Biomarkers for Personalizing Omega-3 Fatty Acid Dosing

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Abstract

Prostaglandin E\(_2\) (PGE\(_2\)) has been linked to a higher risk of colorectal cancer. PGE\(_2\) in colon tissue can be reduced by increasing dietary eicosapentaenoic acid (EPA). The dose-dependent relationships between dietary EPA, serum EPA:arachidonate (AA) ratio, urinary PGE\(_2\) metabolites and colonic eicosanoids were evaluated to develop biomarkers for prediction of colonic PGE\(_2\). Male rats were fed diets containing EPA:ω6 fatty acid ratios of 0, 0.1, 0.2, 0.4, or 0.6 for five weeks. Increasing the dietary EPA:ω6 fatty acid ratio increased EPA:AA ratios in serum and in the proximal, transverse and distal colon (p<0.001). The urinary PGE\(_2\) metabolite was reduced (p=0.006). EPA rich diets reduced colonic tissue PGE\(_2\) concentrations by 58-66% and increased PGE\(_3\) by 19-28 fold. Other arachidonic acid-derived eicosanoids were reduced by 35-83%. The changes were not linear, with the largest changes in eicosanoids observed with the lower doses. A mathematical model predicts colonic tissue eicosanoids from the EPA:AA ratio in serum and the EPA dose. Every 10% increase in serum EPA:AA was associated with a 2% decrease in the (geometric) mean of PGE\(_2\) in the distal colon. These mathematical relationships can now be applied to individualized EPA dosing in clinical trials.
Introduction

Large, population-based studies have indicated that nonsteroidal anti-inflammatory drugs (NSAIDs) can reduce the risk of colorectal adenocarcinoma by approximately 50% (1, 2). This effect of NSAIDs may be mediated by inhibition of the cyclooxygenase (COX) activity of prostaglandin H synthases -1 and 2, also commonly referred to as COX-1 and COX-2. Unfortunately, NSAIDs have unacceptable toxicity profiles that prevent daily use in otherwise healthy populations (3-6). COX is not the only target of NSAIDs for prevention of colorectal and other cancers. NSAIDs indirectly modulate lipoxygenases (LOXs) pathways that can impact tissue concentrations of hydroxyeicosatetraenoic acids (HETEs), leukotriene (LTs) and hydroxyoctadecadienoic acids (HODEs). Lipoxygenases (5-LOX and 12-LOX) and their downstream products have been associated with enhanced carcinogenesis in multiple epithelia (7, 8). 15-LOX expression correlates with increased colonic crypt differentiation and its main downstream product, 13-S-HODE, decreases during the colonic carcinogenesis process (9, 10).

An alternative approach to pharmacological inhibition of COX and LOX may lie in a nutraceutical approach--modulation of the pool of phospholipid-derived fatty acid substrates. The ω3 fatty acid eicosapentanoic acid (EPA) from fish oil is an alternative substrate for COX (11), yielding eicosanoids that are less pro-inflammatory than the products from ω6 fatty acids (12, 13). EPA inhibits COX-2 expression, PGE₂ production and cytokine production in the colon (12-14). Both EPA and docosahexaenoic acids (DHA) from fish oil, inhibit the growth of experimental tumors in tissue culture and in animals, including xenografts of human tumors in nude mice (15), yet only EPA can...
serve as an efficient substrate for COX-2 (16, 17). Although some studies report that DHA can potentially alter the activity of COX-2 (18, 19) and lipoxygenases (20) these effects are via non-metabolic substrate-enzyme competitive interactions. Our *in vitro* data suggest that the EPA is an effective competitive inhibitor of AA for COX-1 (11, 16, 17). Since COX-1 is the source of most prostaglandins in normal mucosa, our data *in vitro* suggest that reduction of local colorectal mucosal PGE$_2$ production *in vivo* would be more contingent upon increasing the colorectal mucosal EPA:ω6 fatty acid ratios as opposed to DHA.

The goal of the current study was to identify mathematical relationships between serum biomarkers that could predict colonic PGE$_2$ concentrations in a rat model with the goal of translating this information to humans. We focused on distal colon. Proximal and distal colon cancers differ in their etiology and biology (21, 22). Chronic inflammation appears to be relatively more important for distal versus proximal colon cancer (23, 24). Here we document the relationships between dietary dosing, serum EPA:AA, EPA:AA in the distal colon, urinary PGE$_2$ metabolites and distal colorectal tissue PGE$_2$ concentrations in an F344 rat model.

**Materials and Methods**

**Animals and diets**

All animal protocols for this experiment were approved by the University Committee on Use and Care of Animals at the University of Michigan. Male F344 rats (5 weeks old) were purchased from Harlan Laboratories (Haslett, MI). The pelleted low fat AIN93-G, high fat control diet (EPA: ω6 fatty acid ratio= 0), and high fat fish oil diets (EPA: ω6 fatty acid ratio= 0.1, 0.2, 0.4, 0.6) were prepared by Dyets Inc. (Bethlehem,
PA). Table S1 shows the composition of the experimental diets. The Western blend oil contained coconut oil (45% by weight), olive oil (30% by weight), corn oil (15% by weight) and soybean oil (10% by weight). The Western blend oil was mixed with menhaden oil to achieve the desired EPA:ω6 ratios.

