dietary supplementation with EPA also has been shown to have anti-lipogenic effects in human adipocytes [38]. In the colonic epithelium, triglycerides are stored in the form of cellular lipid droplets, and lipid droplets have been shown to be active sites of PGE₂ synthesis [39]. Supplementation with ω -3 fatty acids therefore could function to reduce the pro-inflammatory state at least in part by reducing lipid stores in the colon.

The study limitations include the modest number of subjects, measures of PGE₂ which is a labile compound that is challenging to measure (especially in tissues), and it is not known if 12 weeks of ω -3 fatty acid supplementation is long enough for changes in the colon to reach equilibrium. We did not measure body fat which would be a better indicator of excess adiposity than BMI. Another possible caveat is that we cannot account for subtle changes in energy balance that shift metabolic patterns from fatty acid oxidation to fatty acid storage in cellular lipid droplets. The personalized dosing was designed to target a model-determined increase in serum EPA:AA ratios, and this did result in a varying ω -3 fatty acid dose per kg body weight, the latter of which is a more traditional method to gauge dose. The major study strength, however, is the availability of colon biopsies that allowed us to evaluate the impact of previously identified biochemical pathways on colonic PGE₂ formation in humans.

5. CONCLUSIONS

These data show that individuals who have relatively higher baseline mRNA expression of *PTGS2* and who exhibit greater decreases in total colonic fat content after ω -3 fatty acid supplementation are predicted to have a greater reduction in colonic PGE₂. These data are consistent with the allosteric regulation of the COX-2 enzyme by saturated and monounsaturated fatty acids. Further clinical trials are needed to elucidate the factors that regulate the fatty acid milieu of the human colon and how that affects the pro-inflammatory

environment in the colon. Given the central role of COX-2 in colon carcinogenesis [21, 22], this also is likely to impact on colon cancer prevention by other dietary and pharmacological approaches.

Acknowledgements

We thank all the participants of the Fish Oil Study for making this research possible. We thank Dr. Justin A. Colacino for helpful discussions, Jianwei Ren for processing and analyzing samples, and Kirk Herman for study coordination.

Funding

Support for this research was obtained from NIH grant P50 CA130810, Gastro-Intestinal SPORE grant P50 CA130810 (D.E. Brenner, PI), NIH R01 DK107535 (D. Bridges, PI) and Cancer Center Support Grant for the University of Michigan Rogel Comprehensive Cancer Center, P30 CA046592. The research used core resources supported by a Clinical Translational Science Award, NIH grant UL1RR024986 (the Michigan Clinical Research Unit), the Michigan Diabetes Research Center, NIH grant 5P60 DK20572 (Chemistry Laboratory), the Michigan Nutrition and Obesity Research Center, NIH grant P30 DK089503, the Kutsche Family Memorial Endowment (D.E. Brenner) and the Rose and Lawrence C. Page Foundation (D.K. Turgeon). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Abbreviations

AA	Arachidonic acid, ω -6
COX	Cyclooxygenase
EPA	eicosapentaenoic acid, ω -3
PGE₂	prostaglandin E ₂

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Highlights

- Higher baseline *PTGS2* mRNA expression was a significant predictor of decreases in prostaglandin (PG)E₂ in the colon of healthy volunteers after ω -3 fatty acid supplementation that was personalized using blood fatty acid responses.
- Decreases in colon total fatty acid concentrations post-supplementation predicted greater reduction in colonic PGE₂ and point to the importance of lipid stores within the colonic mucosa in governing the anti-inflammatory effects of ω -3 fatty acid supplementation.
- Dose of ω -3 fatty acids, baseline BMI, baseline expression of *PTGS2* and change in colonic total fatty acids accounted for 48% of the inter-individual variability in the change in colonic PGE₂ in linear regression models.

Figure 1.

Linearity of standard curves in the RT-qPCR assay and differences in cycle threshold (CT) values for expression of each gene. The curves were constructed using dilutions of a standard mixture that contained equal aliquots of all samples analyzed, and the curves show that relative expression of PTGS2 was lowest among the genes evaluated in the normal human colon samples (highest cycle threshold).

Table 1

Demographic factors by participants who responded with a 50% reduction in colonic prostaglandin (PG)E₂ or not after dosing with ω -3 fatty acids enriched in eicosapentaenoic acid (EPA). Data on 44 participants with available gene expression are shown as mean (SD) or number (percent).

