Fatty Acid Desaturase Activity, Fish Oil, and Colorectal Cancer Prevention

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1.0 Background

Colorectal cancer (CRC) is the fourth most common cancer and the second leading cause of cancer-related death in the United States. (1) Despite advances in our understanding of the molecular biology of CRC, little progress has been made in improving survival rates for individuals diagnosed in advanced stages.(2) Thus, early identification and prevention are important strategies to reduce CRC mortality.(3)

Chronic Inflammation and Colorectal Cancer

While inflammation is a necessary physiological response to infection and tissue injury, mounting evidence has implicated chronic inflammation as an initiator and promotor of carcinogenesis.(4) Multiple inflammatory conditions are associated with increased cancer risk, and chronic infections can predispose individuals to cancer. Indeed, a recent cohort study including over 10,000 individuals found C-reactive protein (CRP), an acute-phase reactant and biomarker of inflammation, to be associated with an increase hazard for cancer of any type (1.3; 95% CI 1.0-1.6) with a particularly strong association for CRC (HR = 1.9; 0.8-4.6).(5)

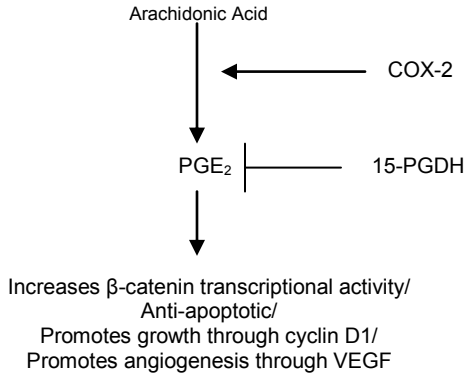


Figure 1: Eicosanoid production and CRC risk

Chronic inflammation can contribute to cancer via several mechanisms including inappropriate gene expression; epigenetic alterations; enhanced cell proliferation; and resistance to apoptosis.(6) One link between cancer and inflammation involves **cyclooxygenase-2**, (**COX-2**) the inducible form of cyclooxygenase, which is overexpressed in multiple malignancies.(7) In the COX pathway, **arachidonic acid** (**ARA**) is released from cellular membranes and then enzymatically converted into various bioactive lipid molecules including prostaglandins. These molecules then exert their cellular functions through binding to cell surface receptors of the seven transmembrane G-coupled rhodopsin-types. Production of **prostaglandin E_2** (**PGE_2**) in particular, may promote carcinogenesis through stimulating cellular proliferation, inhibiting apoptosis, promoting angiogenesis, and/or enhancing invasiveness.(8) (Figure 1) Animal models of CRC have demonstrated the connection between tumor formation and ARA with a positive correlation between increasing tissue levels of ARA and increased PGE_2 production with increased PGE_2 production being associated with tumor number.(9-12) Up-regulation of COX-2 occurs in 50% of colon adenomas and 85% of colon cancers and is considered a key and early oncogenic event in colorectal carcinogenesis.(13) However, while COX-2 is upregulated, **15-hydroxyprostaglandin dehydrogenase** (**15-PGDH**), which is the normal physiological antagonist of COX-2 and inactivates PGE_2,(14) is often down regulated and is considered a tumor-suppressor gene.(15-16)

Chronic inflammation and Obesity/Adipokines/Insulin Resistance

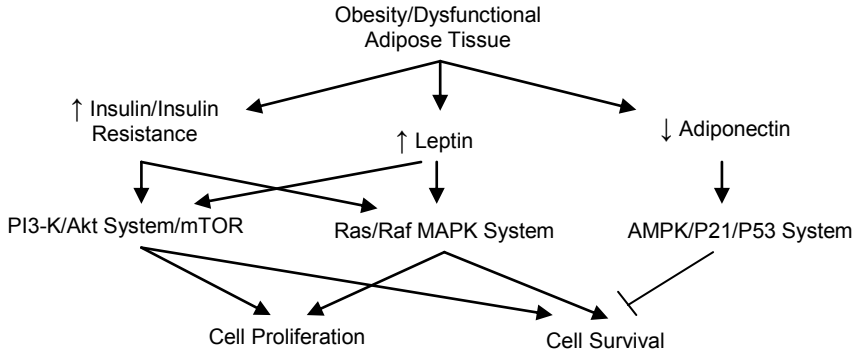


Figure 2: Dysfunctional adipose tissue and CRC risk

Obesity and overweight are strong risk factors for CRC.(17) Potential mechanisms behind this association are beginning to be elucidated, beginning with the identification of adipose tissue as an active endocrine organ that releases a wide variety of biologically active molecules.(18-19) Many of these adipokines, such as **leptin** and **adiponectin,** are related to glucose homeostasis, insulin sensitivity, and energy balance.(20-21) Obesity is associated with increased levels of leptin and decreased levels of adiponectin and reduced insulin sensitivity.(22-24) Insulin activates the PI3K/Akt signal transduction pathway which increases cell proliferation and cell survival. (27) Adiponectin has insulin-

sensitizing effects and may have anti-carcinogenic effects through the activation of the AMP-activated protein kinase (AMPK) system which upregulates the tumor suppressor genes p53 and p21.(25-27) (Figure 2) Conversely, leptin is mitogenic and inhibits apoptosis, likely through the Ras/Raf/ mitogen-activated-protein-kinase (MAPK) system.(27-29) The association between insulin levels and CRC has been well described(30-36) and epidemiological evidence is beginning to emerge suggesting that increased leptin levels(37) and decreased adiponectin levels may be risk factors for colorectal neoplasms although this association has not been consistent across studies.(37-40)