To minimize lipid oxidation, OmegaPure brand menhaden oil from Omega Protein was stabilized against oxidation with a combination of mixed tocopherols and tert-butylhydroquinone (TBHQ). This menhaden oil was then blended with the "Western" blend oil that was also stabilized with TBHQ. The fat blend was then added to the diet and mixed in a planetary mixer in cold conditions with an average temperature change less than 3°C. After pelleting, the diet was air dried (25°C) for 24 hrs. During visual inspection before packaging, any dark or mottled looking pellets that indicated the fat had oxidized were discarded and remade. We stored the diets frozen in a -80°C freezer. Small amounts were thawed in the refrigerator every two days for feeding. We replaced the food in the animal cage on a daily basis to minimize lipid oxidation. The rats were given water ad libitum throughout the experiment. The rats were maintained on a 12 h light/dark cycle.

**Sample collection and the experimental design**

Rats were acclimated to the AIN93-G diet for one week. Sixty rats used for this experiment were randomly divided into five groups (12/group) and provided with one of the five diets: control diet or one of four fish oil diets for five consecutive weeks. Body weight was recorded weekly. During the last week of feeding, the rats were individually housed in metabolic cages for 24 h for urine collection. At the end of the study, animals were euthanized by isoflurane inhalation and decapitation. Blood was collected from the
neck, and serum was separated and stored at -80°C. The animals were not fasted prior to necropsy to preserve the colon biology. The colon was immediately removed from the animal and rinsed with cold PBS containing indomethacin (5.6 μg/mL). The colon was cut into three equal sections horizontally, which were denoted as the “proximal” (cecal end), “transverse” and “distal” (rectal end) colon. The colonic sections were snap frozen in liquid nitrogen, and stored at -80°C prior to processing. Frozen colonic tissue samples were pulverized in liquid nitrogen, and then 1 mL of cold PBS (with indomethacin) was added to the tissue powder to make a tissue homogenate. The suspension was sonicated in ice water for 3 min (20 s sonication, 20 s cooling cycle), and stored in a -80°C freezer until further analysis. An aliquot of the homogenate from each sample was used to determine protein concentrations using the Bradford assay (Bio-Rad Laboratories Inc., Hercules, CA).

The assays were carried out in batches in a balanced manner. The design conformed to a completely randomized block design. There were six batches in all with each batch yielding fatty acid and eicosanoid measurements from all three locations of twelve rats. Two rats from each diet dose group were analyzed in every batch, making an even dose distribution within batch.

**Synthesis of d5-PGE₃**

The internal standard d5-EPA was synthesized as described in a recent report (25). This material was used to synthesize d5-PGE₃ using human recombinant COX-2 as described previously (16), making a 800 μg/ml solution in 50 mM PBS. Human microsomal recombinant prostaglandin E synthase-1 (mPGES-1) was from Cayman Chemical (Ann Arbor, MI). Buffer A (prepared immediately before the reaction) contained
0.5 M TrisHCl, pH 8.3 (200 mL), deionized water (800 mL), 1 mM phenol (10 mL) and 5 mM hemin in DMSO (1 mL). Buffer B contained NaH₂PO₄·H₂O (436 mg), Na₂HPO₄ (971 mg), reduced glutathione (77 mg), EDTA·2Na·2H₂O (37 mg), Triton X-100 (320 mg) and 98 mL of water. The d5-EPA (10 μl of a 6 mM solution in ethanol) was added to Buffer A (900 μL) and the sample mixed well. Recombinant human COX-2 was then added (30 μL of a 0.8 mg/mL solution in 50 mM phosphate buffered saline). The sample was mixed vigorously for 120 sec. Buffer B (180 μL) was added with mixing and then mPGES-1 (6 μL of a 20 mg/mL solution in Buffer B) was added, the sample mixed well and left at room temperature for 10 min. The reaction was quenched with 450 μL of acetonitrile/0.2 M citric acid (4:1, v/v). The reaction mixture was transferred into plastic tubes and centrifuged at 1300 x g for 5 min to remove precipitated protein.

For purification, the supernatant (750 μL) was injected onto an HPLC equipped with C18-RP-HPLC column (Shodex RSpack DE-413L, 250mm x 4.6 mm, 5 mm) operated at 1 mL/min with a binary gradient of solvent A with solvent B. Solvent A was acetonitrile/H₂O/acetic acid (0:70:0.1, v/v/v). Solvent B was acetonitrile/H₂O/acetic acid (90:10:0.1, v/v/v). The gradient was solvent A-100%, 5 min, graduated to solvent B-100% for 15 min, solvent B-100% 8 min, solvent A-100% 6 min. The peak of d5-PGE₃ was collected between 14.5 and 15.5 min. The separation was performed twice to yield 2 mL of the eluate, which was evaporated under vacuum and re-dissolved in ethanol. HPLC-MS (ESI, negative ion mode), m/z (I%) (26): 354.2 (100%) [M (deuterated – d5) - H]-.
Eicosanoid analysis by chiral LC-MS/MS

The d5-PGE$_3$ and d5-EPA used in the assays were synthesized in our laboratory (25). Other eicosanoid standards were obtained from Cayman Chemical (Ann Arbor, MI). The LC-MS/MS method for eicosanoid analysis was adapted from the literature as previously described (27, 28). For extraction of eicosanoids, 300 $\mu$L of the colonic homogenate was added to 12 x 75 mm glass tubes on ice, along with 1 N citric acid (20 $\mu$L), 30 mM disodium EDTA (20 $\mu$L), and deuterated internal standard. The resulting solution was then extracted twice with 2 mL hexane:ethyl acetate (1:1 v/v, containing 0.1% BHT (w/v)). The pooled extracts were evaporated and reconstituted with 100 $\mu$L of HPLC mobile phase. Aliquots (20 $\mu$L) were injected for analysis by LC-MS/MS.

