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Diet and Inflammation in Colorectal Cancer Prevention

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Diet and Inflammation in Colorectal Cancer Prevention

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Abstract

Diet and Inflammation in Colorectal Cancer Prevention
By Carrie R. Daniel

There is no complete agreement as to which aspects of diet prevent or promote colorectal cancer (CRC). Some of the most salient mechanisms are related to growth and inflammation, but biomarkers are needed to bridge the gap between observational and experimental evidence.

We investigated the association between diet and CRC risk on two levels: in normal-appearing colorectal mucosa, where early risk is believed to be expressed, and in a U.S. cohort followed for CRC between 1999 and 2005. In the Markers of Adenomatous Polyps (MAP) II colonoscopy-based, case-control study (n=203), we investigated TGF-α, TGF-$ß_1$, and COX-2 expression as biomarkers of risk for incident sporadic colorectal adenoma. In the Cancer Prevention Study (CPS) II Nutrition Cohort, we prospectively investigated dietary intakes of omega-6 and omega-3 fatty acids, separately and in a ratio, with CRC incidence (778 cases in 96,152 men and women).

In the MAP II study, we found that expression of TGF-α, and the ratio of TGF-α to TGF-β_1, was higher in cases than controls ($P_{diff}<0.05$) and directly associated with adenoma (Odds Ratios: 2.23 and 1.40, respectively). COX-2 and TGF-α expression appeared to vary by nonsteroidal antiinflammatory drug (NSAID)-use; otherwise COX-2 did not differ by case-control status. All markers were associated with inflammatory risk factors for CRC. In the CPS II study, the ratio of omega-6 to omega-3 intake was not associated with CRC risk. In women, marine omega-3 intake was associated with a 20-30% lower risk, particularly in rare NSAID-users. Total omega-6 intake was inversely

associated with CRC risk in men [Relative Risk (RR): 0.79; P_{trend} = 0.07], and alpha-linolenic acid, the primary contributor to total omega-3 intake, was associated with increased risk in women (RR: 1.47; P_{trend}=0.10).

TGF-α showed the most promise as a biomarker of risk for colorectal cancer, although other markers also appeared be modulated by inflammatory risk factors. In the U.S. cohort, marine omega-3 and omega-6 intake were associated with lower risk of CRC in women and men, respectively. Future epidemiologic research should continue to search for means to translate early pathways of risk within the colon to larger studies of diet and CRC.

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CHAPTER 1

INTRODUCTION

In the United States, colorectal cancer is second only to lung cancer in total incidence and mortality (1), but unlike the relatively clear relationship between smoking and lung cancer, the complex interaction of factors involved in the development of colorectal cancer remain a challenge for the research community. Observational evidence suggests that 60 to 70 percent of all cancers are preventable through dietary choices, weight control, physical activity, and by not smoking (2). For colon cancer, it has been estimated that up to 90% of U.S. deaths from this disease could be prevented through feasible dietary and lifestyle intervention in conjunction with screening (3). However, the study of nutritional factors that contribute to colorectal cancer has advanced more slowly than have nutritional studies of other chronic diseases. New advances in the assessment of risk and continued investigation of biological plausible exposures are needed to bridge the gap between laboratory-based evidence and findings in human epidemiologic studies. Research and practice for the prevention of colorectal cancer through diet and lifestyle behaviors is unlikely to progress quickly without improved understanding of the complexities of colorectal carcinogenesis, coupled with advancements in the measurement of early risk, and use of new, integrated methods in human studies (4, 5).

Colorectal cancer appears to be highly correlated with Western diet and lifestyle, but the specific dietary components that prevent or promote the development of colorectal cancer are often disputed. The basic science cancer research community has

identified abnormal patterns of growth and inflammation that drive colorectal carcinogenesis and epidemiologic research is beginning to focus on potential risk factors involved in these mechanisms, but overall findings are inconsistent.

To begin to address the multitude of challenges in studying and elucidating diet and colorectal cancer relationships in humans, we investigated diet, growth, and inflammation pathways involved in colon carcinogenesis on two levels: in the normal-appearing colon mucosa, where early risk is believed to be expressed, and in a large, U.S. cohort followed for colorectal cancer. Herein we explored the mechanisms whereby growth and inflammation may affect risk within the colon; we investigated biologically plausible exposures related to inflammatory pathways with risk of colorectal cancer; and we began to develop biomarkers to measure early, relatively short-term changes in risk. In the future, results may be translated to innovative prospective studies of dietary exposures and colorectal cancer, or short-term chemoprevention trials, ultimately leading to tangible means of colorectal cancer prevention.

BACKGROUND & SIGNIFICANCE

Colorectal Cancer

Cancers of the colon and rectum take many years to develop and begin when a few epithelial cells lining the colon and rectum begin to exhibit abnormal properties (6). Nearly 90% of colorectal cancers arise from polyps of the colon or rectum, which may be simple, benign overgrowths of the cells lining the colon, or early tumor-like growths containing cancer cells. All may become cancerous if not treated. Polyps reoccur, over a period of years, in nearly half the people who have them removed, suggesting normal-

appearing tissue may retain components of risk (5-8). The long, latent, precancerous state characteristic of colorectal cancer and the prevalence of adenomatous polyps makes it well suited to mass screening and presents opportunities to derail the disease before it starts or to treat it in its earliest, detectable stages (8).

The molecular basis of colorectal carcinogenesis, as a multistep process, is becoming clearer and recent evidence pinpoints the importance of regulated cell turnover and regeneration in the gastrointestinal response to injury and inflammation (9-16). Environmental risk factors for colorectal cancer have been reviewed extensively (17), but few single interventions have been effective, with the exception of calcium and NSAIDs, whose anti-carcinogenic properties include suppression of growth and inflammation (18).

Western-style Diet

Colorectal cancer is highly correlated with Western-style diet characterized by a constellation of dietary components including lower intakes of fruit and vegetables and higher intakes of red and processed meats, refined grains, sugars, fats, and a higher ratio of omega-6 to omega-3 fatty acids (2, 19-21). Twenty-fold variations in international colon cancer rates, and migration studies showing acquired high (Western) risk within a generation, emphasize the importance of environmental exposures—especially diet and physical activity—in the etiology of colorectal cancer (22) and thus its preventability. At this time, dietary factors believed to adversely influence colorectal carcinogenesis include red and processed meats, while fruit, vegetables, calcium and vitamin D are thought to protect against the development of colorectal cancer (3). Energy balance may be a central factor in the etiology of colon cancer and ties together other prominent risk factors

such as BMI, physical activity, and total energy intake (19, 23-25). Non-dietary risk factors associated with lower risk of colorectal cancer include use of estrogen replacement therapy among women (26, 27) and use of ant-inflammatory drugs (28-30).

Molecular Targets in Diet and Cancer Prevention: Growth Factors and Inflammatory Mediators, TGF-α, TGF-β_1, and COX-2

Currently, there is no complete agreement as to which dietary factors drive or inhibit colorectal carcinogenesis, nor any functional biomarkers of risk. Further investigation of potential mechanisms whereby diet and lifestyle lead to clinically relevant pathological changes in the colon, and the development of biomarkers of risk derived from such mechanistic understanding, are urgently needed.

The GI tract is in continuous contact with environmental antigens and depends upon an adaptive and selective immune response to avoid a constant inflammatory state. The first line of defense in the colon is the physical barrier provided by the epithelial cells of the colon crypts, which must maintain structural integrity despite constant cell turnover in healthy tissue. To maintain this delicate balance, crypt cells of the colorectal epithelium must be selective for and sensitive to various signals involved in growth control, differentiation, and the immune and inflammatory response.

The earliest phases of colorectal carcinogenesis likely begin in the normal mucosa with a disorder of cell replication and renewal, followed by the subsequent appearance of clusters of enlarged crypts exhibiting proliferative, biochemical, and biomolecular abnormalities (7, 31, 32). Exogenous factors, including dietary and lifestyle modifications, result in adaptive changes in crypt cell proliferation within the rapidly

renewing colon and rectal mucosal crypt epithelium. These exogenous factors work by modulating multiple endogenous factors that are either known to be or are suspected of being involved in the adaptive crypt changes (33-37).

Growth factor independence and loss of the balance between proliferation and apoptosis is a hallmark of malignancy (38). The role of autocrine and paracrine growth factors in the progression of normal colonic mucosa to carcinoma, through the modulation of mucosal homeostasis, proliferation, inflammation, growth inhibition, differentiation, and apoptosis (33, 39-41), is becoming clearer. Transforming growth factor (TGF)-α, an autocrine stimulatory growth factor and member of the epidermal growth factor (EGF) family, is an important mediator of oncogenesis and malignant progression (33, 38). In the gut mucosa environment TGF-α plays a role in multiple pathways, including stimulating cell proliferation, assurance of cell survival, maintenance of cellular integrity, and the response to injury or inflammation (40, 42, 43). TGF-α expression in colon crypts is correlated with the distribution of proliferating cells, and mediated by its interaction with the EGF receptor. Under normal conditions, stem and progenitor cells capable of cell proliferation remain located towards the base of the crypt (zone of proliferation), while daughter cells of newly divided cells migrate up the crypt wall, cease cell proliferation, and differentiate into functionally mature cells as they move towards the crypt mouth (44, 45). The zone of cell proliferation may change in size due to various exogenous factors without a concomitant increase in the zone of differentiated cells (46).

The colon and rectal mucosal crypt epithelium is a rapidly renewing cell population, where cell proliferation is normally balanced by cell loss (33). Growth

regulatory proteins of the transforming growth factor-β family are one of the few classes of endogenous inhibitors of cell growth (41, 47). TGF-β cytokines have been implicated in diverse phenomena including growth control, differentiation, cell adhesion and motility, immune and inflammatory signaling, production of extracellular matrix components, angiogenesis, and alteration of cell phenotype (48, 49). Therefore TGF-β has paradoxical effects during colon carcinogeneis, serving both as an early tumor suppressor and, later, in aspects of tumor promotion. In normal epithelial cells, the major isoform, TGF-β_1 demonstrates an anti-proliferative/pro-differentiation effect (50, 51). Many epithelial cancers demonstrate resistance to TGF-β_1 (39, 49, 52) and a blockade in TGF-β_1 signaling may result in the disruption of tissue architecture, leading to accelerated progression from hyperplasia to adenoma to adenocarcinoma (53).

The inflammation-response includes release of inflammatory cytokines with activation of cyclooxygenase-2 (COX-2) related pathways, which generally delay or suppress apoptosis and modulate angiogenesis (12). COX-2 is usually not detectable under normal conditions, but like the other major cyclooxygenase isoform, COX-1, it has been shown to be constitutively expressed in several tissues including healthy gastric mucosa (54-56). Upregulation of COX-2 expression is an early, key oncogenic event in human colon neoplasia, typifying 85% of colon cancers and 50% of colon adenomas (57), and interventions to block COX action have been effective in the prevention of adenomas (28, 29, 58). However, the mechanisms whereby COX-2 overexpression leads to the formation of colorectal neoplasms are still not fully understood. COX-2 can be induced by a wide variety of growth regulatory and stimulatory signals, including those of the transforming growth factor (TGF) and epidermal growth factor pathway (59-67).