Chemoprevention of Colorectal Cancer with Fish Oil

Eicosapentanoic acid (EPA) is converted to eicosanoids through the same enzymatic pathways as ARA but produces series-3 prostanoids that have less inflammatory actions due to lower receptor affinities when compared to ARA derived series-2 prostanoids.(41-42) In Sprague-Dawley rats, PGE_3 is increased with fish oil supplementation and has pro-apoptotic effect.(43) Similar pro-apoptotic effects of EPA derived PGE_3 have also been reported in human lung cancer A549 cells.(44) Several studies have demonstrated that increased consumption of marine fatty fish results in an increased phospholipid membrane proportion of EPA and a concomitant decreased in ARA and ARA-derived eicosanoids.(45-47) Other potential mechanisms beyond fish oils effect on inflammatory eicosanoids that might contribute to its chemopreventive actions includes downregulation of protein kinase CbII and decreased intracellular Ras levels.(48-51)

Animal models of CRC can involve chemically induced tumors, cancer cell lines transplants, and transgenic animals. With regards to chemically induced tumors a meta-analysis of 14 studies of F344 or Sprague-Dawley rats found a consistently protective effect of fish oil modeled as either percent of total fat or estimated fat calories.(52) In transplant models, fish oil inhibits the growth of transplanted CT-26 colon tumor lines.(53) Several small, randomized controlled trials have been performed to investigate the effect of n-3 PUFAs on colorectal neoplasms with most studies finding fish oil reduces cell proliferation and promote apoptosis. (54-60) While most of these studies have used surrogate endpoints, such as rectal epithelial cell proliferation or apoptosis, a recent 6-month RCT study found that EPA supplementation (2 g per day) reduced rectal polyp number and size in individuals with familial adenomatous polyposis.(61) Taken together these studies strongly support the CRC chemopreventive potential of fish oil.

Eicosanoid Synthesis and the Importance of the n-6 to n-3 PUFA ratio

We hypothesize that diets with a high ratio of ARA/ [EPA + DHA] result in a pro-inflammatory milieu and that these pro-inflammatory effects might contribute to colon carcinogenesis. This hypothesis is supported by animal models of CRC, which have investigated dietary ratios of polyunsaturated fatty acids (PUFAs) and tumor burden. In the $Apc^{Min/+}$ mouse model of colon carcinogenesis, mice fed EPA and ARA simultaneously had similar tissue levels of ARA and intestinal tumor growth compared to mice feed ARA without EPA.(11) Boudreau et al. investigated the effect of fish oil consumption on the production of ARA derived eicosanoids on Sprague-Dawley rates fed at a constant ratio of n-3 to n-6 PUFAs. (62) As the investigators increased the absolute dose of n-3 PUFAs, they also increased the dose of n-6 PUFAs to maintain a fixed ratio. There was no apparent dose effect of n-3 PUFAs on ARA-derived eicosanoids under the conditions of a fixed ratio of n-3 to n-6. Thus, the investigators concluded that the ratio of n-3 to n-6 PUFA must be taken into account when investigating dietary interventions of n-3 PUFAs.

One of the most compelling animal models supporting the benefits of modulating the ARA to EPA + DHA ratio is the *FAT-1* transgenic mouse. These mice have been engineered to carry the *fat-1* gene from *C. elegans* and are able to convert n-6 PUFAs to n-3 PUFAs in vivo, a function all mammals are unable to perform.(63) Subsequently, *FAT-1* mice preferentially convert dietary n-6 PUFAs to n-3 PUFAs. Gravaghi et al. compare *FAT-1* transgenic mice to wild type mice in the DSS-induced experimental colitis model.(64) Both groups of mice were fed the same n-6 PUFA rich diet. *FAT-1* mice had a significant attenuation of colonic inflammation along with lower colonic cell ratios of n-6 to n-3 PUFA and a significant decrease in PGE_2 production and COX-2 expression.

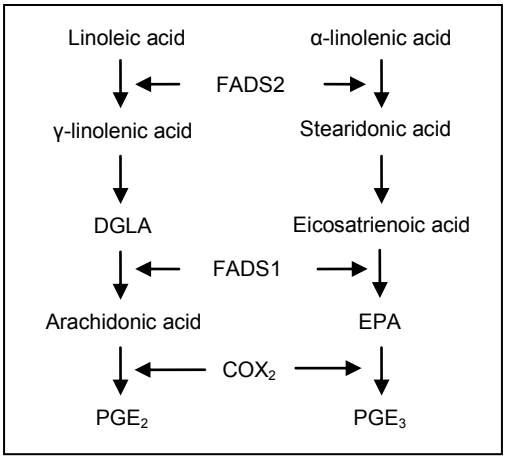
Very limited controlled trials have investigated the impact of varying amounts of PUFAs and CRC risk. Bartram et al. randomized 12 volunteers to either fish oil supplementation (4.4 g n-3 fatty acids/day) versus placebo and found no differences in proliferation indexes after 30 days.(65) This study was designed to determine the effects of fish oil supplementation in subjects eating a controlled high fat diet (50% of energy). The null results of this study contrasted with an earlier study by Bartram where volunteers maintained their usual dietary fat intake (30% of total energy).(59) Comparing the results of these two trials, Bartram et al., concluded that the ratio of n-3 to n-6 PUFAs likely plays an important mediating role in fish oils anti-proliferative effects.(65)

Taken together these animal and human studies strongly emphasize the important of the ratio of n-6 PUFAs to n-3 PUFAs on modulating inflammation and eicosanoid production in the colon. However while dietary manipulation can result in changes in tissue levels of n-6 and n-3 PUFAs, genetic factors may also play a key role in determining this ratio.