HPLC separation was performed on a Waters 2695 separations module, using a Chiral-Pak AD-RH analytical column (2.1 x 150 mm, 5 $\mu$m particle size) (Chiral Technologies, West Chester, PA). The column was maintained at 40$^\circ$C. Mobile phase A was 10 mM ammonium acetate pH 4.1 and mobile phase B was acetonitrile. The flow rate was 0.2 mL/min. The linear gradient program was as follows: 30-50% B (0–5 min), 50-100% B (5–24 min), 100% B (25-30 min), and return to 30% B (30–37 min). The effluent was introduced into a Finnigan TSQ Quantum Ultra triple quadrupole mass spectrometer by electrospray ionization (ESI) and detection of negative ions was as previously described (27). Eicosanoid concentrations in colonic tissue were normalized to protein concentrations.

Urinary metabolite analysis by LC-MS/MS

Rat urine samples (1 mL) were processed following the method of Murphey et al. (26). Briefly, LC was performed on a Zorbax Eclipse XDB-C18 column (Agilent
Technologies, Palo Alto, CA, USA) attached to a Surveyor MS Pump (Thermo-Finnigan, San Jose, CA, USA). Detection was with a ThermoFinnigan TSQ Quantum triple quadrupole mass spectrometer operating in the selected reaction monitoring (SRM) mode. Quantification of urinary metabolite of PGE$_2$, tetranor PGE-M (9,15-dioxo-11α-hydroxy-2,3,4,5-tetranor-prostan-1,20-dioic acid, PGE-M) as done using an isotopically-labeled internal standard (using m/z 336 and 339 ions). Urinary 2,3-dinor-6-keto-PGF$_{1α}$, a metabolite product of PGI$_2$, also was determined in the urine samples following procedures documented previously (29). Urinary creatinine was measured using an Autoanalyzer (Technicon, Buffalo Grove, IL, USA). Data were expressed as nanograms per milligram creatinine.

**Fatty acid analysis by GC-MS**

For quantitation of fatty acids, 10 μL of internal standard (17:0, 1 mg/mL in hexane) was added to 150 μL of colon homogenate or serum. The samples were then extracted with 1.5 mL of Folch reagent (chloroform: methanol 2:1), vortexed for 2 minutes, and centrifuged (200 x g for 5 min). The organic layer was removed to a 10 x 75 mm glass tube and dried in a SpeedVac. The samples were solubilized in 150 μL of hexane:chloroform (1:1), and vortexed. Fatty acid methyl esters (FAMEs) were prepared by adding 10 μL of METH-PREP II derivatization reagent (0.2 N methanolic (m-trifluoromethylphenyl) trimethylammonium hydroxide, Alltech, Deerfield, IL). GC-MS was conducted as previously described using selected ion monitoring (30).

**Data analysis**

Descriptive plots and summary measures (mean and SD) were calculated for fatty acids percentages and eicosanoid concentrations obtained in serum as well as in...
different segments (transverse, proximal, and distal) of the colon. The primary statistical framework adopted was that of a linear mixed effect regression model that properly accounted for the clustering within the sections of the same colonic tissue. In the first set of such models, eicosanoids and EPA:AA ratios were compared across the different sections in analysis of covariance (ANCOVA) models with section as a 3-level factor, and a linear and quadratic dose effect as covariates. The model was further adjusted for variation across analytical batches by using batch as a categorical variable. A random subject effect incorporated clustering within the different sections of the colon. Mathematically, the model for a given measure assumed the analytical form

\[ y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + \delta_1 d_{ij} + \delta_2 d_{ij}^2 + \epsilon_{ijk} \]  

(1)

where \( y_{ijk} \) was the natural logarithm of the outcome measure (eicosanoid) from the \( k \)-th section of the colon of \( i \)-th animal in the \( j \)-th batch, \( i=1,\ldots,10; j=1,\ldots,6; k = 1, 2, 3 \). In the model, \( \mu \) denote the overall mean, \( \alpha_i \) denotes the \( i \)-th subject effect, \( \beta_j \) denotes the \( j \)-th batch effect and \( \gamma_k \) denotes the \( k \)-th section effect. Further \( d_{ij} \) indicates the dose level for the \( i \)-th subject in the \( j \)-th batch and \( \delta_1, \delta_2 \) reflect the linear and quadratic dose effects. The random error \( \epsilon_{ijk} \) and the subject effect \( \alpha_i \) were both assumed to have normal distributions with mean zero and constant variance, independently of each other. Post-hoc pairwise comparison between sections was carried out with Bonferroni adjustment for multiple comparisons. In a separate set of analogous analysis, the association between eicosanoids and, the PGE\(_2\), PGE\(_3\) ratio, was investigated after controlling for the batch and section effects. A random subject effect was used in the models as before. Logarithmic transformation was used for all the eicosanoid measures based on the behavior of the residuals and other model selection criteria such as AIC and BIC.
In order to graphically assess the goodness of fit, plots of fitted mean curve of PGE$_2$ and PGE$_3$ as a function of dietary EPA:ω6 ratio were superimposed on the observed values at the proximal and distal sections of colon. A similar plot depicted the relationship between PGE$_2$ and PGE$_3$ and EPA:AA in serum. Each plot was further accompanied by 95% point wise confidence intervals of the regression function. We illustrate the construction of the confidence interval by means of the dose-response curve that was explained by a regression function of the form

\[ y = a + b \times \text{dose} + c \times \text{dose}^2 \]  