The role of TGF-α and TGF-β_1 in the gastrointestinal response to injury and inflammation and their interaction with COX-2 signaling is becoming more evident (9, 11, 59-66, 68). TGF-α may serve an important modulatory function in regulating prostaglandin release under inflammatory conditions (69) and aspirin treatment has been shown to significantly reduce TGF-α expression in the colorectal mucosa (60). In inflammatory bowel disease models, TGF-α and TGF-β play a role in the cellular response to injury, through activation and modulation of a variety of lamina propria cell populations, and recruitment of cells to the site of disease activity (9). The presence of TGF-α containing cells is increased both in active inflammation and during remission (11). TGF-α promotes mucosal wound healing (68) and is normally mediated by the action of COX-2 and eicosanoid products (64). However, pro-TGF-α signaling may also contribute to epithelial hyperproliferation and increased risk of malignancy in an errored attempt at epithelial regeneration in the face of mucosal injury and chronic inflammatory conditions (9). Conversely, TGF-β, also key during periods of active inflammation and epithelial cell restoration modulated by COX-2, acts as an anti-inflammatory cytokine (70). High COX-2 expression has been shown to block TGF-β_1 signaling (59, 63), and similarly, inhibition of COX-2 appears to lead to an increase in TGF-β_1 (54, 62).

Physical inactivity, obesity, and increasing age are all risk factors associated with both colorectal cancer and systemic inflammation (71), yet the mechanisms remain unclear (Figure 1). Human studies suggest that inhibition of inflammation via COX-2 may be one mechanism whereby physical activity reduces the risk of incident adenoma (72). The molecular mechanism by which obesity, an indicator of long-term energy balance, augments colon carcinogenesis is unknown, but there is substantial evidence that

modulation of the multi-step colon carcinogenic process is associated with altered expression of a number of pro-growth and inflammatory cytokines, including TGF-α (19, 72, 73). TGF-β_1 is potently induced by vitamin D_3 (51, 74), a potentially strong protective dietary factor for colorectal cancer. Beyond their regulatory roles in calcium homeostasis and gut absorption, vitamin D and the vitamin D receptor (VDR) are otherwise involved in protection against oxidative damage and modulation of inflammation and immunity, through regulation of growth factor and cytokine synthesis and signaling (53, 75-85). Recent research also suggests that bioactive vitamin D (calcitriol) can block the pro-inflammatory response via inhibition of COX-2 and degradation of prostaglandin products (86).

To our knowledge, no other study has quantified the expression of all three of these proteins (COX-2, TGF-β_1, and TGF-α) in the normal colon of persons at varied risk for colorectal cancer. To begin to address this need, we investigated phenotypic expression of these markers involved in the regulation of growth and inflammation in rectal biopsies of normal-appearing mucosa in persons with (cases) or without (controls) incident sporadic colorectal adenoma upon colonoscopy. A biomarker of risk's utility in prevention is largely dependent on whether it is modifiable; therefore, in addition to exploring these markers' associations with adenoma or case-status, we also investigated their associations with risk factors for colorectal neoplasms. Clarity on these issues and early diet-cancer-risk mechanisms may guide new investigations of biologically plausible exposures in larger prospective studies.

Inflammation, Fatty Acids, and Colorectal Cancer Risk

Comparisons of high colorectal cancer rates in the U.S. with relatively low rates in Mediterranean and Asian countries (20, 87) suggest a possible link between both type and quantity of dietary fat intake and colon cancer risk. Inconsistent and/or null early observational data for total fat led many to abandon the possibility that a relationship between fat and colorectal cancer may exist (88). Highly publicized, but inconclusive results of the Women's Health Initiative low-fat dietary pattern trial (89) raised more questions than answers on the subject of dietary fat and colorectal cancer risk. Few epidemiologic studies have been able to address the multitude of complexities involved in studying fat, such as quantity, origin (animal vs. vegetable), type (saturated, polyunsaturated, etc.), or composition of specific fatty acids. Recently, more detailed and comprehensive dietary questionnaires and updated nutrient databases have enabled researchers to investigate these questions. While several major cardiovascular disease studies investigating fatty acids have been published (90-99), comparatively little epidemiologic research has been done with fatty acids and colorectal cancer.

Proposed fatty acid and colorectal cancer mechanisms involve inflammation (prostaglandin metabolism, COX-2, TNF-alpha, immune function), oxidative stress (lipid peroxidation, ROS), signaling (nuclear receptor activation, apoptosis, differentiation, cell interaction, metastasis cascade), membrane properties (structure, fluidity, lipid rafts), bile acid metabolism, and lipid and glucose metabolism (cholesterol, IGF) (37, 100-102). Perhaps the most salient mechanism is modulation of inflammation. As one of the body's normal physiological responses to injury or disease, chronic inflammation or chronic inflammatory conditions have been shown to increase the risk of colorectal

cancer by facilitating the initiation and progression of normal cells to malignancy through the production of inflammatory cytokines and oxidative stress (12). Thus, while collective epidemiologic research (17) has not corroborated the relationship between total fat intake and colorectal cancer suggested by ecologic comparisons (2, 20), polyunsaturated fatty acids (PUFAs) are of particular interest due to their key role in inflammatory pathways.

The two major classes of PUFAs, omega-3 and omega-6 fatty acids, differ only by the position of a single double bond (Figure 2). The marine or long-chain omega-3's (20 carbons) include eicosapentaenoic (EPA), docosahexaenoic (DHA), and docosapentaenoic acid (DPA). Alpha-linolenic acid (ALA), the only short-chain omega-3 (18:3), is the predominant omega-3 in U.S. diets. Omega-6 fatty acids include linoleic (LA) (18:2) and arachidonic acid (AA) (20:4). Both ALA and LA are essential fatty acids (EFA), as they cannot be synthesized endogenously by mammals and must be acquired through the diet. The other PUFAs can be formed from EFA substrates through enzymatic processes of elongation and desaturation (Figure 1). However, endogenous conversion of ALA from plant sources to EPA is highly variable and in some individuals can be largely inefficient (103).

Following conversion to EPA and AA, PUFAs directly feed into the inflammatory pathway, serving as substrates and competing with each other for COX enzymes, which produce prostaglandins and other eicosanoid signaling molecules (Figure 3). Omega-3 fatty acids are hypothesized to reduce the risk of colorectal cancer by inhibiting the production of pro-inflammatory, omega-6 derived eicosanoids via the cycolooxgenase-2 (COX-2) enzyme (37, 104-106). Anti-inflammatory drugs are designed to block the

synthesis of eicosanoids (Figure 3), as these hormones mediate a variety of responses including inflammation, pain, immune response, platelet aggregation, vasoconstriction, vasodilation, and mobilization of intracellular calcium.

Experimental studies report anti-inflammatory and anti-carcinogenic effects in the colon for omega-3 PUFAs [eicosapentaenoic (EPA), docosahexaenoic (DHA), and alpha-linolenic (ALA) acid] highest in fish and seed oils, and adverse effects for omega-6 PUFAs, [linoleic (LA) and arachidonic (AA) acid] found in commercially popular oils and animal products (106-117). Despite experimental evidence supporting the omega-6 to omega-3 ratio as a biologically relevant target (37, 105, 110, 115, 117, 118), observational studies have generally not found an association with colorectal cancer (101) and prospective data for the ratio is sparse (119, 120). Prospective studies and relatively short-term clinical trials have shown that omega-3 fatty acids, particularly the long-chain or marine fatty acids (DHA and EPA), decrease both biomarkers of inflammation (96, 121, 122), and rectal cell proliferation (108, 111-114). Such evidence, coupled with the efficacy of nonsteroidal anti-inflammatory drug (NSAID), strong COX inhibitors, to reduce risk of colorectal neoplasia (28-30, 123) supports the promise for omega-3 PUFAs in the prevention of colorectal cancer through modulation of similar mechanisms.

However, of all the fatty acids, PUFAs are the most susceptible to lipid peroxidation (oxidative stress inducing process) due to their multiple double bonds where free radicals can “attack”. This is not much of a concern unless particularly high levels of PUFAs are ingested, such as may be the case with high-dose supplement use or a very high fat diet. Adequate intake of antioxidants, particularly the chain-breaking antioxidant

vitamin E, should keep this process in check in most healthy individuals, but an imbalance of prooxidants to antioxidants is more likely to occur in high risk groups such as smokers and heavy drinkers. Chronic conditions, such as diabetes and CVD may also lead to alterations in normal fatty acid metabolism (oxidation, utilization, transport, and/or storage) as do cholesterol-lowering medications and estrogen levels (124). Statins and PUFAs, particularly the omega-3 fatty acids, exert similar actions: both inhibit the production of pro-inflammatory cytokines and lower circulating cholesterol levels. Statins also enhance the conversion of linoleic acid and eicosapentaenoic acid (Figure 1) to their long chain derivatives (92, 124-126). Hormone replacement therapy (HRT) has been shown to improve fatty acid profiles in postmenopausal women and, to some extent, attenuate the effects of diet (92, 127, 128).

Review of Epidemiologic Evidence

A comprehensive analysis of international cancer incidence and dietary fat (1990) determined that total fat and saturated fat intakes were strongly associated with cancers of the colon (20). At the time, this was in line with international food disappearance data, cancer incidence rates, ecologic comparisons, and experimental findings in animal studies, shoring up the theory of an underlying biological relationship between fat intake and human cancer. Several years later, a meta-analysis of 13 case-control studies found no energy-independent association between dietary fat intake—total, saturated, mono or polyunsaturated--and colorectal cancer risk (88). Null or weak associations emerged from the first few early observational studies attempting to address specific fatty acids [reviewed in (101, 129)]. A recent international review of

prospective cohort studies investigating associations of fish and omega-3 intakes with colorectal cancer risk suggested sex and population-specific differences (130). Inconsistent findings and differential associations by sex for both omega-6 and omega-3 fatty acids are apparent across both biomarker- (131-133) and dietary assessment- (119, 134-138) based prospective studies. Of the seven prospective studies deriving marine omega-3 intake from food frequency questionnaires, two found an inverse association (134, 136) while five found no association with colorectal cancer (120, 132, 135, 138) or adenoma (119). Prospective data for omega-6 fatty acids are largely inconsistent (120, 131, 133, 137, 138) and studies investigating short-chain omega-3 fatty acids are sparse (101, 131, 139). In countries like the United States, where fish intake is low but meat, dairy, and vegetable/seed oil intake is relatively high, one must consider the effects of high intakes of both LA (an omega-6 PUFA which competes with omega-3 fatty acids for the same metabolic enzymes) and ALA, which can be converted to long-chain omega-3 fatty acids (EPA and DHA), particularly when fish intake is low (103). Five (115, 118, 139-141) of nine (119, 120, 142, 143) previous observational studies reviewed supported direct associations between a ratio of omega-6 to omega-3 fatty acids and colorectal cancer. Although prospective studies and short-term trials have shown that omega-3 fatty acids, particularly the long-chain or marine fatty acids (DHA and EPA), decrease rectal cell proliferation (108, 111-114), no consistent or strong associations with risk of adenoma have been found in larger studies (99, 142).