Genetic Determinants of Polyunsaturated Fatty Acid Levels

PUFA are chains of 18 to 22 carbon atoms that contain two or more sequential double bonds. In the Western diet, linoleic acid (18:2n-6) accounts for almost 89% of total PUFA energy intake.(66) Linoleic acid (LA) is metabolized to ARA (20:4n-6) through a series of desaturation and elongation reactions. The first step in this metabolic pathway is ***fatty acid desaturase* 2** (**FADS2**) that converts LA to γ-linolenic acid [GLA] (18:3n-6).(67) GLA is elongated to dihomo-γ-linolenic acid [DHLA] (20:3n-6) and then desaturated by ***fatty acid desaturase 1*** (***FADS1***) to ARA. Both the genes for *FADS2* and *FADS1* are localized in chromosome 11 (11q12-q13.1) as a cluster.(68) While EPA can be produced in vivo from α-linolenic acid through this same pathway, in humans, this process is extremely inefficient and most tissue level EPA derives directly from dietary consumption of fatty fish.(69-71)

Evidence that genetic factors could play an important role in tissue levels of PUFA comes from several sources. In a study of 80 families conducted in kibbutz settlements in Israel the heritability estimated for red blood cell (RBC) phospholipid membrane concentrations were 0.53 ± 0.11 for ARA, 0.56 ± 0.11 for EPA, and f 0.71 ± 0.08 for DHA, suggesting a strong genetic component influencing tissue levels of long chain PUFAs(72). Similarly, genomic studies have established that variants in *FADS1* strongly influence RBC membrane and plasma levels of ARA.(73) Three recent genome-wide association studies (GWAS) found a strong association between single nucleotide polymorphisms (SNPs) in *FADS* and plasma and RBC phospholipid PUFA levels (74-76) with 18 to 28% of the additive variance in tissue ARA levels explained by *FADS* genotypes.(77-78) Tanaka et al. in a GWAS study investigating genetic factors associated with plasma and RBC PUFA levels found a strong association between the rs174537 SNP in *FADS1* and ARA levels.(74) In this study, participants in the InCHIANTI study with the GG genotype had higher plasma concentrations of ARA and this level decreased with increasing copy of the T allele (8.76% for GG, 7.39% for GT, 6.35% for TT, *P*-value <0.0001). This finding was replicated in the GOLDN cohort with a similar statistically significant decrease in ARA concentration.(74) This SNP is included within haplotype blocks for two prior studies that found strong associations between *FADS1* gene variation and tissue levels of ARA.(77-78) Thus the results of familial aggregation studies, haplotype studies, and GWAS studies have all supported that genetic variants in *FADS1* are important determinates of tissue levels of ARA and have led some investigators to call for the "*inclusion of FADS genotypes as well as diet and lifestyle factors in future randomized control trials addressing biological effects of PUFAs..".* (79)



2.0 Rationale and Specific Aims

Colorectal cancer (CRC) is the third most common cancer and the second most frequent cause of cancer related mortality in the United States. Animal and human studies have suggested that the marine-derived n-3 polyunsaturated fatty acids (PUFAs), **eicosapentanoic acid** (**EPA**) and **docosahexanoic acid (DHA),** have cancer

inhibitory properties while conversely, n-6 PUFAs such as **arachidonic acid (ARA)** may promote tumorigenesis. The mechanism behind these opposing effects is likely due to differences in the biological activity of their eicosanoids end products and their effects on chronic inflammation. **Prostaglandin E_2 (PGE_2)** is a pro-inflammatory eicosanoid that is aberrantly produced in both colorectal adenomas and cancer and is derived from ARA via the cyclo-oxygenase pathway. EPA is converted through the same pathway into prostaglandin E_3, which has 4 to 7- fold less prostaglandin E receptor affinity, is less inflammatory, and may even be pro-apoptotic compared to PGE_2. As such, it may be the *ratio of ARA to EPA and DHA* rather than the absolute levels of marine-derived n-3 PUFAs that contribute most towards their antiproliferative and pro-apoptotic effects.

The ratio of ARA to EPA + DHA can be manipulated through fish oil supplementation, however; genetic factors may play a key role on determining this ratio. Recent genome-wide association and haplotype studies have demonstrated that up to 28% of the additive variance in tissue levels of ARA is explained by variants in a single gene, ***fatty acid desaturase 1* (*FADS1*)**. *FADS1* is the rate-limiting enzyme in the conversion of linoleic acid (LA), the most commonly consumed PUFA, to ARA, and homozygotes for the T allele (population frequency of 13%, HapMap –CEU) in **rs174537** have lower fatty acid desaturase activity and subsequently lower tissue levels of ARA. While EPA can be produced in vivo from α-linolenic acid, in humans, this process is extremely inefficient and most tissue level EPA derives directly from dietary consumption of fatty fish. Thus, high activity of *FAD1* and subsequently increased tissue levels of ARA may offset some of the potential benefits of dietary supplementation with fish oil. To date, no previously published studies have investigated how genetic variants that influence fatty acid desaturase activity might modify the beneficial effects of fish oil supplementation.

Our hypothesis is that the individuals with genetically determined lower activity of FADS1 will derive greater benefit from fish oil supplementation than individuals with higher FADS1 activity because of lower tissue levels of ARA and subsequently a more favorable ARA to EPA + DHA ratio. To test this hypothesis we will recruit 150 participants with recently identified adenomatous polyps and conduct a 6-month double blind 3 X 2 factorial randomized controlled trial. Our first factor will be the rs174537 genotype (GG, GT, and TT) in the *FADS1* gene and our second factor will be fish oil supplementation (fish oil versus placebo). Our primary study outcome will be the change from baseline in rectal epithelial cell proliferation as measured by Ki-67 labeling and rectal crypt apoptosis as measured by TUNEL. Secondary endpoints will include rectal epithelial cell expression of COX-2 and 15-PGDH, rectal cell production of PGE_2 and PGE_3, rectal cell fatty acid concentrations, as well as, changes from baseline in biomarkers of inflammation (C-reactive protein), adipokines (leptin, adiponectin), and markers of insulin sensitivity (HOMA-IR).

Our Specific Aims for this research proposal are:

1) to determine the efficacy of fish oil supplements on rectal epithelial cell proliferation indexes and markers of rectal crypt apoptosis; and,

2) to determine the effect of genetically determined fatty acid desaturase activity on fish oil supplementation for markers of colorectal cancer risk.