(2)

where \( y \) was the response (log(PGE$_2$) or log(PGE$_3$)) at a given colonic section, and \( a, b, c \), were fitted regression coefficients. At a given dose \( d \), the lower and upper confidence limits of the regression function were calculated as

\[ y(d) \pm 1.96 \times \text{s.e.}\{y(d)\} \]  

(3)

with the standard error \( \text{s.e.}\{y(d)\} \) given by

\[ \text{s.e.}\{y(d)\} = \sqrt{\text{var}(a) + d^2 \text{var}(b) + d^4 \text{var}(c) + 2d \text{cov}(a,b) + 2d^2 \text{cov}(a,c) + 2d^3 \text{cov}(b,c)} \]  

(4)

With var and cov denoting variance and covariance terms, respectively. The confidence limits for PGE$_2$ or PGE$_3$ were obtained by exponentiating the expressions in Equation (1).

Results

Diets and body weight

The modified AIN93-G diet contained EPA to ω6 fatty acids at ratios of 0, 0.1, 0.2, 0.4 and 0.6, while keeping total fat content constant at 34% of calories using a Western blend fat (Table S1). The fatty acid composition of the diets given in Table S1 was independently verified by GC-MS (data not shown). Rats consumed these experimental diets for five weeks. The body weight of the rats was measured weekly. No differences were found in body weight among the means of the five rat groups (Figure S1).

Colonic and serum fatty acid concentrations

Total fatty acids were assayed in the colonic sections and in the serum from the rats without separation of lipid sub-fractions (Table S2). Since blood was obtained in the non-fasting state and fatty acids were extracted directly from serum, one limitation of the study is that much of the serum fatty acid will have been from chylomicrons. We found that percentages of AA in total fatty acids varied in different anatomic colonic segments; but, the percentages of EPA did not differ among colonic segments (Figure 1A and 1B). Distal colon had a higher percentage of AA than the proximal or transverse colon. The percentage of AA in the serum reflected that of the colon. The percentage of EPA in the serum was higher than in the three colonic tissue sections in rats fed with EPA-containing diets.

The percentage of EPA in the total fatty acid pool increased in all three colonic segments as well as in the serum when the dietary EPA: ω6 ratio dose was increased (Figure 1B). We also observed a dose dependent increase in docosahexaenoic acid (DHA, 22:6) and a decrease in lauric acid (12:0) (Table S2). EPA:AA ratios for the
tissues and sera were calculated, and there was a consistent increase in the EPA:AA ratio upon increasing dietary EPA with changes in serum being the largest and changes in distal colon being the smallest (Table 1).

Similar to AA, increasing the dose of dietary EPA caused no change in total \( \omega_6 \) polyunsaturated fatty acids (PUFAs) in any of the colonic sections or in the serum (Figure 1C). Dietary EPA caused a dose dependent increase of \( \omega_3 \) PUFAs in both colon and sera (Figure 1D). Although serum \( \omega_6 \) PUFAs were similar to colonic tissue \( \omega_6 \) PUFAs levels, \( \omega_3 \) PUFAs were consistently higher in serum than in the three colonic tissue segments in the rats fed EPA-enriched diets (Figure 1C and 1D). The average EPA:AA ratios were quite similar in proximal and transverse sections, while the EPA:AA ratio in the distal section was significantly lower than either of the other two sections (Figure 1E).

**Colonic prostaglandin concentrations**

The proximal colonic PGE\(_2\) concentrations were three times higher than the distal PGE\(_2\) concentrations, regardless of the dietary EPA:\( \omega_6 \) fed (Table S3); the same relative relationship was detected for PGE\(_3\). Statistical analyses showed that adjusting for the dose and batch effects, both PGE\(_2\) and PGE\(_3\) exhibited the following significant pattern decreasing the average concentration: Proximal > Transverse > Distal (Table S3). Interestingly, even though the highest EPA dose was well above most human dietary intakes, the PGE\(_3\) concentrations in the rat colon were less than 10% of the PGE\(_2\) concentrations. The major PGE (and eicosanoid) product in colonic tissue was PGE\(_2\) despite large increases in dietary \( \omega_3 \) fatty acids.
Increased dietary EPA caused a non-linear, dose dependent reduction in colonic tissue PGE$_2$ concentrations. The reduction in PGE$_2$ concentrations was maximal with an EPA:ω6 diet ratio of 0.4 but small incremental reductions occurred with the 0.6 diet in proximal, transverse and distal colon (Figure 2A). PGE$_2$ concentrations decreased 66% in the proximal colon, 58% in the transverse and 61% in the distal colon with the 0.4 EPA:ω6 diet relative to the diet with no EPA (Table S3). Colonic PGE$_3$ concentrations increased with increasing dietary EPA:ω6 fatty acid ratios (p<0.0001) in all three colonic sections (Figure 2B and Table S3). The highest dose EPA diet increased the concentrations of PGE$_3$ by 19-fold in the proximal colon, 28-fold in the transverse colon and 19-fold in the distal colon as compared to rats fed no EPA.