Sample size, study design, the quality of dietary assessment, the ability to adequately control for plausible confounders and potential effect modifiers, and the handling of fatty acid variables is highly variable for case-control and cohort studies

conducted over the past 10 years (101, 129, 130, 144). Overall, epidemiologic studies suggest that AA may increase risk and that DHA and EPA may decrease risk. Collective results for LA and ALA are inconclusive, although experimental data suggests that omega-3 (DHA, EPA, ALA) and omega-6 (LA, AA) fatty acids would be associated with decreased and increased risk, respectively. Several large studies did not control for anti-inflammatory drug use, cholesterol-lowering drug use, nor hormone use in women (101, 120, 135, 138). Few studies (119, 133, 134) tested the interaction between anti-inflammatory drug-use, marine omega-3 intake, and colorectal cancer risk and only one found that blood levels of marine omega-3 fatty acids were associated with lower risk of colorectal cancer in men not taking aspirin (P interaction = 0.04) (133). Weak and/or inconsistent results may also be caused in part by failure to account for the combination of fatty acids consumed (109). Very few colorectal cancer studies have looked at the competitive interactions between these fatty acids using a ratio of omega-6 to omega-3 fatty acids.

The ideal omega-6 to omega-3 ratio to reduce risk of cardiovascular disease and cancer is unclear (109, 145-147). The typical Western diet contains 10-20 times more omega-6 than omega-3 polyunsaturated fatty acids (PUFAs) with cellular levels readily influenced by diet. In contrast, human beings evolved on a diet with a ratio of ~1, and current recommendations extend from an "optimal range" of 1-4:1 to an "acceptable range" of 1-8:1(21, 109). Therefore, as high omega-6 and low omega-3 fatty acid intakes are characteristic of a higher-risk Western-style diet and may play opposing roles in inflammation-driven colorectal carcinogenesis, we examined the relationship of these fatty acids, and the ratio of their intake, with colorectal cancer risk in a large U.S.

prospective cohort. Regular anti-inflammatory drug use is common in older U.S. populations; therefore we also evaluated effect modification by NSAID or aspirin-use, as these drugs are designed to block the inflammatory response and may circumvent the upstream effects of fatty acids in this pathway (Figure 3) (133).

Summary

Herein, we translated pathways of risk to human epidemiologic studies of diet and colorectal cancer prevention. Much of the progress in reducing mortality and preventing heart disease in this country has been related to making biological measurements (or "biomarkers of risk"), such as blood cholesterols, as such measurements tell us who is at risk, what they need to do about it, and whether or not their efforts (diet, lifestyle modification, medications, etc.) are working. To date, there have been no such measurements to help cancer prevention efforts, as few studies have been able to address the multitude of complexities, both environmental and molecular, involved in studying diet-cancer relationships in humans. To our knowledge, no human study has explored the associations of adenoma and diet with TGF-α, TGF-β_1, and COX-2 in the normal-appearing colon. Investigating biomarkers of risk may generate new hypotheses regarding diet-colorectal cancer mechanisms and pathways, and provide guidance for new prospective analyses and chemoprevention trials. Despite relevant biochemical mechanisms and strongly supportive experimental data linking polyunsaturated fatty acids to inflammation and colorectal carcinogenesis, very few large, prospective studies of diet and colorectal cancer have investigated the ratio of omega-6 to omega-3 fatty acids. Prospective findings for individual polyunsaturated fatty acids and colorectal

cancer risk also remains inconsistent and few studies have addressed potential variations by sex and NSAID-use suggested by the literature.

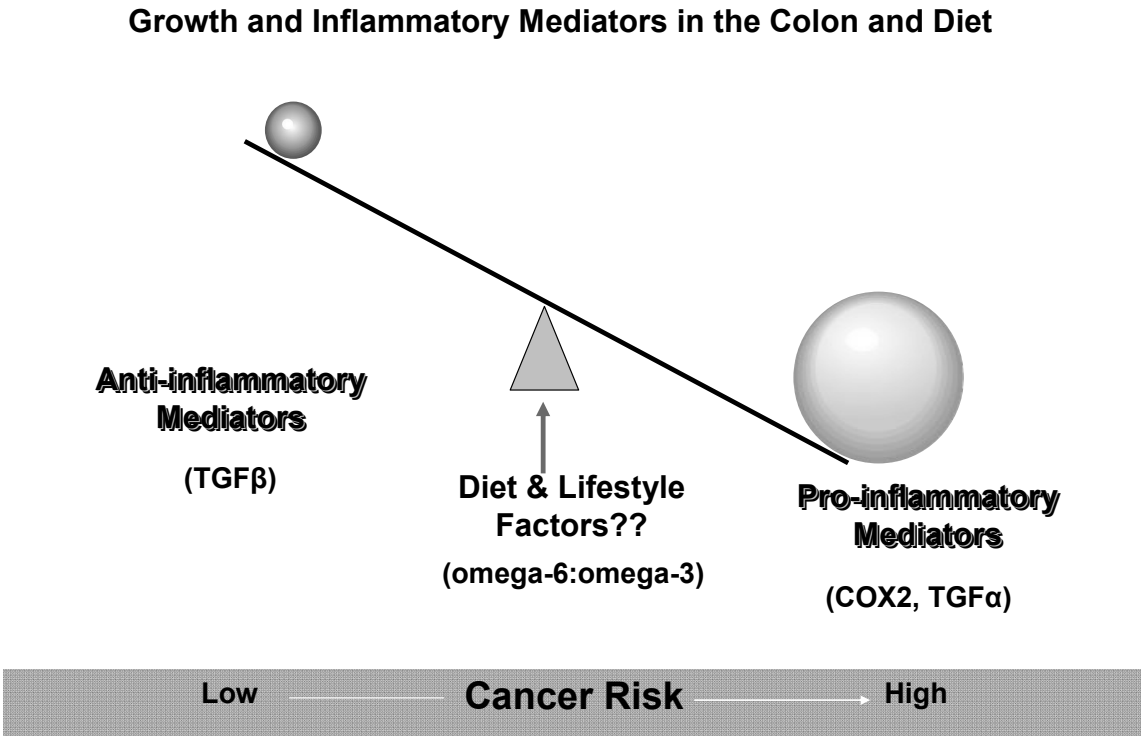

Figure 1: A proposed mechanism for the balance between pro-inflammatory and growth factors in the colon, potentially modulated by diet and lifestyle factors.

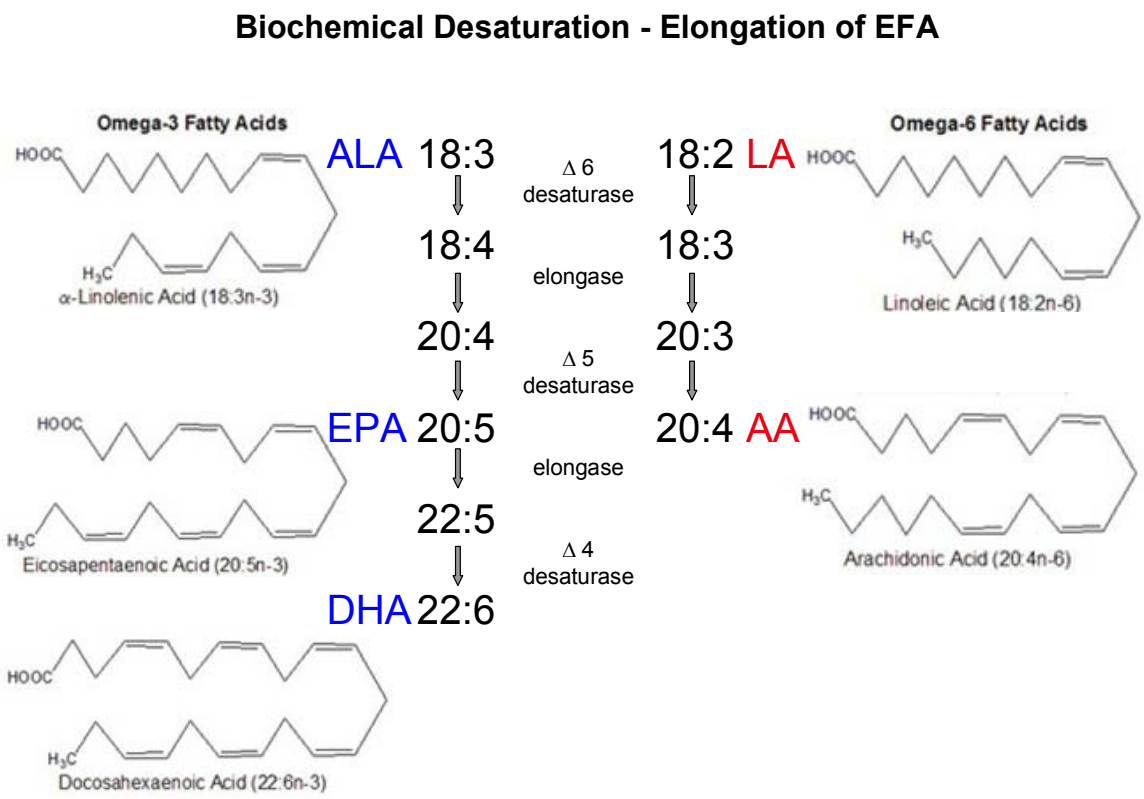

Figure 2: Desaturation and elongation of the nutritionally essential fatty acids (EFA), alpha-linolenic acid and linoleic acid, to their non-essential derivatives (both endogenously produced and acquired through diet). Desaturase and elongase enzymes are shared by both pathways leading to substrate competition.

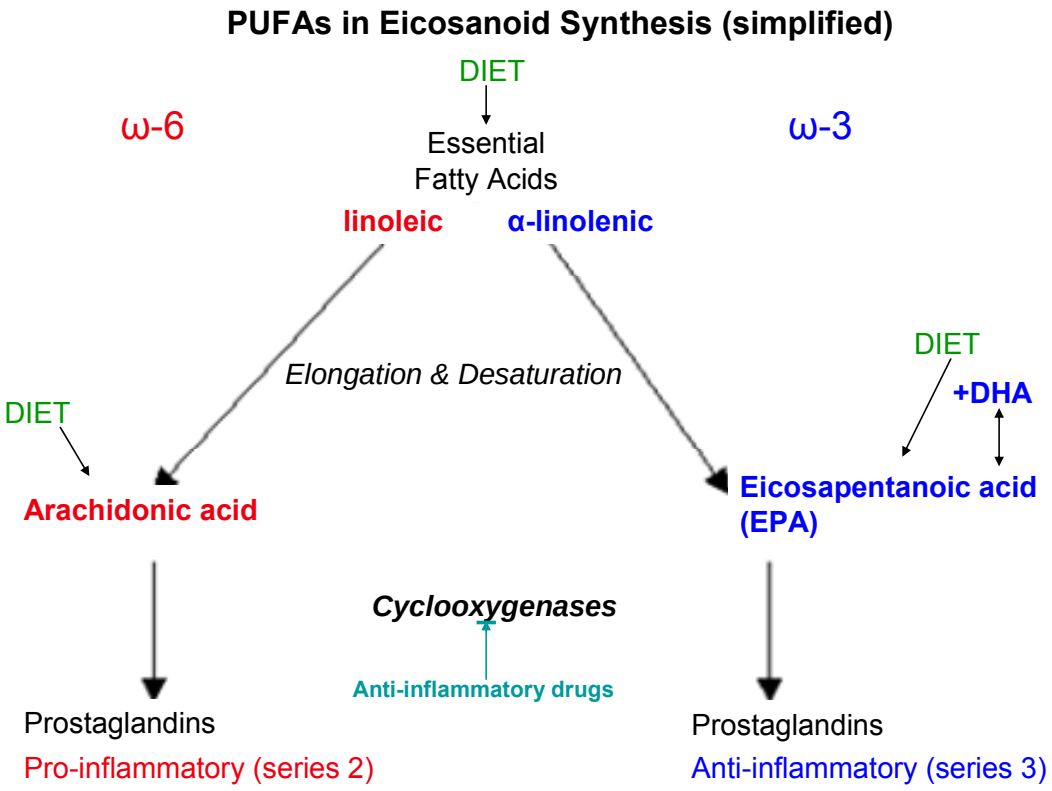

Figure 3: Circulating free fatty acids and fatty acids contained in lipoproteins or membrane phospholipids are utilized for eicosanoid synthesis, with fatty acid levels readily influenced by diet. Linoleic acid is elongated and desaturated to arachidonic acid. Alpha-linolenic acid competes for the same enzymes to produce EPA with interconversion between EPA and DHA. The action of cyclooxgenase (COX) and lipoxygenase enzymes generate the final eicosanoid products: prostaglandins, thromboxanes, and leukotrienes. Anti-inflammatory drugs (like aspirin and ibuprofen) inhibit COX-1 or COX-2 action to block production of pro-inflammatory prostaglandins.