Our long-term objectives are to determine genetic factors that might influence the efficacy of fish oil supplementation in order to conduct a more definitive adenoma recurrence trial using marine-derived n-3 PUFAs. We anticipate that fish oil will have anti-neoplastic effect and individuals with low *FADS1* activity will have a greater response compared to individuals with high *FADS1* activity. Our study will be the first to investigate the nutrigenomics of fish oil supplementation in colorectal cancer chemoprevention and may have implications beyond cancer prevention as fish oil is being actively investigated for its anti-inflammatory effects in cardiovascular and psychiatric diseases as well as diabetes mellitus and the metabolic syndrome.

3.0 Animal Studies and Previous Human Studies

Please see above for relevant description of animal studies and previous human studies.

4.0 Inclusion/Exclusion Criteria

Inclusion Criteria:

1) ≥ 40 and < 80 years of age
2) History of 1 or more adenomatous polyps
3) Consent to be contacted for future studies
4) Participants with known genotype for rs174535 in *FADS1*

Exclusion Criteria:

1) Previously resected colorectal cancer
2) Congestive heart failure
3) Current metabolic or life-threatening disease
4) Currently taking fish oil supplements
5) Allergic to fish products
6) Diagnosis of inflammatory bowel disease
7) Diagnosis of any cancer (except non-melanoma skin cancer)
8) Diagnosis of cirrhosis
9) Diagnosis of kidney disease requiring dialysis
10) Pregnant or breast feeding
11) Previous partial of total colectomy
12) Inability to come to Vanderbilt GRCR for research procedures

5.0 Enrollment/Randomization

Recruitment and Retention

We will identify eligible participants based on the inclusion criteria by reviewing study data and medical record data collected in the TCPS. Participants still eligible after record review will be mailed an introductory letter inviting them to participate. One week after the letter is mailed; a trained interviewer from the SRSR will call the potential participants to provide more detailed information about the study, answer questions about the study, and to see if they may be interested in participating. At that time if the participant appears interested verbal consent will be obtained and the SRSR interviewer will administer the baseline survey and the first 48 hour dietary recall interview. At the end of the first 48-hour dietary recall interview, an appointment will be made with the participant for the baseline in-person visit. After this initial visit,informed consent is obtained. At the baseline visit, we will re-genotype rs174535 to confirm the accuracy of our imputation process. This strategy will allow us to efficiently and accurately identify appropriate candidates for our study.

Eligible subjects will present to the Vanderbilt General Clinical Research Center (GCRC) for the initial visit and baseline study procedures. Participants who are eligible for the study and provide written consent for enrollment will have blood obtained; an adipose tissue biopsy performed, and undergoes the baseline rectal mucosal biopsy procedure. Treatment assignment will be obtained from the Vanderbilt Investigational Pharmacy by a coordinator. The first dose of the study medication will be given to patients at the initial visit and the date and time recorded. This date and time will be considered the time of randomization.

Randomization

Randomization will be performed according to a permuted block randomization scheme stratified on the three genotypes. Randomization will proceed within these three strata with a block size of balancing interval, varying randomly according to the outcome of a computer generated random number. This ensures that the cumulative proportion of assignments to each treatment will be balanced after each block of assignments has been made.

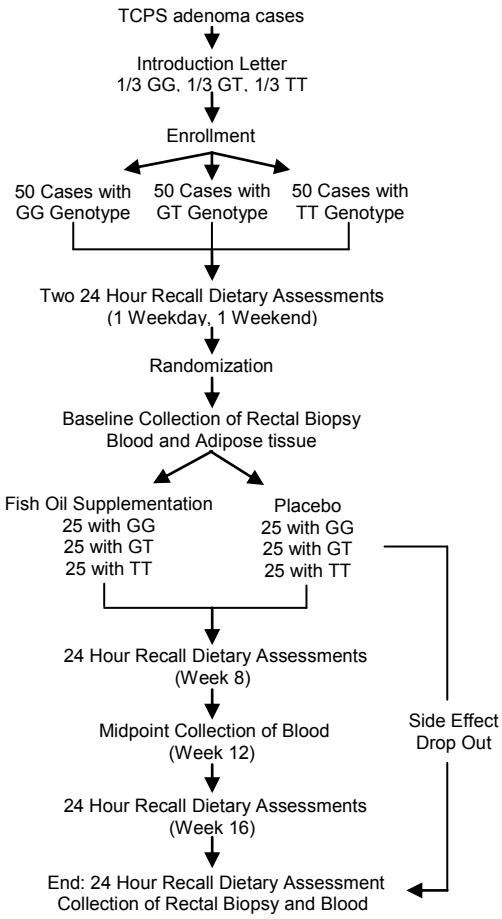
6.0 Study Procedures

Data Collection
Because outside dietary exposure to both n-6 and n-3 PUFAs could possibly confound the effect of fish oil supplementation we will perform a total of three 48-hour dietary recall studies for each participant over the course of the study. At enrollment, we will conduct the initial 48-hour dietary assessments.(93-94) In addition we will conduct one 48-hour dietary recall at week 12 and at week 24. We will use data collected from these 48-hour dietary assessments along with standard food composition tables to calculate dietary exposure to PUFAs. We have chosen to use 48-hour dietary recalls as opposed to food frequency questionnaires as 48-hour dietary recalls are considered the most accurate survey based method of determining food exposure.(95) We will determine adherence to study drug at each in-person visit during the study. Medication and medication changes will be recorded at these visits. Adherence to fish oil will also be determined through RBC phospholipid membrane fatty acid analysis performed at month 3 and month 6. Prior studies have confirmed that this measure is a valid method to determine compliance with fish oil supplementation and have documented increased levels of erythrocyte phospholipid membrane concentrations of EPA in subjects randomized to fish oil supplements. (96-100) In addition, to determine whether fish oil supplementation also influences fatty acid membrane concentration at our target tissue, we will determine the change in rectal epithelial cell phospholipid membrane fatty acid concentration.