**Colonic concentrations of HETEs and HODEs**

We also measured the concentrations of LOX products in colonic tissues from rats fed the different diets. The proximal colon consistently had the highest concentrations of 5-S-HETE and 15-S-HETE (Figure 2C, 2D, Table S3). The concentrations of 12-S-HETE and 13-S-HODE did not differ among the three colonic segments from rats in any dietary group (Figure 2E, 2F, Table S3). For the 5-S-HETE, 12-S-HETE, 15-S-HETE, the pattern of significant differences among the colonic sections was not consistent (Table S3). Among the four HETEs and HODE, increased dietary EPA:ω6 fatty acid ratios caused the largest reduction in 12-S-HETE, by more than 80% in all colonic segments (Figure 2E and Table S3). Similarly, consistent decreases by dietary EPA were found in all three colonic segments for 15-S-HETE (Figure 2D and Table S3) and a trend of decrease in 13-S-HODE (Figure 2F and Table S3). The largest reduction for both 12-S-HETE and 15-S-HETE occurred with
dietary EPA:ω6 fatty acid ratios between 0 and 0.1. Further increases in dietary EPA:ω6 fatty acid ratios did not cause changes in 5-S-HETE in the three colonic segments. In rats fed the control diet, the relative amount of $R$ to $S$ isomers of 5-HETE was 1.2 in the proximal colon, 1.9 in the transverse colon and 1.7 in the distal colon; for 12-HETE it was 0.1 in the proximal colon, 0.06 in the transverse colon and 0.06 in the distal colon; for 15-HETE it was 0.4 and for 13-HODE it was 0.2 in all three anatomic colon segments. Increased dietary EPA:ω6 fatty acid ratios did not cause changes in concentrations of the $R$-enantiomers for any of these eicosanoids in the colonic samples (Table S3).

**Urinary metabolites**

Increasing the dietary EPA:ω6 fatty acid ratio from 0 to 0.4 caused a dose-dependent decline in urinary PGE-M with little further change at the highest EPA:ω6 dose (Figure 3 and Table 1). A maximal 43% reduction was seen in urinary PGE-M in rats fed the 0.6 EPA:ω6 diet (Figure 3A). The concentration of the urinary metabolite of prostacyclin (PGI$_2$-M), 2,3-dinor-6-keto-PGF$_{1α}$, was not altered by increased dietary EPA (Figure 3B).

**Mathematical relationship between fatty acids and eicosanoids**

Both the linear and the quadratic terms corresponding to EPA dose in the diet were significant for EPA:AA, PGE$_2$, and PGE$_3$ as well as their ratio (Table 1). Due to the existence of the quadratic term, the rate of change in the eicosanoids was dose dependent. This is consistent with the plateauing pattern demonstrated in Figure 2A. While the coefficient in Table 1 corresponding to dose indicated the initial change in the
eicosanoid measurement per unit dose, the dose squared coefficient contributed to the plateauing effect.

Dose was significantly associated with urinary PGE-M but not 2,3-dinor-6keto-PGF1α. For the HETE’s and HODE’s the patterns of associations were mixed. The linear dose effect was significantly associated with both 12-S-HETE and 15-S-HETE, while the association was not significant for 5-S-HETE or 13-S-HODE. On the other hand, 12-S-HETE was the only monohydroxy acid that that exhibited any significant association with the quadratic effect of dose.

Key fatty acid percentages (EPA, ω3HUFA, ω3PUFA, ω6HUFA and ω3PUFA:ω6PUFA) demonstrated significant associations with PGE2 and PGE3 (Table 2) both in the colonic tissue and the serum. The coefficients corresponding from the linear regression on the log-log scale can be translated into an interpretation of percent change. For the PGE2 and serum EPA:AA relationship (adjusting for batch and location effect), the relationship reduced to the equation

$$\frac{(PGE_2)_I}{(PGE_2)_I} = \left[\frac{(EPA:AA)_I}{(EPA:AA)_I}\right]^{-0.2}$$

(5)

where the ratios of both sides of Equation (5) represent fold changes between dose-levels I and II. Thus to achieve a 10% increase in EPA:AA, we set the right hand side of Equation (5) to 1.1, which yields $(PGE_2)_I = 0.98(PGE_2)_I$, or equivalently a 2% decrease in the (geometric) mean of PGE2, the association being significant with $p<0.0001$. Similarly, a 10% increase in EPA:AA corresponds to a $(1.1)^{0.74} - 1 = 0.07$ or a 7% increase in the (geometric) average of PGE3 ($p<0.0001$). Similar interpretations apply to other coefficients.
The dose response curves in Figure 4A-D tracked the observed data quite well both for PGE$_2$ and PGE$_3$. A dose close to 0.4 seemed optimal in any colon section. The curves for PGE$_2$ showed a tighter cluster than that for PGE$_3$. The fit through the scatter of serum EPA:AA and PGE$_2$ showed close conformity to the data (Figure 4E-H). By contrast there seemed to be high variability in PGE$_3$ values over a short range of EPA:AA and the fitted curve was driven by a few outlying observations.