CHAPTER 2

MANUSCRIPTS

TGF-α expression as a potential biomarker of risk within the normal-appearing colorectal mucosa of patients with and without incident sporadic adenoma

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Running Title: TGF-α expression as a biomarker of risk in normal colon

Key Words: TGF-α, colorectal mucosa, case-control

ABSTRACT

Background: Transforming growth factor (TGF)-α, an autocrine/paracrine stimulatory growth factor and member of the epidermal growth factor (EGF) family, is a mediator of oncogenesis and malignant progression in colorectal carcinogenesis. Limited evidence suggests its utility as a proliferation-related biomarker of risk for colorectal cancer.

Methods: We measured expression of TGF-α in biopsies of normal-appearing colorectal mucosa using automated immunohistochemistry and quantitative image analysis in a random sample of 29 cases and 31 controls from a colonoscopy-based case-control study (n=203) of biomarkers of risk for incident sporadic colorectal adenoma. Diet, lifestyle and medical history were assessed with validated questionnaires.

Results: TGF-α expression in the rectum was 51% proportionally higher in cases than controls, p=0.05; and statistically significantly associated with accepted risk factors for colorectal neoplasms (36% lower among nonsteroidal anti-inflammatory drug [NSAID] users, 49% lower among women using hormone replacement therapy [HRT], 79% higher among persons with a family history of colorectal cancer).

Conclusions: TGF-α expression in the normal-appearing rectal mucosa shows promise as an early, potentially modifiable biomarker of risk for colorectal cancer.

INTRODUCTION

Cancers of the colon and rectum take many years to develop and begin when a few epithelial cells lining the colon and rectum begin to exhibit abnormal properties (6). Nearly 90% of colorectal cancers arise from polyps, which reoccur over a period of years in nearly half the people who have them removed, suggesting that normal-appearing tissue may retain components of risk (5-8). The long, latent, precancerous state characteristic of colorectal cancer and the prevalence of adenomatous polyps makes it well suited to mass screening and presents opportunities to derail the disease before it starts or to treat it in its earliest, detectable stages (8). Better understanding the complexities of colorectal carcinogenesis coupled with advancements in the measurement of early risk are needed to progress research and practice in the prevention of colorectal cancer (4, 5).

The earliest phases of colorectal carcinogenesis likely begin in normal mucosa with a disorder of cell replication and renewal, followed by the subsequent appearance of clusters of enlarged crypts showing proliferative, biochemical, and biomolecular abnormalities (7, 31, 32). Transforming growth factor (TGF)-α, an autocrine/paracrine stimulatory growth factor and member of the epidermal growth factor (EGF) family, is an important mediator of oncogenesis and malignant progression (33, 38, 148, 149). In the gut mucosa environment TGF-α plays a role in multiple pathways, including stimulating cell proliferation, assurance of cell survival, maintenance of cellular integrity, and response to injury or inflammation (40, 42, 43).

Within colon crypts, TGF-α expression is correlated with the distribution of proliferating cells and mediated by its interaction with the EGF receptor (EGFR). Under

normal conditions, stem and progenitor cells capable of cell proliferation remain located towards the base of the crypt (zone of proliferation), while daughter cells of newly divided cells migrate up the crypt wall cease cell proliferation and differentiate into functionally mature cells as they move towards the crypt mouth (Figure 1) (44, 45). The zone of cell proliferation may change due to various exogenous factors (46).

Dietary and lifestyle modifications have been shown to cause adaptive changes in crypt cell proliferation within the rapidly renewing colon and rectal mucosal crypt epithelium. These exogenous factors work by modulating multiple endogenous factors involved in adaptive crypt changes (33, 34, 36, 37, 117, 150). To date, limited *in vivo* evidence is available to suggest that TGF-α may be a potential marker of proliferation and cancer risk (43, 151) and that it may also be modifiable through aspects of diet and lifestyle (60, 152). Mechanisms whereby changes in TGF-α expression within the colon modulate risk remain unclear. To begin to address these issues, we characterized the expression of TGF-α protein within the normal-appearing colorectal mucosa and assessed its association with adenoma and other risk factors for colorectal cancer in a pilot case-control study.

MATERIALS AND METHODS

The Markers of Adenomatous Polyps II study (MAP II) (2002) was a community- and colonoscopy-based, case-control study of incident, sporadic colorectal adenomas designed to investigate whether the expression patterns of various genes and cell cycle markers in normal-appearing rectal mucosa are associated with adenomas and thus be possible biomarkers of risk for colorectal neoplasms. Participants in the study

were recruited upon referral for routine outpatient elective colonoscopy at Consultants in Gastroenterology, PA, a large private practice gastroenterology group in Columbia, SC. English-speaking adults, aged 30-74 years and capable of signing informed consent, were eligible to participate. Subjects were excluded if they had previous adenomatous polyps, familial adenomatous polyposis, Gardner's syndrome, ulcerative colitis, Crohn's disease, incident colorectal cancer, or prevalent cancer other than non-melanoma skin cancer. Of the 351 patients identified over a five-month period, 305 (86.6%) were eligible to participate upon initial recruitment screening. Of these, 232 (76%) were successfully contacted and provided informed consent prior to colonoscopy. Of the 203 (87.5%) who met final eligibility criteria, 87 (42.8%) had polyps, of whom 49 (56.3%) had adenomas (overall adenoma prevalence 24.1%). Thus, the final sample size of 203 was composed of 49 cases (24.1%) and 154 controls (75.9%). Five participants were later excluded due to missing questionnaire data or implausibly low (<600 kcal/day) or high (>5,000 kcal/day) self-reported total energy intake. The final sample for the main study included 198 participants of whom 49 were incident sporadic adenoma cases. Of these, 31 cases and 29 controls were available for laboratory analysis of biopsy specimens for TGF-α.

Data collection: Prior to undergoing colonoscopy, participants completed mailed questionnaires eliciting self-reported demographics, medical history, anthropometrics (153) (height, weight, waist and hip circumference), diet, and lifestyle characteristics. Information collected included family history of polyps or colon cancer, reproductive and hormonal history, alcohol and tobacco use, use of medications such as aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs), medical conditions, and reasons for

and the sequence of events leading to colonoscopy. Physical activity was assessed using a modified Paffenbarger (154) questionnaire. Diet and nutritional supplement information was obtained using a modified Willett Food Frequency Questionnaire (155, 156), a 153-item paper questionnaire designed to capture individuals' usual dietary intake by asking respondents to report their frequency of consumption of a list of foods over the last year.

Colonoscopy, Pathology, and Tissue Collection: Colonoscopy of all participants was performed in the usual manner following a 12-hour fast and polyethylene glycol bowel cleansing preparation. All participants had six "pinch" biopsies taken from the normal-appearing rectal mucosa (10 cm above the anus). On 20% of the participants, biopsies from the mid-sigmoid and proximal ascending colon were also collected. No biopsies were taken within 4 cm of a polyp or tumor. For any polyp removed at colonoscopy, colon site, *in vivo* size (measured the largest diameter using fully opened forceps as a reference), and shape were recorded. The following histologic information was further reviewed by the study index pathologist according to the protocol developed by the National Polyp Study (157): polyp type (adenoma, hyperplastic, mixed, other), and if an adenoma, the subtype (villous, tubular, tubulovillous, serrated) and degree of atypia. Blood was drawn and stored for future analysis.

Laboratory methods: All biopsy specimens were fixed with 10% normal buffered formalin for 24 hours then stored in 70% ethanol. Within a week, the specimens were processed and embedded in paraffin blocks with three biopsies per colon site per block.

The paraffin blocks were then cut into three micron thick sections with each level 40 microns apart. Five slides with four biopsy levels each were processed and stained within seven days of being cut, yielding a total of 20 biopsy levels per patient. The slides underwent immunohistochemical processing using a DAKO Automated Immunostainer (Dako Corp., Carpinteria, CA; further referred to as DAKO), Leica H&E Autostainer and Leica CV500 Coverslipper (Leica Microsystems, Inc., IL). First, TGF-α antigen was unmasked via a heat-induced epitope retrieval procedure by placing the slides in a preheated Pretreatment (PT) Module (Lab Vision Corp., CA) with 100x Citrate Buffer pH 6.0 (DAKO S1699) and steamed for 40 minutes. Then the slides were immunohistochemically processed using an anti-TGF-α antibody (Neomarkers MS1000P) in a 1:50 dilution, a DAKO LSAB2 detection kit (DAKO K065), and 3,3' diaminobenzidine (DAB; DAKO K3466) as the chromogen. Slides were not counterstained. Positive and negative control tissue (tonsil) slides were processed and stained with each batch of patient samples and treated identically with one exception: antibody diluent was used rather than primary antibody on the negative control slide.

Image Analysis: The basic scoring method (Figure 2) used to describe and quantify various characteristics of the labeled antigens in the colon crypts is an image analysis scoring procedure for antigens that are labeled with a wide range of intensities in gradient distributions along the crypt axis (158). Methods and staining protocols are fine-tuned to achieve ideal staining quality within the tissue for quantification using our custom-designed plug-in into ImagePro Plus image analysis software (Media Cybernetics, Inc., MD), a light microscope, digital camera, and drawing board. The scoring and analysis

unit is a "hemicrypt", defined as one side of a colonic crypt bisected from its base to its gut luminal surface. Intact crypts (i.e., extending from the muscularis mucosae to the gut lumen) are analyzed in a systematic and reliable process by a trained technician (intra-rater reliability, intra-class correlation $r \geq 0.9$).

In brief, the technician reviews the slides and selects two of three biopsies on which 16 to 20 "scorable" hemicrypts per biopsy (32 per patient) can be obtained. The technician is guided through the scoring protocol by the computer software with background correction images obtained for each slide. Hemicrypts are manually traced by the technician and divided by the software into a number of segments corresponding in width to an average normal crypt epithelial cell (Figure 2). Overall hemicrypt- and segment-specific optical signal densities are then calculated by the software and stored into a Microsoft Access database along with various dimensional parameters of the hemicrypt. All images are obtained at 200x magnification and stored as 16-bit grayscale 1,600 × 1,200 pixel images.