Fish oil Capsules
Participants allocated to fish oil supplementation will be instructed to take three Lovaza® capsules each containing 465 mg of EPA and 375 mg DHA daily; this will provide a total daily dose of 1395 mg EPA plus 1125 mg DHA for a total daily dose of fish oil of 2.5 grams. Patients will take one tablet three times a day with meals. The rationale for this dose is that it has successfully lowered indexes of rectal mucosal proliferation in previously trials and is well tolerated without any reported adverse effects.(54, 56) Prior studies have demonstrated that 2.5 mg of fish oil daily is able to decrease the rectal epithelial cell ratio of ARA to EPA + DHA by 49 to 57%.(54, 56) Lovaza® capsules are the only FDA approved preparation of fish oil, and as such, the quality of the drug is monitored and assured. Pharmacological grade fish oil capsules have the advantage of providing high concentrations of PUFAs, low levels of contaminants, such as mercury, and almost no fish odor.

Placebo Capsules
We will use oleic acid as our placebo. The reason for the use of oleic acid is several-fold. First, oleic acid (olive oil) capsules have a similar texture, size, color, and consistency to fish oil capsules. More importantly, oleic acid does not undergo conversion to an eicosanoid or any other metabolically active product. This is opposed to corn oil, which has also been used as a placebo in fish oil studies but is primarily linoleic acid and could subsequently increase tissue levels of ARA and confound the results of our study. Oleic acid has been used as a placebo in several prior studies of fish oil supplementation and is well tolerated.(54-56)

Figure 3: Study Flow Diagram



Assessment Visits
Patients will attend the GCRC clinic at the baseline (**initial visit**), after 3 months of study treatment (**mid-point visit**), and after 6 months of study treatment (**end visit**). The study coordinator will contact participants every 4 weeks over the course of the study to encourage adherence to the study protocol. At the 3-month and 6-

month visits, compliance with treatment will be monitored by capsule count and measurement of RBC phospholipid fatty acid concentrations. Adverse events will be recorded at these visits.

Primary Endpoint and its Determination

The primary outcome of interest is rectal epithelial cell proliferation, as measured by Ki67 (mib-1) labeling and apoptosis as measured by TUNEL (TdT-mediated dUTP Nick-End Labeling). At the initial visit and the end visit, eight mucosal biopsies will be taken 30 cm from the anal margin with 2.4 mm disposable biopsy forceps (Cook Medical, Charlotte, NC). Biopsies will be taken from normal appearing mucosa. Four biopsies will be formalin-fixed and paraffin embedded and processing will begin within 1 hour of collection. Four additional biopsies will be snap frozen in liquid nitrogen and stored at -70˚C until analysis of mucosal fatty acids. Rectal biopsies offer numerous advantages to sigmoid colonic biopsies as no bowel preparation is required and no analgesia or sedation is required.

Serial sections of 5 μm thick will be prepared from the rectal tissue. The sections will be placed sequentially on positive-charged slides and numbered from 1 up to 26. The sections numbered 1, 11, and 21 will be used for H&E staining. For each biomarker, we will stain three sections spaced 50 μm apart to avoid double counting of crypts in biopsy specimens. Thus, for example, sections 2, 12, and 22 may be used for one biomarker but adjoining sections (e.g. 2-4) may not be used for the same biomarker. We will collect multiple biopsies and prepare multiple sections for quality control or to repeat assays in case of failure. Since we produce tissue sections that are 34 microns thick, literally hundreds of sections could be prepared from any tissue block that is 2.4 mm thick. If there is remaining tissue, the block face will be re-sealed with paraffin to prevent degradation of tissue components through oxidation. Sections will also be coated with a thin layer of paraffin. All blocks and slides will be stored in a vacuum chamber at 4°C in a cold room for long-term storage.

Expression of Ki-67 in colon epithelial cells will be detected following the standard IHC protocol of EnVision™+ System, HRP (DAKO). After deparaffinization of the tissue slides, antigen retrieval is performed by heating the slides with a pressure cooker in the optimized buffer solutions: Retrievagen A pH6.5 (BD Pharmingen) for Ki-67 and using the primary antibodies of mouse monoclonal anti-Ki-67(BD Biosciences Pharmingen). The TUNEL assay is conducted to measure apoptosis of colon epithelium using DeadEnd Colorimetric TUNEL System (Promega). After all fields of each sample are measured, the final immunoreaction indices are generated automatically by setting algorithms as ‘‘total positive area / total nuclear area. Apoptotic activity is also scored using standard morphologic criteria applied to H&E stained sections. Briefly, a total of 24 to 30 longitudinal crypts at three section levels are scored per biopsy. The cells are considered apoptotic if the shrinkage of the cell from its neighbors, chromatin condensation and nuclear fragmentation (karyorrhexis) are presented. Cells manifesting these criteria are included in apoptosis scores only when observed in isolated, single cells not associated with an inflammatory response. The number of apoptotic cells for each crypt is combined to calculate a mean apoptosis score per crypt for each specimen

All biomarkers will be scored using imaging analysis. We use a system composed of an Olympus BX40 microscope, a Retiga FAST 1394 color digital camera and BioQuant NOVA Prime imaging software (BioQuant, Nashville, TN). The video images are captured using a 10X objective lens under a constant state of exposure control. Approximately 24-30 well-oriented longitudinal crypts at three section levels per biopsy specimen are quantified. The distribution of positive cells in the upper, middle, and basal zones is evaluated separately by a zonal quantitative analysis procedure established in our lab. When each field is measured, region of interest tools are used to exclude blank, folding, hemorrhage, necrosis, poorly stained, and stromal areas, whereas threshold tools are used to precisely define and measure total epithelial area, total nuclear area, positively stained area, and average gray density. After all fields of each sample are measured, the final immunoreaction indices are generated automatically by setting algorithms as ‘‘total positive area / total nuclear area’’ for nuclear staining markers.