**Discussion**

Omega-3 fatty acids in the form of dietary fish oil appear to have cancer preventive effects; but all studies do not agree and taking high doses can be problematic in terms of both compliance and toxicity (31, 32). Beneficial effects of fish oil may be limited by dietary intakes of other fats (33, 34). Individualized dosing is an attractive approach for optimizing the beneficial, preventive effects of ω3 fatty acid formulations. Development of mechanism-based, rapidly assayed, accurate, analytically cost-effective biomarkers will facilitate such individualized dosing to allow anti-carcinogenic efficacy while minimizing toxicity.

We translated our *in vitro* data demonstrating that the ω3 fatty acids inhibit the catalytic activity of COX-1 (11, 16, 35). EPA and DHA can both serve as substrates for ovine and human COX-1 (16, 35); but, they are much poorer substrates than AA. Both ω3 FA can inhibit the oxygenation of AA by COX1 and 2 (16). In a non-neoplastic epithelium, COX-1 is the primary cyclooxygenase form present and is the likely target for reducing PGE$_2$. Both DHA and EPA were present in the diet and were incorporated into colon tissue with similar dose-dependent kinetics. EPA is oxygenated by COX-1
but at 10% of the efficiency of AA (35). EPA competes directly with AA for the catalytic site of COX-1 (36). DHA is a poor substrate and modest inhibitor of AA oxygenation by COX-1 (35, 37). We therefore chose to model PGE$_2$ reduction in colon using the EPA:AA FA ratio.

While less relevant to the current model, EPA is metabolized by COX-2 to form PGE$_3$, which has anti-proliferative effects in cancer (38). The small amounts of PGE$_3$ found in the colon are likely due to EPA metabolism via COX-2.

We were able to establish in F344 rats a quantitative, statistically valid relationship *in vivo* between serum fatty acids using the EPA:AA ratio, urinary PG metabolites, and colonic tissue concentrations of PGE$_2$ (Table 2). Dietary intake of EPA reduced colonic tissue PGE$_2$ concentrations in a log-linear relationship while increasing PGE$_3$ concentrations. Previously published data suggest that an increased dietary ω3:ω6 ratio is more important than the total amount of ω3 fatty acid intake in altering eicosanoid production in rodents (39); and in reducing human colonic epithelial proliferation (33). The doses of EPA or fish oil used in human studies for cancer prevention ranged from over 7 g/day to as low as 2 g/day, but none of these trials employed a dose-response with biomarker approach to individualize human dosing (33, 40-44). The data presented here provide a working model that predicts the relationship between dietary ω3 fatty acids, serum and tissue fatty acid concentrations, and resulting eicosanoids in an epithelial target—the distal colon. Such relationships, based upon known enzymatic substrate-product relationships were translated to *in vivo* rodent models systems in preparation for future human applications (11, 16, 17). Our data support a mathematical model that may be useful for designing an individualized...
human dosing strategy. Currently, our model only incorporates EPA supplementation in healthy individuals without known colonic inflammatory stress because of our data suggesting the EPA inhibits COX-1 metabolism of AA more efficiently than DHA. We recognize that DHA has other anticarcinogenesis effects and may have a role in cancer risk reductive interventions with ω3 fatty acids (15, 45). In future studies, we would need to model DHA modulation of CYP metabolism to epoxides and COX-2 interactions (19, 20).

Human metabolism may differ from rodents. Published data to date suggest that fatty acids in both rodents and humans after feeding ω3 fatty acids correlate with intake but few direct comparisons with comparable doses are known. For example, Stark assayed bioavailability of ω3 fatty acids in rats, pigs, and humans, but none were dosed equivalently (46). Previous data suggest a pharmacodynamic relationship between fatty acid concentrations in blood or tissue compartments and eicosanoids in rodents (13) and in humans (41); but a direct comparison with pharmacodynamics models using rat data and applying it to humans has not been published. The data presented here provide a rodent-based pharmacodynamic model that we are currently testing in a human clinical trial of EPA supplementation in humans using a colon eicosanoid endpoint. Such a pharmacodynamic trial will permit rodent to human comparisons in the future.

A diet high in EPA from fish oil reduced colonic concentrations of PGE$_2$ by at least 50% in all three regions of colon, but did not alter percentages of AA or ω6 PUFA in the colon or in serum. This may be important for the required physiologic functions of AA. The increased concentrations of ω3 PUFA can exert their anti-carcinogenic effects
on both initiation and promotion independent of ω6 PUFAs, as opposed to substituting for them (47). The present data demonstrate that incremental increases in the dietary intake of EPA reduced colonic PGE$_2$ by 60%, an effect that is likely to be reproduced in other epithelial tissues.

In addition to the potential utility of serum fatty acids as biomarkers of colon PGE$_2$, urinary PGE-M has been reported to be associated with CRC risk in a large prospective cohort study (48). In our study, dietary EPA induced a dose-dependent reduction of PGE-M; however, neither dietary EPA nor PGE-M were as highly correlated with tissue eicosanoid concentrations as the EPA:AA ratio in tissue and serum. Changes in urinary PGE-M represent not only colonic COXs but also systemic metabolism. Kakutani et al. (49) demonstrated that an AA-enriched diet did not increase urinary PGE-M concentrations and did not modulate PGE$_2$ in blood in humans. However, our data suggest that urinary PGE-M may be a useful biomarker for epithelial pro-inflammatory state. We observed that an ω3 fatty acid diet did not modulate urinary 2,3-dinor-6-keto PGF$_{1α}$ similar to what Kakutani et al. reported (49).