Statistical Analysis: Cases included participants with pathology-confirmed, incident, sporadic colorectal adenomas, regardless of their number, shape, type, degree of dysplasia or location. Participants free of adenoma upon colonoscopy were considered controls. Baseline characteristics for the MAP II study population (49 cases and 149 controls) and a random subset of participants for whom slides were immunohistochemically processed for TGF-α (31 cases and 29 controls) were examined to assess the potential for confounding and comparability between the main study and biomarker subpopulation. Analysis of covariance (ANCOVA) was used for continuous

variables and the Fisher's exact test or χ^2-test was used for categorical variables, as appropriate. All values were adjusted for age. Nutrients were energy-adjusted according to the residual regression method of Willett and Stampfer (159). All statistical analyses were conducted using SAS version 9.1 software (SAS Institute, Cary, NC).

The primary biomarker variable of interest was the staining optical density for TGF-α expression within crypts of the rectal mucosa and its association with adenoma. While the immunohistochemical procedures were fully-automated, there was some variability between staining runs or batches. Therefore, biomarker values were standardized for staining batch by taking the value in each individual divided by the mean of the staining batch in which the individual's sample was processed. The batch-standardized primary biomarker variables adequately met multiple regression assumptions as evaluated by residual linear regression plots and diagnostics. No influential observations were detected by examining studentized residuals to detect outlying response values, DFFITS values to determine influence on a single value, and DFBETAS values to determine influence on regression coefficients (160).

We investigated the distribution of TGF-α expression graphically along the axis of colon and rectal crypts. Crypt divisions or cells (Figures 1 and 2) were first standardized to 50 divisions per hemicrypt, averaging measurements within each division across all crypts separately for each patient, and adjusted for possible batch effects as described previously. Descriptive plots were created using non-parametric smoothed LOESS (locally weighted scatterplot smoothing) procedures separately in cases and controls to depict TGF-α distribution from crypt base to apex.

The mean marker amount refers to the overall expression across rectal crypts for each patient and was calculated by summing the staining densities from all analyzed crypts from the biopsy specimens and dividing by the number of crypts analyzed. Measures of within-crypt expression or distribution of the marker (e.g., expression in sections from the lower 60% or upper 40% of the crypt) were also calculated for each patient by taking the mean of the biomarker densities from sections in the lower 60% of crypts, or in the upper 40% of crypts, and constructing ratios of these means.

We used ANCOVA methods to determine adjusted proportional differences in mean marker amount, standardized for batch, in cases vs. controls. Results reported herein by these methods were comparable to those using methods where batch was adjusted for as a fixed covariate or in mixed modeling procedures with batch treated as a random covariate. Regardless of method, all estimates were adjusted for age and sex as fixed covariates. In secondary analyses, we also examined means in a subgroup of patients (10 cases and 10 controls) with biopsy samples from the sigmoid and ascending colon in an equivalent manner.

Diet and lifestyle covariates considered in analyses were selected following a literature review of risk factors for colorectal neoplasms. We also assessed current observational literature and experimental evidence related to risk factors and markers of inflammation, oxidative stress, and energy balance suspected to be involved in colorectal carcinogenesis. Non-dietary exposures considered as potential covariates included age, sex, race, body mass index (BMI), waist-to-hip ratio (WHR), recreational physical activity (METs), smoking history (never, current, former), history of alcohol consumption (never, current, former heavy drinker), history of first-degree relative with

colorectal cancer (yes/no), regular use (more than once per week) of NSAIDs (yes/no), regular use of aspirin (yes/no), multivitamin use (never, current, former), and hormone replacement therapy (HRT) use among women (never, current or former). Dietary exposures examined as potential covariates included daily intake of total energy, intake of fats (total, monounsaturated, saturated, polyunsaturated, omega-3, omega-6) and fat ratios (omega-6:omega-3), intake of protein (total, animal, vegetable), carbohydrates and sugars (sucrose, fructose), total intake of calcium, vitamin D, antioxidants (E, C, carotenoids), fiber, dairy products (total, high fat, low fat), red and processed meats, fruits and vegetables (not including juice or potatoes). Continuous exposures were converted to a dichotomous (high, low) variable based on the median value (sex-specific for nutrients) in all 149 controls from the main MAP II study.

Bivariate analyses to evaluate TGF-α expression in the rectum according to diet and lifestyle characteristics was conducted in all 60 individuals, as well as among cases and controls separately. We used ANCOVA to compare adjusted means for TGF-α expression according to high vs. low levels of risk factors. All values were standardized for staining batch as described previously and adjusted for age, sex, energy, and case-control status as appropriate. Diet and lifestyle risk factors with notable differences in bivariate analyses were further evaluated as confounders when investigating adjusted associations between biomarker levels and adenoma.

The odds ratio with 95 percent confidence interval was calculated as a measure of association of incident sporadic adenoma with continuous batch-standardized biomarker expression using standard logistic regression modeling methods for case-control studies.

We also examined associations with a categorical (high vs. low) variable for TGF-α expression above or below the batch-standardized median in controls, as well as associations with batch-specific median cut points of unstandardized values. Results were similar when using these methods, although the analysis using a categorical TGF-α variable had less statistical power. Covariates in small multivariate logistic regression models of the association of incident, sporadic adenoma with TGF-α expression in the rectum were carefully selected based on biological plausibility, strength of confounding, and consideration of highly correlated variables.

RESULTS

Selected characteristics of cases and controls are presented in Table 1. On average, cases were more likely to be smokers, less likely to take NSAIDs regularly (once per week or more), and had higher total energy intake than controls. Unexpectedly, controls had lower physical activity levels and were more likely to have a first degree relative with a history of colorectal cancer. Female cases were more likely to use HRT. None of these differences were statistically significant. Baseline characteristics in all cases and controls (not shown) were comparable to those of this subpopulation.

The distribution of TGF-α expression (staining optical density) within normal-appearing rectal crypts for cases and controls is presented in Figure 3. TGF-α expression appeared highest at the base of the colorectal crypt and appeared to decrease moving up the crypt toward the colon lumen (Figures 1 and 3). Throughout the crypt, expression of TGF-α appeared to be higher in cases than controls.

Average TGF-α expression throughout the rectal crypts (Table 2) was proportionally 51% higher in cases than controls (adjusted for age and sex, p diff=0.05). There was a greater difference in total TGF-α expression among female cases and controls than among males, but associations in both sexes were in the same direction (p interaction NS, data not shown). There was also some evidence of differential risks by NSAID use, but the test for interaction was not statistically significant (p interaction=0.28). Among persons not using NSAIDs, TGF-α expression was 57% higher in cases than controls (p diff=0.12), while among persons using NSAIDs once per week or more there was no difference between cases and controls (p diff=0.98, data not shown). Strong, statistically significant case-control differences were apparent both in the lower 60% and in the upper 40% zones of the crypt (Table 2). TGF-α expression in the normal rectal epithelium was directly associated with case-control status (adenoma) both in the entire crypt (Odds Ratio (OR): 2.23; 95% Confidence Interval (CI): 0.98-5.07), as well as in the lower 60% (OR: 2.16; 95% CI: 1.11-4.17) and upper 40% (OR: 2.12; 95% CI: 1.03-4.38) of the crypts. Analyses involving the 10 cases and 10 controls on whom sigmoid and ascending colon biopsies were processed for TGF-α proved not meaningful due to the small sample size (data not shown).

When investigating the potential relationship between diet, lifestyle, and TGF-α expression (Table 3), we found statistically significant differences in TGF-α expression according to categories of several key risk factors for colorectal cancer. While primarily driven by differences in cases, overall differences adjusted for case-control status suggested that TGF-α expression was 79% higher among persons with a positive family history of colorectal cancer (p=0.02), 36% lower in persons regularly taking NSAIDs

(p=0.05), and 49% lower among women who used HRT (p=0.06). Differences in persons found to be free of polyps at colonoscopy (controls) were in the same direction, although smaller and not statistically significant. In contrast, dietary associations appeared to be somewhat stronger among controls, as TGF-α expression was 28% lower among persons with a high physical activity level (p=0.46), 58% higher in persons with high sucrose intake (p=0.07), and 31% lower in persons with high fruit and vegetable intake (p=0.16). In general, differences across cases and controls, examined separately, appeared to be in the same direction as with cases and controls combined and adjusted for case-control status.

We investigated associations between TGF-α expression and risk of adenoma in multivariate models adjusted for risk factors that were suggested as potential confounders by the analyses presented in Tables 1 and 3. Case-control differences were attenuated when adjusted for NSAID-use and strengthened when adjusted for total energy intake and/or family history of colorectal cancer (Table 4). Estimates were not strongly affected by inclusion of other risk factors in the models. In the multivariate-adjusted model, higher TGF-α expression in normal-appearing rectal tissue was associated with a 3- to 4-fold increased risk of adenoma (OR: 3.75; 95% CI: 1.18-11.98).

DISCUSSION

In the MAP II study, expression of TGF-α, an autocrine/paracrine growth factor of the EGF family (33, 38, 148, 149), was statistically significantly higher in normal-appearing rectal tissue from adenoma cases than from controls. These associations persisted following adjustment for potential confounders (i.e., risk factors for colorectal

neoplasms that also appeared to be associated with TGF-α levels). TGF-α expression varied significantly across key risk factors for colorectal neoplasms. Although our results are preliminary and require further validation, they suggest that higher TGF-α expression in the normal-appearing colorectal mucosa may indicate an at-risk phenotype, which may also be modulated by risk factors or behaviors believed to be important for preventing colorectal neoplasms. These findings support the potential for TGF-α as a modifiable biomarker of risk for colorectal cancer. Although not directly addressable by our study design, our results also suggest the possibility that TGF-α signaling may be involved in the progression of normal colorectal mucosa to adenoma.

Basic science evidence strongly supports a pro-growth and hyper-proliferative mechanism in colon carcinogenesis with elevated TGF-α expression in both colon adenoma and adenocarcinomas (43). Identifying at-risk molecular phenotypes is critical to advance the understanding of how colorectal cancer develops and how to target risk at a reversible stage. Early proliferative changes in the normal colorectal mucosal crypt epithelium appear to precede or at least accompany the development of polyps or cancer and, thus, may have value as a predictive or diagnostic marker (161-166), yet there are few human studies of TGF-α expression in normal-appearing colon or rectal crypts in persons at varied risk for colorectal neoplasms (60, 152). To begin to address these needs, we characterized and quantified phenotypic expression patterns of TGF-α in normal-appearing rectal tissue and detected significantly higher levels in persons at increased risk for colorectal cancer (persons with adenomatous polyps as compared to polyp-free controls). While there was a clear difference in the magnitude of total TGF-α expression between cases and controls, the distribution of expression within crypts in

normal-appearing tissue from adenoma cases did not appear different than that of normal-appearing tissue from adenoma-free controls. We found TGF-α staining in the cytoplasmic region of colonocytes in the rectal crypts to be slightly higher at the base of the crypt and throughout the proliferative zone, but strong case-control differences in TGF-α expression were maintained throughout the crypt, including the upper portion of the crypt, believed to be the zone of differentiation. Preliminary studies from other groups showed denser TGF-α staining expression in the upper one-third to two-thirds of colonic crypts (33, 152, 167). Discrepancies in characterization of the localization of TGF-α in colon crypts may be due to differences in quantification methods, as we are the first to use our custom image analysis program, which allows us to quantify both average expression and section-specific expression for each crypt analyzed (32 hemicrypts per patient x 60 patients). Another explanation may be the use of different antibodies with variable specificities, which may or may not be able to differentiate between the inactive and active form of TGF-α. Only the active form is believed to bind to the EGFR and stimulate proliferation of epithelial cells (149, 168, 169). Our findings seem more plausible for TGF-α, an early stimulator of growth and pro-proliferative marker, as we found the greatest level of expression near the base of the crypt or proliferative zone.