Rationale for Using Ki-67 and TUNEL as a Primary Endpoint

The rationale for using cell proliferation and apoptosis labeling indexes is five-fold. First, there is an association between proliferation and apoptosis indexes and colorectal adenomas and cancer.(101-106) Second, intra-observer correlations of blinded recounts are reported at 0.80 or greater suggesting good reproducibility for Ki-67 labeling.(107) Third, for TUNEL, the balance between crypt proliferation and apoptosis is important in colorectal cancer tumorigenesis and TUNEL methodology to date has been the most employed biomarker of colon crypt apoptosis.(108-109) Fourth, proliferation, and apoptosis indexes have been commonly used in prior studies investigating fish oils versus placebo. Finally, the use of proliferation and apoptosis indexes allows for a smaller sample size and shorter duration of study than using an outcome such as adenoma recurrence. (107) This will allow a more efficient experiment to test our hypotheses that fish oil will reduce markers associated with an increase risk of colorectal cancer before performing the more resource intense trial using adenoma recurrence as an outcome.

Samples and Laboratory Measurements
At the initial visit, mid-point visit, and end visit participants will be asked to provide 10 ml venous blood for measurements of erythrocyte phospholipids fatty acid content, to determine our secondary biomarker endpoints, and to provide plasma and serum that will be stored. Over the entire course of the study this will result in the collection of 30 ml of blood. At the initial visit, subjects will undergo an adipose tissue biopsy of the upper buttock to obtain approximately 20 mg of adipose tissue for fatty acid analysis. Adipose tissue reflects dietary exposure to fatty acids over a period of 2-3 years and is considered the best biomarker of long term dietary fatty acid exposure.(110) Survey studies have found that research volunteers consider this procedure no more or less uncomfortable when compared to venipuncture.(134)

Secondary Endpoints- Immunohistochemistry:
The rationale for including the secondary outcome measurements of COX-2 and 15-PGDH is several fold. Our secondary markers includes expression levels of two growth factors that are often aberrantly expressed in the early stages of CRC and are important factors in the production of eicosanoids.(111) COX-2 converts ARA to PGE_2 and 15-PGDH is the key enzyme responsible for the biological inactivation of these eicosanoids.(112) Thus, aberrantly production of PGE_2 could be related to over-expression of COX-2 or inactivation of 15-PGDH and indeed both of these findings have been described as important contributors toward colorectal carcinogenesis.(111) Expression of COX-2 and 15-PGDH in rectal epithelial cells will be detected following the standard IHC protocol of EnVision™+ System, HRP (DAKO). After deparaffinization of the tissue slides, antigen retrieval is performed by heating the slides with a pressure cooker in the optimized buffer solutions: R-buffer A for COX-2 and Tris-glycine for 15-PGDH, The primary antibodies of mouse monoclonal anti-COX-2 and rabbit polyclonal anti-HPGD are applied with proper work dilutions. After all fields of each sample are measured, the final immunoreaction indices are generated automatically by setting algorithms as ‘‘total positive area / total epithelial area X average density’’ for cytoplasmic/membranous staining markers (COX-2, 15-PGDH).

Determination of Erythrocyte and Rectal Cell Phospholipid Membrane Fatty Acid Analysis
Lipids will be extracted using the method of Folch-Lees.(113) Fatty acid methyl esters are identified by comparing the retention times to those of known standards. Inclusion of the internal standard, dipentadecanoyl phosphatidylcholine (C15:0), permits quantitation of phospholipid amount in the sample. The lowest level of detection for individuals’ fatty acids is less than 0.5% of the total profile. In our laboratory, the inter-assay coefficient of variation (CV) is 1.1% and the intra-assay CV is 1.2% for measuring the mass of erythrocyte phospholipids. The variation for the percent fatty acid is less than 1%

Determination of C-reactive protein:
The rationale for including measures of CRP into the study design is two-fold. First, CRP is a well-known and validated biomarker of systemic inflammation, which has been associated with an increased risk for many cancers. (5, 114) Second, prior literature has suggested that fish oil supplementation may affect the production of CRP however; this finding has not been entirely consistent and may be modified by genetic factors. (114-116) Plasma samples will be transported from the GCRC Core Laboratory. CRP measurements

were performed using the High Sensitivity C-Reactive Protein Reagent (Pointe Scientific, Inc, Canton, MI) by means of latex particle enhanced immunoturbidimetric assay (ITA). The minimal detectable concentration of CRP is 0.1 mg/L. The coefficients of variation are 1.99 to 5.74% for within day samples and 1.23 to 6.97% for day-to-day sampling.

Determination of Adipokines
The rationale for determining the effect of fish oil supplementations on adipokines is two-fold. First, emerging evidence has suggested that these adipose-derived hormones may be an important factor linking obesity with CRC risk. (19, 27, 37-38, 117-118) As such, we plan to determine baseline levels of these factors in individuals at increased risk for CRC and determine if levels of these factors can be manipulated through fish oil supplementation. Second, although not entirely consistent, several studies have suggested that fish oil supplementation may reduce leptin levels while increasing adiponectin levels and thus reduce obesity-related chronic inflammation.(119-126) Measurement of adipokines levels will be performed in the Vanderbilt Hormone Assay and Analytical Services Core, which has over 30 years experience in measuring circulating levels of hormones. Leptin and adiponectin will be assayed via radioimmunoassay (RIA) using a double antibody procedure. Final analysis is accomplished by quantifying the bound radioactive counts with a Packard Gamma counter connected to a computerized data reduction station.