Previously, our group developed a chiral LC/MS-MS method to quantify HETEs and HODEs in the colonic tissue of rodents (27). The technology permits more efficient interrogation of eicosanoid products beyond COX products. Among all the AA derived eicosanoids measured, colonic PGE$_2$ was the most abundant form followed by 12-S-HETE, and 5-S-HETE was the lowest. ω3 fatty acid competition for eicosanoid metabolizing proteins extends beyond COX. 5- and 12-LOX products have mitogenic effects, inducing DNA synthesis while inhibiting cell cycle arrest and caspase release, and promoting angiogenesis (7). The role of 15-LOX-1 and -2 metabolites appears less
clear with the predominance of data supporting anti-inflammatory and anti-
carcinogenesis mechanisms via PPAR γ induced apoptosis and DNA-dependent protein
kinase activity activation of p53 (7). Our data suggest that ω3 fatty acid
supplementation will have useful anticarcinogenesis activity as much via LOX pathway
modulation as with cyclooxygenase pathway modulation.

Acknowledgements

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Gretchen Bentz, for help with the animal study.
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**Table 1** Effect of dietary EPA dose and EPA dose squared on fatty acid and eicosanoid outcome variables. Data shown as regression coefficients, standard error (SE) and statistical significance (P-value).

<table>
<thead>
<tr>
<th>Variables</th>
<th>Dose of EPA</th>
<th>EPA Dose×EPA Dose</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Coefficient</td>
<td>SE</td>
<td>P-value</td>
<td>Coefficient</td>
<td>SE</td>
<td>P-value</td>
</tr>
<tr>
<td>EPA:AA</td>
<td>1.370</td>
<td>0.169</td>
<td>&lt;0.0001</td>
<td>-0.140</td>
<td>0.027</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PGE₂</td>
<td>-0.431</td>
<td>0.076</td>
<td>&lt;0.0001</td>
<td>0.048</td>
<td>0.012</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PGE₃</td>
<td>1.431</td>
<td>0.148</td>
<td>&lt;0.0001</td>
<td>-0.155</td>
<td>0.023</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PGE₂:PG₂</td>
<td>2.054</td>
<td>0.193</td>
<td>&lt;0.0001</td>
<td>-0.228</td>
<td>0.031</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>5-S-HETE</td>
<td>-0.276</td>
<td>0.157</td>
<td>0.080</td>
<td>0.041</td>
<td>0.025</td>
<td>0.099</td>
</tr>
<tr>
<td>12-S-HETE</td>
<td>-0.694</td>
<td>0.122</td>
<td>&lt;.0001</td>
<td>0.072</td>
<td>0.019</td>
<td>0.000</td>
</tr>
<tr>
<td>15-S-HETE</td>
<td>-0.351</td>
<td>0.126</td>
<td>0.006</td>
<td>0.035</td>
<td>0.020</td>
<td>0.085</td>
</tr>
<tr>
<td>13-S-HODE</td>
<td>0.126</td>
<td>0.158</td>
<td>0.427</td>
<td>-0.020</td>
<td>0.025</td>
<td>0.421</td>
</tr>
<tr>
<td>Serum EPA:AA</td>
<td>1.835</td>
<td>0.089</td>
<td>&lt;0.001</td>
<td>-0.208</td>
<td>0.014</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Log tetranor PGE-M*</td>
<td>-0.141</td>
<td>0.051</td>
<td>0.006</td>
<td>0.008</td>
<td>0.008</td>
<td>0.302</td>
</tr>
<tr>
<td>Log 2,3-dinor-6keto-PGF₁₆*</td>
<td>0.028</td>
<td>0.045</td>
<td>0.538</td>
<td>0.000</td>
<td>0.007</td>
<td>0.968</td>
</tr>
</tbody>
</table>

1 All outcome variables were transformed using natural logarithm. Unless indicated otherwise, the variables measured were in colonic tissue.

2 Coefficients, SE and p-values are from linear mixed effect regression models, adjusted for analytical batch and colon section. Serum and urine variables were not adjusted for colonic sections. The dose squared models were used to account for the curvature in the dose-response data, as used in equation 2 (see methods).
Table 2 Effect of colon and serum fatty acid variables colon prostaglandins. Data shown as regression coefficients, standard error (SE) and statistical significance (P-value)