Variable	Responder		P-value ^a
	(N=18)	Non-Responder (N=26)	
BMI, kg/m ²	25 (4)	28 (5)	0.053
Age, years	47 (12)	48 (15)	0.472
Male, n (%)	5 (28%)	11 (42%)	0.325
Caucasian, n (%)	17 (94%)	25 (96%)	1.000
Smoker, n (%)	1 (6%)	4 (15%)	0.390
Family history of colon cancer, n (%)	1 (6%)	0 (0%)	0.409
Dose consumed, g/day ^b	6.1 (1.8)	4.9 (2.3)	0.080
Dose, g/kg body weight	0.084 (0.024)	0.062 (0.030)	0.014
Baseline PGE ₂ , pg/mg protein	9.5 (7.8)	5.0 (3.4)	0.030
Baseline colonic fatty acids, μ g/mg protein	306 (124)	338 (156)	0.483
Baseline colonic EPA:AA ratio	0.14 (0.09)	0.20 (0.09)	0.043
Baseline <i>PTGS1</i> ^c	0.78 (0.21)	0.86 (0.25)	0.261
Baseline <i>PTGS2</i> ^c	1.04 (0.39)	0.72 (0.44)	0.019
Baseline <i>HPGD</i> ^c	1.02 (0.38)	1.08 (0.33)	0.552

^aP-values are from independent independent-samples t-tests for continuous variables, from Pearson Chi-Square tests (two-sided) for gender, and from Fisher's Exact Test (two-sided) for race, smoking and family history.

^bAdherence to the prescribed dose over 12 weeks averaged 97% (SD 5%, range 77–100%) and the actual dose consumed from pill counts is shown.

^cBaseline values for gene expression relative to a standard mixture of all samples are shown normalized to actin.

Table 2

Fold change in gene expression after ω -3 fatty acid supplementation relative to baseline. Shown is the mean and 95% confidence interval for relative gene expression that was normalized to actin prior to determining fold change in mRNA expression.

Gene	Fold Change	P-value ^a
All subjects (n=44)		
<i>PTGS1</i>	1.25 (1.06, 1.44)	0.01
<i>PTGS2</i>	1.30 (0.97, 1.63)	0.08
<i>HPGD</i>	1.20 (1.04, 1.36)	0.02
Responders (n=18)		
<i>PTGS1</i>	1.23 (0.94, 1.52)	0.14
<i>PTGS2</i>	1.13 (0.59, 1.67)	0.64
<i>HPGD</i>	1.29 (1.09, 1.49)	0.01
Non-Responders (n=26)		
<i>PTGS1</i>	1.27 (1.01, 1.53)	0.05
<i>PTGS2</i>	1.41 (0.99, 1.83)	0.07
<i>HPGD</i>	1.13 (0.90, 1.36)	0.27

^a P-values are from one-way t-tests to determine whether fold changes are significantly different from 1.00.

Table 3

Predictors of the natural log of fold change in colonic PGE₂ concentrations in 44 healthy individuals after ω -3 fatty acid supplementation (post/pre) in stepwise linear regression models.

Predictor	β -Coefficient in Final Model	SE of β -Coefficient	P-Value for F-change	R ²
ω -3 Fatty Acid Dose (g/kg body weight) ^a	-7.44	4.09	0.082	0.072
+ Baseline BMI (kg/m ²) ^a	0.04	0.03	0.030	0.161
+ Baseline <i>PTGS2</i> Expression ^b	-0.82	0.28	0.003	0.297
+ Change in Total Colonic Fatty Acid Concentration ^c	1.00	0.27	<0.001	0.484

^a In the first step, the actual dose consumed based upon pill counts was calculated and entered in the model to with natural log of fold change in colonic PGE₂ as the outcome variable. The negative β -coefficient indicates that a higher dose predicted a larger decrease in colonic PGE₂. Conversely, higher baseline BMI, entered into step 2, predicted a smaller decrease in colonic PGE₂. These effects together accounted for 16.1% of the variance in colonic PGE₂ change after ω -3 fatty acid supplementation.

^b Stepwise selection was used to enter relative expression of *PTGS1*, *PTGS2* and *HPGD* in baseline biopsies in the third step. Expression of *PTGS2* was the only significant predictor and was retained in the final model.

^c Stepwise selection also was used in step 4 to enter fold change in colonic total fatty acid (post/pre) content and fold change in colonic EPA:AA ratio. Change in colonic total fatty acid content was significant and retained in the final model. The R² indicates the fraction of the variance in fold change of colonic PGE₂ explained by the model, which was 48.4%, with p<0.001 for the overall model.