Little is known regarding the effects of diet and lifestyle behaviors on TGF-α signaling in the colon; therefore we investigated whether the expression of TGF-α in normal-appearing rectal tissue varied across plausible risk factors for colorectal neoplasms. We found TGF-α expression to be correlated with a family history of colorectal cancer or adenomatous polyps in a first-degree relative(s), NSAID-use, and HRT-use in postmenopausal women. Despite strong, plausible evidence for these risk

factors (18, 26, 170), the mechanisms whereby they modulate risk within the colon remain unclear. Animal and human studies have shown that manipulation of diet and other lifestyle exposures result in adaptive changes in the colon crypt epithelium and in TGF-α expression specifically (34, 46, 60, 117, 152). Our preliminary findings suggest that TGF-α levels may vary according to modifiable dietary risk factors (intake of sucrose, low-fat dairy, fruits and vegetables), and although in this small sample few differences were statistically significant, many are consistent with current hypotheses regarding diet, growth, and colorectal cancer (32, 40, 46, 60, 73, 82, 150, 152, 162, 171-173). Furthermore, associations between TGF-α expression and risk of adenoma were strengthened following adjustment for risk factors, suggesting that a person with elevated TGF-α levels could further increase or decrease their probability of being a case (i.e., risk of incident, sporadic colorectal adenoma) based on their history of risk behaviors, such as use of NSAIDs or total energy intake. The efficacy of NSAIDs in preventing colorectal adenoma is well-documented (28, 29, 58), and other investigators found decreased staining for TGF-α in the rectal mucosa of patients with a history of adenoma treated with aspirin (60). Rodent studies support total energy restriction to lower the risk of spontaneous and induced tumors (174) with inhibitory effects on DNA synthesis translating to reduced cellular growth and tumorigenesis in the colon (175). The reduced number of dividing cells in the colons of these animal models may explain why many are "resistant" to the induction of colon cancer (176). Beyond modifiable risk factors like diet and lifestyle, elevated expression of TGF-α in normal-appearing tissue from persons with a family history of colorectal cancer emphasizes the importance of regular monitoring and screening for high risk persons and the imminent need for early

biomarkers of risk. Our group is currently evaluating the effect of supplemental calcium and/or vitamin D on TGF-α expression and other markers to further develop and validate our biomarkers of risk panel and determine whether markers are treatable.

The MAP II project had several major strengths due to the use of new technologies and integration of laboratory, clinical, and epidemiologic methods. Moving beyond cell culture studies and gene arrays, expression of genes (phenotype) in colon tissue, particularly within the structure of colon epithelial crypts, provides more relevant information regarding early tissue-level changes and the progression toward carcinogenesis, which is the result, not only of genotype, but of epigenetic influences, gene-gene interactions, gene-environment interactions, and complex multi-gene-multi-environment interactions. Nonetheless, results of this novel, but small pilot study should be viewed and interpreted with caution. We were not able to address multiple confounding effects or interactions, which was particularly problematic in analyses with risk factors. Few of these risk factors can be appropriately considered in isolation and may be masking a more complex pattern of diet and lifestyle behaviors. Some covariate categories, such as those for current smoker and positive family history of colorectal cancer, were restricted to a small number of individuals. Larger samples, representative of the population at-large, will be needed to validate these findings. Nor were we able to adequately address TGF-α expression in more proximal sites of the colon. However, the study size was sufficient to begin to accomplish our objectives of obtaining preliminary estimates to provide direction for future study. Other limitations included those inherent to case-control studies in general, and colonoscopy-based case-control studies of adenomas in particular. Cases and controls may not be representative of the population at

large as they were selected from among individuals referred for routine colonoscopy screening and thus may have been at potentially higher risk for colorectal neoplasms. Knowledge of the postulated diet and lifestyle associations with colorectal cancer are likely to have been available and of interest to this population. The most likely product of these biases is that controls were more similar to cases, leading to an underestimate of true risk. Although, a major strength of this investigation is that controls were derived from the population that gave rise to cases, and the potential for differential reporting bias was limited by obtaining self-reports of exposure *prior* to colonoscopy and diagnosis of adenoma.

As a retrospective evaluation of risk factors and cross-sectional investigation of the biomarker-disease relationship, the design of our pilot study does not allow us to determine causal associations with diet, or with adenoma progression (i.e., which came first, the biomarker or the neoplasm). However, to learn whether or not the biomarker is associated with the presence of a neoplasm, temporality is irrelevant, and there is negligible misclassification of neoplasm status with a colonoscopy-based design. Although it is of relevance primarily to etiology and risk assessment, it is not unlikely that, considering that patients going to colonoscopy are likely at higher risk, some patients who received colonoscopies and are classified as controls will later develop neoplasms and therefore have biomarker profiles similar to those of cases. The latter limitation would tend to attenuate true associations; however, despite this potential limitation, our preliminary data suggest that there may be substantial, biologically plausible, and statistically significant differences between cases and controls. While our results seem promising and informative of the potential mechanisms involved, further

research is needed to clarify whether TGF-α expression in colorectal crypts is predictive of relevant precancerous changes leading to the appearance of colorectal neoplasms.

Results illuminating potential associations between TGF-α and diet and lifestyle risk factors suggest that larger studies of similar design to assess biomarkers of risk may elucidate poorly understood diet-cancer mechanisms. Although convincing human evidence is needed, large prospective studies are challenging and costly in cancer research due to the temporality of the exposure-disease relationship and the lack of biomarkers of risk to advance research in this area. In the future, a panel of biomarkers of risk for colorectal cancer could serve as an end point in relatively short-term prospective studies and chemoprevention trials to investigate promising diet and lifestyle exposures and to make vital progress in colorectal research and prevention.

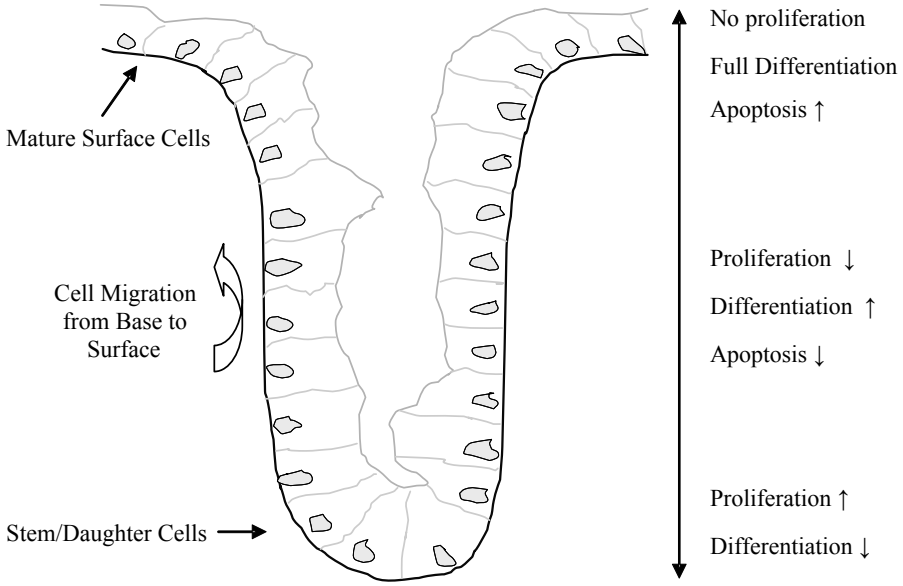

Figure 1. Colon Crypt Model

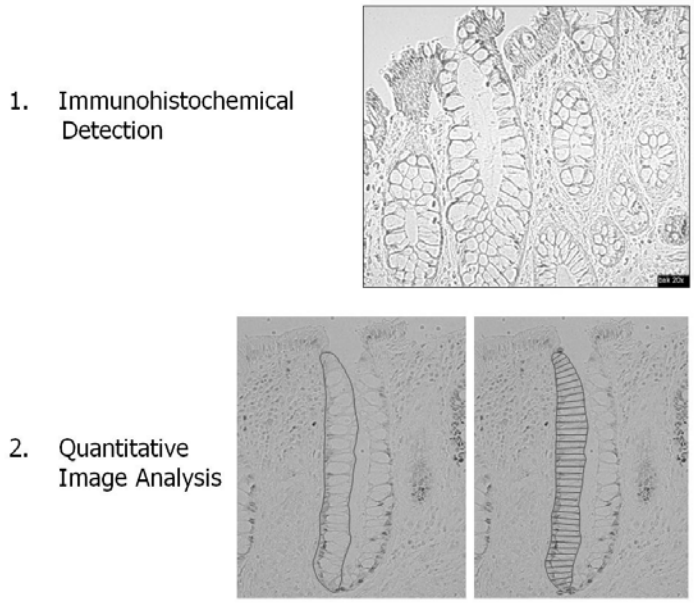

Figure 2. Molecular phenotyping is conducted by detecting the expression of various genes using automated immunohistochemistry (1). The detected expression of the genes in the tissues is quantified using custom designed quantitative image analysis software. Note that this image analysis measures and quantifies total expression as well as the architecture or tissue distribution of the expression (2).