<table>
<thead>
<tr>
<th>Samples</th>
<th>Variables</th>
<th>Log PGE&lt;sub&gt;2&lt;/sub&gt;:PGE&lt;sub&gt;2&lt;/sub&gt;</th>
<th>Log PGE&lt;sub&gt;3&lt;/sub&gt;</th>
<th>Log PGE&lt;sub&gt;3&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Coefficient</td>
<td>SE</td>
<td>p-value</td>
</tr>
<tr>
<td>Colonic tissue</td>
<td>Log AA</td>
<td>0.055</td>
<td>0.139</td>
<td>0.692</td>
</tr>
<tr>
<td></td>
<td>Log ω6HUFA</td>
<td>-2.456</td>
<td>0.525</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td></td>
<td>Log ω6PUFA</td>
<td>0.073</td>
<td>0.282</td>
<td>0.797</td>
</tr>
<tr>
<td></td>
<td>Log EPA</td>
<td>0.670</td>
<td>0.099</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td></td>
<td>Log ω3HUFA</td>
<td>2.327</td>
<td>0.174</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td></td>
<td>Log ω3PUFA</td>
<td>0.738</td>
<td>0.162</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td></td>
<td>Log (EPA:AA)</td>
<td>0.911</td>
<td>0.098</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td></td>
<td>Log (ω3:ω6PUFA)</td>
<td>1.509</td>
<td>0.172</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Serum</td>
<td>Log AA</td>
<td>0.382</td>
<td>0.908</td>
<td>0.674</td>
</tr>
<tr>
<td></td>
<td>Log ω6HUFA</td>
<td>-4.064</td>
<td>0.352</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td></td>
<td>Log ω6PUFA</td>
<td>-0.773</td>
<td>1.494</td>
<td>0.606</td>
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<tr>
<td></td>
<td>Log EPA</td>
<td>0.961</td>
<td>0.079</td>
<td>&lt;.0001</td>
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<tr>
<td></td>
<td>Log ω3HUFA</td>
<td>2.711</td>
<td>0.189</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td></td>
<td>Log ω3PUFA</td>
<td>1.773</td>
<td>0.124</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td></td>
<td>Log (EPA:AA)</td>
<td>1.021</td>
<td>0.076</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td></td>
<td>Log (ω3:ω6PUFA)</td>
<td>1.786</td>
<td>0.118</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

1 Coefficients (with SE and p-value) are from linear mixed effect regression models adjusted for analytical batch and, if appropriate, colon section.

2 Coefficients represent rate of change per 0.1 unit of dose. All outcome variables were transformed using natural logarithm.
**Figure Legends**

**Figure 1** Fatty acid levels in colonic tissue and sera. Levels of arachidonic acid (AA), eicosapentaenoic acid (EPA), ω6 polyunsaturated fatty acids ((PUFA); sum of linoleic acid, dihomo-gamma-linolenic acid and AA), ω3 PUFA (sum of alpha-linolenic acid, docosahexaenoic acid and EPA) and EPA:AA ratio in proximal, transverse and distal rat colon and serum samples from the rats fed with diets having different EPA:ω6 ratios. Error bars represent SEM. Note the different scales for AA and EPA. The asterisks (*) denote values significantly different from the control diet (p<0.01). Detailed results of the statistical analyses are presented in the supplementary data (Table S2).

**Figure 2** Eicosanoid production in different segments of the colon. Concentrations of PGE₂, PGE₃, S-HETEs and S-HODE in different segments of the colon from the rats fed with different dietary EPA:ω6 fatty acid ratios. Error bars represent SEM. Note the different scales for PGE₂ and PGE₃. The absolute amount of R isomers (data not shown) did not change appreciably with fish oil feeding. 2-way ANOVA was conducted for each eicosanoid. Diet was a significant factor for all the eicosanoids except 5-S-HETE, 5-R-HETE and 15-R-HETE. Colon section was a significant factor for all except 5-R-HETE, 12-S-HETE, 12-R-HETE, and PGE₃/PGE₂ ratio. The asterisks (*) denote values significantly different from the control diet (p<0.01). Detailed results of the statistical analyses are presented in the supplementary data (Table S3).

**Figure 3** Urinary tetranor PGE-M and 2,3-dinor-6-keto-PGF₁α. Quantification of tetranor PGE-M and 2,3-dinor-6-keto-PGF₁α in 24 h urine samples from the rats fed diets with different EPA:ω6 fatty acid ratios. The asterisks (*) denote values significantly different from the control diet (p<0.01).

**Figure 4** Dose-response relationship of PGE₂ and PGE₃ with diet and serum EPA:AA ratios. A-D: The plots show a fitted mean curve of PGE₂ and PGE₃ as a function of dietary EPA:ω6 ratio superimposed for the proximal and distal sections of colon. E-H: Relationship between colonic PGE₂ or PGE₃ and serum EPA:AA ratios.
Figure 1 Fatty acid levels in colonic tissue and sera

A

AA

B

EPA

C

ω-6 PUFA

D

ω-3 PUFA

E

EPA:AA ratio

F

ω-3PUFω-6 PUFA ratio
Figure 2 Eicosanoid production in different segments of the colon.

A

PGE$_2$

- - Proximal
- - Transverse
- - Distal

B

PGE$_3$

- - Proximal
- - Transverse
- - Distal

C

5-S-HETE

- - Proximal
- - Transverse
- - Distal

D

15-S-HETE

- - Proximal
- - Transverse
- - Distal

E

12-S-HETE

- - Proximal
- - Transverse
- - Distal

F

13-S-HODE

- - Proximal
- - Transverse
- - Distal
Figure 3 Urinary tetranor PGE-M and 2,3-dinor-6-keto-PGF1α

A

Tetranor PGE-M

B

2,3 dinor-6-keto-PGF1α
Figure 4 Dose-response relationship of PGE$_2$ and PGE$_3$ with diet and serum EPA:AA ratios

A. Distal

B. Proximal

C

D

E

F

G

H
Biomarkers for Personalizing Omega-3 Fatty Acid Dosing

Yan Jiang, Zora Djuric, Ananda Sen, et al.


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