Determination of Insulin Sensitivity:
The rationale for including the homeostasis model assessment-insulin resistance (HOMA-IR) within our study design is that the HOMA-IR is strongly correlated to euglycemic clamp estimates of insulin sensitivity in individuals with normal glucose tolerance, individuals with impaired glucose tolerance and individuals with Type 2 diabetes mellitus.(91, 127-128) The HOMA-IR independently predicts the development of diabetes. (129-130) Diabetes mellitus and elevated insulin levels are well-described risk factors for colorectal cancer (131) and knowledge as to whether *FADS1* activity might modify the effect of fish oil on glucose homeostasis could be relevant towards both prevention of colorectal cancer and metabolic diseases. We will calculate HOMA-IR using a method described by Matthews et al.(91) The HOMA-IR derives an estimate of insulin sensitivity from the mathematical modeling of fasting plasma glucose and insulin concentrations. The formula is [fasting glucose*fasting insulin]/22.5. Plasma glucose will be determined based on the Hexokinase method measuring the absorbance of NADPH spectrophotometrically and measures on the Hitachi 911 analyzer using Roche Diagnostics regents (Indianapolis, IN). Plasma insulin level will be determined by radioimmunoassay (RAI) using a double antibody procedure. Both plasma glucose and plasma insulin determination will be performed in the GCRC Core Laboratory that has extensive experience in determining plasma glucose and insulin levels for research studies.

Data management and quality control
The Vanderbilt GCRC Informatics Core will be used as a central location for data processing and analysis. Vanderbilt University has developed software tools and workflow methodology for electronic collection and management of research study data. (132) REDCap (Research Electronic Data Capture) is a secure, web-based application that provides an intuitive interface for users to enter validated data remotely (with automated data type and range checks), data manipulation audit trails and reporting, and an export mechanism for end-of-study export of data to common statistical packages.

7.0 Risks

Potential Risks
The potential risk to the subjects related to the drawing of venous blood, adipose tissue biopsy, the biopsy procedure for rectal tissue, and the use of fish oil supplements.

Sampling of venous blood includes risk such as bruising, bleeding, and infection. These complications are uncommon and phlebotomy is a routine part of general clinical care.

Adipose tissue biopsies are associated with several risks. We will obtain a subcutaneous adipose biopsy from the upper buttock with a 16-gauge needle and disposable syringe. These procedures have been successfully used in multiple nutritional epidemiological studies as well as clinical trials, are well tolerated and do not require local anesthetics. These procedures do not require any specialized equipment and can be performed with standard 10-ml vacutainer tube and butterfly needle. Survey studies have found that research volunteers consider this procedure to be no more or less uncomfortable compared to venipuncture.(134) The risks associated with this procedure are similar to those of venipuncture and include bruising, bleeding and infection.

Rectal mucosal biopsies are associated with several potential risks. There is a risk of patient discomfort associated with the insertion of the anoscope. However, this discomfort is generally mild, such that when anoscopy is utilized in clinical care, for example visualizing internal hemorrhoids, sedation and analgesia is not required. In addition, no bowel preparation is needed reducing the risk of abdominal discomfort associated with cathartic agents. Infection and bleeding are also potential risk associated with rectal biopsy procedures. Infection however should be uncommon as all equipment, including the anoscope, obturator and surgical forceps are sterile and disposable so there is no risk of contamination between subjects. Bleeding is minimized by the shallow depth of the biopsy and small circumference of the biopsy. When bleeding does occur with the procedure it is generally well controlled through the application of gentle pressure or silver nitrate.

Omega-3 fatty acids have been part of the human diet for millennia and have uncommon and generally trivial side effects. In 1997 the Food and Drug Administration rules that an intake of up to 3 g/day of marine omega-3 fatty acids are Generally Recognized as Safe (GRAS)(135) (http://www.epa.gov/fedrgstr/EPA-IMPACT/2002/February/Day-26/i4327.htm). This ruling specifically considered the possible effects of fish oil on bleeding time, glycemic control, and LDL cholesterol. Fish oil supplements have been used in several large randomized controlled studies of cardiac patients.(136) In some trials doses as high as 12 g/d for durations of 2 years have been well tolerated.(137) The most common side effect of fish oil is a fishy after-taste which is less of a problem with pharmaceutical grade supplements. Minor gastrointestinal symptoms occur in 5 percent of patients. (135) Fish oil has been approved by the FDA for treatment of hypertriglyceridemia.

8.0 Reporting of Adverse Events or Unanticipated Problems involving Risk to Participants or Others

Data and Safety Monitoring Plan

Adverse event (AE) grading and attribution scale
For the purposes of this study and adverse event is defined as any untoward medical occurrence in a subject, not necessarily having a causal relationship with the study. A serious adverse event (SAE) is any untoward medical occurrence that a) results in death, b) is life-threatening, c) requires inpatient hospitalization or prolongation of existing hospitalization, d) results in persistent or significant disability/incapacity, or e) is a congenital anomaly/birth defect. AE's are graded as Mild (no limitation of usual activities), Moderate (some limitation) or Severe (inability to carry out usual activities) and attributes according to the relationship to the study drug and/or procedure as Not related, Unlikely, Possible, Probable, or Definite.

Plan for unanticipated AE reporting
SAEs and unanticipated AEs will be reported immediately by telephone to the Vanderbilt University IRB, the Nashville VA IRB, the GCRC and the DSMB. An Adverse Event Report form will be completes and returned to the VU and VA IRB, GCRC, and DSMB within 5 working days.

Plan for annual reporting of AEs
Annual reports are submitted to the VU and VA IRBs and will contain a) the number of adverse events and an explanation of how each event was handled, b) the number of complaints and how each complaint was

handled, c) the number of subject withdrawals and an explanation of why the subject withdrew or was withdrawn, and d) the number of protocol violations and how each was handled.

Plan for safety review
Patients will be monitored by both a study physician and a nurse during rectal biopsy procedures. Subjects will be monitored for 1 hour after the procedure is complete within the GCRC to ensure to immediate adverse events and will be contacted by the Research Assistant over the next 24 hours to assess for pain or any other symptoms which might be possible post-procedure complications.