Table 1. Selected characteristics of patients with (cases) and without (controls) incident, sporadic colorectal adenoma, MAP II

Characteristic[1]	Cases n=31	Controls n=29	*P*[2]
Age, years	55.0 ± 1.4	54.7 ± 1.5	*0.9*
Male (%)	41.9	44.8	*1.0*
Caucasian (%)	93.5	96.6	*1.0*
Current smoker (%)	16.1	10.3	*0.1*
College graduate (%)	35.5	27.6	*0.8*
HRT use among women (%)	87.5	66.7	*0.2*
Consumes alcohol currently (%)	64.5	65.5	*1.0*
Regular NSAID use (%)	32.3	55.2	*0.1*
Regular aspirin use (%)	32.3	37.9	*0.8*
1st degree relative with CRC (%)	10.0	25.9	*0.2*
Recreational physical activity, METs/day	27.9 ± 3.5	21.6 ± 3.6	*0.2*
Body mass index, kg/m^2	30.5 ± 1.4	32.3 ± 1.4	*0.4*
Daily intakes			
Total energy intake, kcal	1,943.8 ± 121.3	1,624.3 ± 125.5	*0.1*
Total fiber intake[3], g	14.1 ± 4.9	15.3 ± 6.6	*0.4*
Total calcium intake[3], mg	928.8 ± 85.3	867.9 ± 88.3	*0.6*
Total vitamin D intake[3], IU	335.7 ± 52.1	321.0 ± 54.0	*0.8*
Total folate intake[3], mcg	471.9 ± 45.8	491.0 ± 47.37	*0.8*
Sucrose intake, g	40.3 ± 2.93	35.8 ± 3.03	*0.3*
Whole fruit & vegetable servings/day	3.5 ± 2.1	3.4 ± 1.5	*0.9*
Weekly intakes			
Processed meat servings/week	2.2 ± 0.30	2.6 ± 0.37	*0.4*
Red meat servings/week	6.2 ± 2.46	8.2 ± 2.54	*0.6*
Low-fat dairy servings/week	4.5 ± 0.95	4.1 ± 0.98	*0.8*

Abbreviations: HRT=hormone replacement therapy, NSAID=nonsteroidal antiinflammatory drug, CRC=colorectal cancer, MET=metabolic equivalents,

[1] Mean ± standard error (SE) presented unless otherwise specified

[2] Fishers exact for categorical variables; Pdiff (ANOVA) for continuous variables; adjusted for age and total energy intake, as appropiate

[3] Total intake (diet + supplements) adjusted for total energy intake

Figure 3. Distribution of TGF-α in normal-appearing rectal mucosa, cases and controls

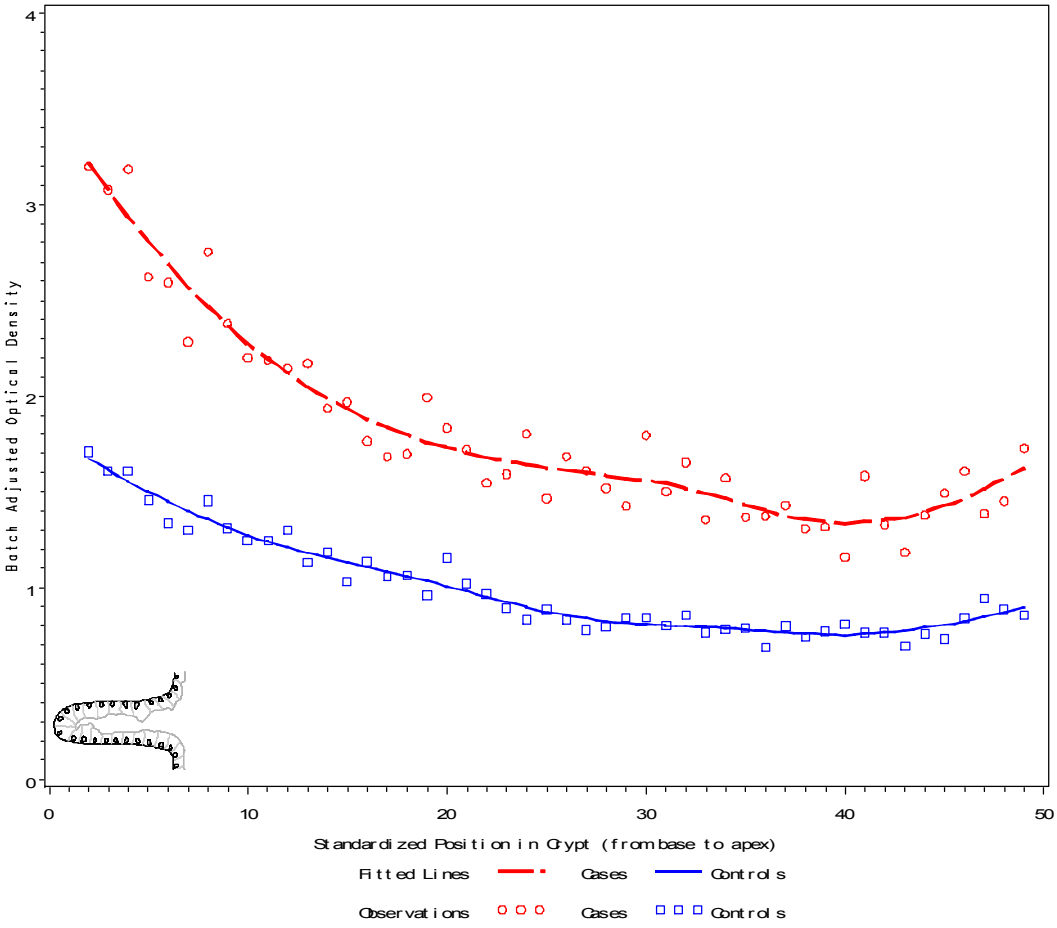

Figure 3. Distribution of TGF-α expression throughout colon crypts in incident, sporadic colorectal adenoma cases and controls, MAP II. Cells from base to top of crypt (proliferative zone to zone of differentiation) are presented on the X-axis. The Y-axis refers to the batch-standardized staining optical density. Circles refer to measures in controls and squares refer to measures in cases. Each point represents the average of all patients' hemicrypts at a standardized crypt section or location. The smoothed line represents the best fit for the data.

Table 2. TGF-α expression (staining optical density[1]) in the normal-appearing rectal mucosa of incident, sporadic colorectal adenoma cases and controls, MAP II

Crypt Section[2]	Case[3] (n = 29)	Control[3] (n = 31)	Proportional Difference %[4]	P_{diff}[5]	Odds Ratio[6] (95% Confidence Interval)
Full Crypt	1.18 (0.14)	0.78 (0.14)	51.2	0.05	2.23 (0.98-5.07)
Lower 60%	2.12 (0.32)	1.01 (0.31)	109.9	0.02	2.16 (1.11-4.17)
Upper 40%	2.31 (0.41)	0.98 (0.39)	135.7	0.02	2.12 (1.03-4.38)
L60%/FC	1.66 (0.11)	1.34 (0.11)	23.9	0.04	3.75 (1.01-13.91)
U40%/FC	1.81 (0.22)	1.40 (0.22)	29.2	0.19	1.52 (0.80-2.90)

[1] Batch-standardized as individual value divided by batch mean; all estimates adjusted for age and sex

[2] Full Crypt (FC) = average expression throughout entire crypt; Lower (L) = sections from base of crypt; Upper (U) = sections from crypt apex or mouth; Ratios of section-specific expression

[3] Mean staining densities and standard errors

[4] Difference between means (cases - controls) divided by mean in controls x 100%

[5] Difference p-value for comparison of means (ANCOVA)

[6] Association between TGF-α expression (as a continuous variable) and adenoma (relative odds of being a case) adjusted for age and sex

Table 3. TGF-α expression (staining optical density[1]) according to diet and lifestyle risk factors, MAP II

Characteristic[2]	*n*	All[3] (n = 60)	Prop Diff %[4]	P_{diff}[5]	*n*	Controls (n = 29)	Prop Diff %[4]	P_{diff}[5]	*n*	Cases (n = 31)	Prop Diff %[4]	P_{diff}[5]
Lifestyle												
Age < 55 years	*32*	0.94			*15*	0.75			*17*	1.12		
Age ≥ 55 years	*28*	1.06	12.8	*0.55*	*14*	0.84	12.0	*0.68*	*14*	1.28	14.3	*0.66*
NSAID-use never to < once/wk	*34*	1.16			*13*	0.83			*21*	1.40		
NSAID-use ≥ once/wk	*26*	0.74	-36.2	*0.05*	*16*	0.76	-8.4	*0.74*	*10*	0.66	-52.9	*0.05*
HRT-use never (women)	*7*	1.68			*5*	1.11			*2*	2.72		
HRT-use ever (women)	*24*	0.86	-48.8	*0.06*	*10*	0.66	-4.1	*0.23*	*14*	1.07	-60.7	*0.07*
BMI < 30 kg/m^2	*31*	1.06			*13*	0.78			*18*	1.34		
BMI ≥ 30 kg/m^2 (obese)	*29*	0.93	-12.3	*0.50*	*16*	0.81	3.8	*0.89*	*13*	1.00	-25.4	*0.33*
Physical activity[6] < 22 METs/day	*37*	1.05			*20*	0.85			*17*	1.24		
Physical activity > 22 METs/day	*23*	0.99	-5.7	*0.75*	*9*	0.69	-18.8	*0.46*	*14*	1.27	2.4	*0.92*
No family history of CRC	*47*	0.82			*20*	0.72			*27*	0.97		
1° relative with CRC	*10*	1.47	79.2	*0.02*	*7*	0.78	8.3	*0.78*	*3*	2.53	160.8	*0.01*
Dietary intakes[7]												
Total energy[6] low	*20*	1.13			*10*	0.79			*10*	1.43		
Total energy high	*40*	0.92	-18.6	*0.32*	*19*	0.75	-5.1	*0.84*	*21*	1.08	-24.4	*0.36*
Total vitamin D low	*33*	1.02			*16*	0.82			*17*	1.24		
Total vitamin D high	*27*	0.95	-6.9	*0.72*	*13*	0.77	-6.1	*0.82*	*14*	1.13	-8.9	*0.75*
Total calcium low	*33*	1.02			*17*	0.79			*16*	1.28		
Total calcium high	*27*	0.96	-5.9	*0.76*	*12*	0.80	1.3	*0.97*	*15*	1.10	-14.1	*0.67*
Total folate low	*31*	1.02			*15*	0.85			*16*	1.18		
Total folate high	*29*	0.97	-4.9	*0.81*	*14*	0.73	-14.1	*0.53*	*15*	1.21	2.5	*0.92*
Total fiber low	*35*	0.98			*16*	0.78			*19*	1.17		
Total fiber high	*25*	1.01	3.1	*0.85*	*13*	0.81	3.8	*0.89*	*12*	1.23	5.1	*0.86*
Sucrose low	*28*	0.89			*15*	0.62			*13*	1.15		
Sucrose high	*32*	1.08	21.3	*0.33*	*14*	0.98	58.1	*0.07*	*18*	1.22	6.1	*0.84*
Red & processed meat low	*31*	1.11			*15*	0.82			*16*	1.41		
Red & processed meat high	*29*	0.87	-21.6	*0.29*	*14*	0.77	-6.1	*0.87*	*15*	0.97	-31.2	*0.25*
Low fat dairy low	*34*	1.07			*16*	0.88			*18*	1.26		
Low fat dairy high	*26*	0.89	-16.8	*0.38*	*13*	0.69	-21.6	*0.35*	*13*	1.10	-12.7	*0.68*
Fruit & vegetables low	*26*	1.07			*12*	0.97			*14*	1.17		
Fruit & vegetables high	*34*	0.93	-13.1	*0.53*	*17*	0.67	-30.9	*0.16*	*17*	1.21	3.4	*0.91*

Abbreviations: NSAID=nonsteroidal antiinflammatory drug, HRT=hormone replacement therapy, BMI=body mass index, CRC=colorectal cancer

[1] Batch-standardized as individual value divided by batch mean; full crypt values

[2] All estimates (except age) adjusted for age; cut-points based on medians in all MAP II controls

[3] Adjusted for case-control status

[4] Proportional Difference = difference between means [high - low (reference)] divided by low or mean in reference group x 100%

[5] Difference p-value for comparison of means (ANCOVA)

[6] Physical activity and total energy intake mutually adjusted for each other

[7] Nutrient values residual energy-adjusted + total energy intake covariate; cut-points based on sex-specific medians in all controls

Table 4. Adjusted associations[1] for TGF-α expression (staining optical density[2]) in the normal-appearing rectal mucosa of incident, sporadic colorectal adenoma cases and controls, MAP II