9.0 Study Withdrawal/Discontinuation

Participants may be withdrawn from the study if they are not able to provide blood, urine or rectal samples, or they are not able to take their supplements as instructed.

If the intervention proves clearly harmful, the study will be stopped ahead of schedule. Furthermore, a single life-threatening condition related to the intervention, or a single life-threatening event from biospecimen collection, would cause consideration for study termination. Moderate complications occurring in more than one subject from any protocol would likewise cause consideration for study termination.

10.0 Statistical Considerations

General Approach
Patients will be followed for a maximum of 6 months and will complete the study on the last day of treatment after rectal mucosal biopsy. The following categories of patients may fail to complete follow-up: (1) patients who discontinue the study before 6 months due to adverse events or other reasons not related to the endpoint will be considered withdrawals, (2) patients who are observed at least once during follow-up but die or do not return for subsequent visit or do not return for scheduled visits, (3) patients who die before a scheduled visit or do not return for any scheduled visits.

Approach to Specific Aim 2
Hypothesis
Specific Aim 2 will test the hypothesis that genetically-determined fatty acid desaturase activity will modify the effect of fish oil supplementation on biomarkers of colorectal cancer risk

General Approach for Specific Aims 2
The 150 patients enrolled in Specific Aim 1 will provide data for Specific Aims 2. Thus, the inclusion/exclusion criteria, recruitment and retention, randomization, study design, data collection, intervention, determination of the end points, and assessment visits have been described in Specific Aim 1.

Statistical Analysis: Specific Aim 1 and Aim 2
General approach
The study is a double-blinded 3 X 2 factorial randomized controlled trial where patients will be randomized to the fish oil intervention and placebo group within each genotype group. The primary outcomes are rectal epithelial cell proliferation measured by Ki-67 labeling and rectal crypt apoptosis measured by TUNEL. The main secondary outcome variables will include: rectal mucosal expression of COX-2 and 15-PGDH, production of PGE_2 and PGE_3, rectal mucosal tissue levels of fatty acids, and changes in biomarkers of inflammation (C-reactive protein), adipokines (leptin, adiponectin), and markers of insulin sensitivity.

In Aim 1, the primary analysis will compare baseline and 6-month Ki-67 proliferative indexes and TUNEL labeling indexes in participants allocated to fish oil supplements compared to those allocated to placebo. In Aim 2, the primary analysis will compare baseline to 6-month Ki-67 proliferation indexes and TUNEL labeling indexes in participants in *FADS1* genotypes (GG, GT, and TT) allocated to fish oil supplements. The

secondary analysis will follow a similar plan as the primary outcome with the secondary outcomes of rectal mucosal tissue expression of COX-2 and 15-PGDH and the rectal epithelial cell production of PGE_2 and PGE_3, serum levels of C-reactive protein, leptin, adiponectin, and HOMA-IR scores.

Statistical analysis plan
Linear regression models will be used to test whether there are differences in the primary outcomes between the fish oil intervention group and placebo group (Aim 1) as well as the interaction between the two groups and *FADS1* genotypes (Aim 2) after controlling for the baseline measurement for each outcome and potential confounding factors. For Aim 2, we will also test differences in the primary outcomes among the genotypes within the fish oil intervention group. For our secondary analyses, a similar regression modeling approach will be employed to test differences in all the outcomes among comparison groups described above. The potential confounders included in the models will be polyp type, size, and location for primary adenoma as well as body mass index, age, total energy intake, intakes of other nutrients (average of four 24-hour recalls), and baseline PUFA levels derived from adipose tissue fatty acid biomarkers.

Descriptive statistics, including means, standard deviation, and ranges for continuous variables, as well as percents and frequencies for categorical variables, will be presented. Standard graphing and screening techniques will be used to detect outliers and to ensure data accuracy. Distributions of continuous outcomes will be assessed for normality. If normality is violated, an appropriate data transformation will be employed. Hypotheses will be tested at the level of α=0.05. This data analysis plan will be carried out using statistical software SAS® (Cary, North Carolina) or statistical package R (R Development Core Team, 2008).

Sample size estimation and power analysis
Our sample size estimates are derived using previously described clinical trial information using similar primary outcomes and doses of fish oil supplements. Courtney et al found a change in the mean level of Ki67 labeling of -5.86 (SD ± 8.8) for individuals allocated to fish oil supplements (n=14) and 3.62 (SD $\pm$ 8.0) for the placebo group (n=14).(57, 60) With a sample size of 22 per group for each genotype would provide at least 90% of power to detect 1 SD (8.4) difference in ΔKi67 between the two groups within each genotype with type I error rate = 5%. Assuming approximately 10% of drop-out rate, the total of 150 patients (25 patients per group per genotype $\times$ 2 groups $\times$ 3 genotypes = 150) would provide excellent power to detect a clinically significant difference between the intervention and placebo groups.

11.0 Privacy/Confidentiality Issues

Confidentiality and ethical considerations will be addressed as follows. All identifying documents, data and specimens collected as a result of this study will be retained by the investigator. Access to this material will be available only to the research investigator and his staff. Paper (hard) copies of study documents will be kept in a locked file cabinet. Electronic copies of documents will be stored in a password protected database on a secured server. If results of this study are to be published, only code numbers will be used for identification. Participants will not be identified by name.

12.0 Follow-up and Record Retention

Hard copies of all records will be stored in locked filing cabinets located in locked office space. Electronic databases are located in restricted access folders within the Vanderbilt University Medical Center computer network. Access to database is restricted by password or permission-based access. All identifying information will be removed and/or deleted from the participant records at 30 years following the conclusion of the investigation. Biological samples are coded and retained in a locked location with access limited to specific study personnel. DNA extracted will be stored for 30 years. After this period DNA samples will be destroyed.

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Appendix A Study Procedure Calendar

Table D summarizes proposed research tasks during a 5-year study plan.