Case[3] (n = 29)	Control[3] (n = 31)	Proportional Difference %[4]	P_{diff}[5]	Odds Ratio[6] (95% Confidence Interval)	Model Covariates[7]
1.10 (0.14)	0.80 (0.14)	37.5	0.14	2.26 (0.91-5.64)	NSAID-use
1.22 (0.14)	0.74 (0.14)	64.9	0.02	2.83 (1.11-7.24)	total energy intake
1.41 (0.17)	0.88 (0.15)	60.2	0.01	3.00 (1.17-7.70)	family history of CRC
1.39 (0.16)	0.84 (0.15)	65.5	0.01	3.75 (1.18-11.98)	NSAID-use, energy, family history of CRC

[1] Adjusted for risk factors or potential confounders (Table 1, Table 3)

[2] Batch-standardized as individual value divided by batch mean; full crypt values

[3] Mean staining densities and standard errors

[4] Difference between means (cases - controls) divided by mean in controls x 100%

[5] Difference p-value for comparison of means (ANCOVA)

[6] Association between TGF-α expression (as a continuous variable) and adenoma (relative odds of being a case)

[7] All estimates adjusted for age and sex and other covariates as specified; total energy intake modeled as a continuous variable

Phenotypic expression of inflammation and growth mediators COX-2, TGF-β_1, and TGF-α in human rectal mucosa

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ABSTRACT

The role of TGF-α and TGF-β_1 in the gastrointestinal response to injury and inflammation and their interaction with COX-2 signaling in colorectal carcinogenesis is becoming more evident. We measured expression of COX-2, TGF-β_1, and TGF-α in biopsies of normal-appearing rectal mucosa using automated immunohistochemistry and quantitative image analysis in a subsample of participants from a colonoscopy-based case-control study (n=203) of biomarkers of risk for incident, sporadic colorectal adenoma. We found the ratio of TGF-α to TGF-β_1 (n=31 cases, 26 controls) to be 163% higher in cases than controls (p=0.04). There was no significant overall case-control difference in COX-2 (n= 33 cases, 32 controls) expression (3.1%; p=0.85), but stratified analyses suggested differential associations by NSAID-use (e.g., 15.6% higher COX-2 in cases relative to controls among those who did not take NSAIDs [p=0.47]). Expression of COX-2 and TGF-β_1 (n=30 cases, 29 controls) were directly correlated in cases (r^2=0.69). Expression of these markers was associated with colorectal cancer risk factors that may modulate inflammatory pathways: COX-2 was directly associated with polyunsaturated fat intake ($p<0.05$), TGF-β_1 was directly associated with the ratio of omega-6 to marine omega-3 fatty acid intake ($p<0.05$), and COX-2 expression was lower and TGF-β_1 higher among those with high calcium intakes. These preliminary findings 1) suggest that TGF-α, TGF-β_1, and COX-2, alone or in combination, may be useful as modifiable biomarkers of risk for colorectal neoplasms, 2) provide support for a role for these biomarkers in inflammation-mediated carcinogenesis in humans, and 3) support the need for a larger, confirmatory study.

BACKGROUND

In the United States, colorectal cancer is second only to lung cancer in total incidence and mortality (1). Non-steroidal anti-inflammatory drugs (NSAIDs) and aspirin, which target and suppress inflammation via the cylcooxygenase (COX)-2 pathway, are effective in preventing colorectal neoplasms (28-30), but comparatively little is known about what drives phenotypic variation in risk within the colon epithelium, which provides the physical barrier and first line of defense within the colon.

Autocrine/paracrine transforming growth factors (TGF)-α and TGF-β, modulators of growth and differentiation, interact with COX-2 signaling to maintain the structural integrity of healthy tissue despite constant cell turnover and exposure to environmental antigens (9, 11, 59-68). However, increased expression of COX-2 has been associated with both premalignant and malignant changes in the colorectal epithelium (57, 177) and TGF-α signaling may contribute to epithelial hyperproliferation and increased risk of malignancy in an errored attempt at epithelial regeneration in the face of mucosal injury and chronic inflammatory conditions (9, 11). Conversely, TGF-β, an early tumor suppressor and anti-inflammatory cytokine, may be a key regulator of COX-2 during periods of active inflammation and epithelial cell restoration (62, 70).

Previously, we reported that TGF-α may serve as a modifiable biomarker of risk for colorectal cancer, as expression of this pro-growth marker was higher in the normal-appearing colorectal mucosa of persons with adenoma (178). Given the complex interaction of factors involved in the development of colorectal neoplasms, it is unlikely that a single biomarker alone will progress research and prevention. To our knowledge, no other study has quantified the expression of all three proteins, COX-2, TGF-β_1, and

TGF-α, in the normal-appearing colorectal mucosa of persons at varied risk for colorectal cancer. To begin to address this need, we investigated phenotypic expression of these potential markers involved in the regulation of growth and inflammation in rectal biopsies of normal-appearing mucosa in persons with or without incident sporadic colorectal adenoma.

METHODS

Rectal biopsy samples were procured from participants of The Markers of Adenomatous Polyps II study (MAP II) (2002), a community- and colonoscopy-based, case-control study of incident sporadic colorectal adenomas described in detail elsewhere (178). In brief, the MAP II study was designed to investigate whether the expression patterns of various genes and cell cycle markers in normal-appearing rectal mucosa are associated with adenomas and thus be possible biomarkers of risk for colorectal neoplasms. Adults aged 30-74 were recruited upon referral for routine outpatient elective colonoscopy at Consultants in Gastroenterology, PA, a large private practice gastroenterology group in Columbia, SC. Subjects were excluded if they had previous adenomatous polyps, familial adenomatous polyposis, inflammatory bowel disease, incident colorectal cancer, or prevalent cancer other than non-melanoma skin cancer. Prior to undergoing colonoscopy, MAP II participants (n=203; 49 cases, 154 controls) completed mailed questionnaires eliciting self-reported demographics, medical history, anthropometrics, diet, and lifestyle characteristics. Colonoscopy of all participants was performed in the usual manner following a 12-hour fast and polyethylene glycol bowel cleansing preparation. All participants had six "pinch" biopsies taken from the normal-

appearing rectal mucosa (10 cm above the anus). No biopsies were taken within 4 cm of a polyp or tumor.

Laboratory Procedures: All biopsy specimens were fixed with 10% normal buffered formalin for 24 hours then stored in 70% ethanol. Within a week, the specimens were processed and embedded in paraffin blocks with three biopsies per colon site per block. The paraffin blocks were then cut into three micron thick sections with each level 40 microns apart. Five slides with four biopsy levels each were processed and stained within seven days of being cut, yielding a total of 20 biopsy levels per biomarker per patient. Using automated procedures, the slides underwent immunohistochemical processing and staining [described in detail elsewhere (178)] with COX-2 (Oxford PG27B, 1:200 dilution), TGF-β_1 (Santa Cruz sc-146, 1:75), or TGF-α antibody (Neomarkers MS1000P, 1:50).

Image Analysis: An image analysis procedure was used to quantify the optical density of the staining of the immunohistochemically detected markers in the epithelium of intact, full length colorectal crypts such that both the overall expression of the markers as well as their distributions along the crypt axis could be assessed. To do this we used our custom-designed plug-in into ImagePro Plus image analysis software (Media Cybernetics, Inc., MD), a light microscope, digital camera, and drawing board. The scoring and analysis unit was a hemicrypt, defined as one side of colonic crypt bisected from its base to its gut luminal surface. For each patient, a total of 32 intact hemicrypts (i.e., extending from the muscularis mucosae to the gut lumen) were analyzed from two

biopsies in a systematic and reliable process by a trained technician (intra-rater reliability, intra-class correlation $r \geq 0.9$) described in detail elsewhere (178).

Statistical Analysis: Cases included participants with pathology-confirmed, incident, sporadic colorectal adenomas. Participants free of adenoma upon colonoscopy were considered controls. Baseline characteristics for the MAP II study population and a usable subsample of participants for whom slides were immunohistochemically processed and analyzed for each marker were examined to assess the potential for confounding and comparability between the main study and biomarker subpopulation. Analysis of covariance (ANCOVA) was used for continuous variables and the Fisher's exact test or χ^2-test was used for categorical variables, as appropriate. All statistical analyses were conducted using SAS version 9.1 software (SAS Institute, Cary, NC).

The primary variable of interest was the staining optical density for biomarker expression within crypts of the rectal mucosa and its association with adenoma. Biomarker values were standardized for staining batch by taking the value in each individual divided by the mean of the staining batch in which the individual's sample was processed. Other methods for batch-adjustment considered included adjusting for staining batch as a fixed covariate or in mixed modeling procedures with batch treated as a random covariate. The mean marker amount refers to the overall expression across rectal crypts for each patient and was calculated by summing the staining densities from all analyzed crypts from the biopsy specimens and dividing by the number of crypts analyzed. From these means ratios of markers (i.e., mean expression of TGF-β_1 divided

by mean expression of COX-2) were also constructed based on relationships suggested by the literature.

We used ANCOVA methods to determine adjusted proportional differences (for age and sex) in mean marker amount and marker ratios, standardized for batch, in cases vs. controls. The odds ratio with 95 percent confidence interval was calculated as a measure of association of incident sporadic adenoma with continuous batch-standardized biomarker expression using standard logistic regression modeling methods for case-control studies. We also examined whether associations between biomarker expression and adenoma varied by NSAID-use (taken never to less than once per week vs. once per week or more).

Bivariate analyses to evaluate biomarker expression in the rectum according to diet and lifestyle characteristics were conducted in cases and controls separately and combined. We used ANCOVA to compare adjusted means for biomarker expression according to high vs. low levels of risk factors suspected to be involved in inflammation-driven colorectal carcinogenesis. All values were standardized for staining batch as described previously and adjusted for age, sex, energy (179), and case-control status as appropriate.

RESULTS

Selected characteristics of cases and controls among persons with usable COX-2 and TGF-β_1 samples are presented in Table 1 [characteristics of TGF-α sample presented in reference (178)]. On average, cases were more likely to be smokers, less likely to take NSAIDs regularly, and had higher total energy intake than controls. With the exception

of total energy intake and smoking status, none of these differences were statistically significant. Baseline characteristics in each biomarker subpopulation were comparable to those in all MAP II study participants (data not shown).

Correlations between markers in cases and controls are presented in Table 2. In cases, COX-2 and TGF-β_1 expression were significantly correlated (r^2=0.69). No other correlations were statistically significant. All correlations were positive and appeared to be relatively similar in cases vs. controls, with the exception of COX-2 and TGF-β_1, which were only weakly correlated in controls, as compared to cases.

Proportional differences in marker expression between cases and controls, and associations with adenoma are presented in Table 3. TGF-β_1 was proportionally 30% lower (p=0.28) and the ratio of TGF-α to TGF-β_1 was 163% higher in cases than controls (p=0.04). There was no apparent overall case-control difference in COX-2 expression (p=0.85). In cases compared to controls, the ratio of TGF-β_1 to COX-2 was 31% higher (p=0.59). Odds ratios (OR) for associations with adenoma suggested that the ratio of TGF-α to TGF-β_1 was directly associated with risk (OR: 1.40, 95% CI: 0.96-2.06).

Because of the potential for an overwhelming influence of NSAIDs on inflammation-related biomarkers, associations of the biomarkers with adenomas were examined according to NSAID use (data in text only). Associations of COX-2 with adenomas differed according to NSAID use; among never or rare NSAID-users, COX-2 expression was, proportionally, 16% higher in cases than controls, whereas among regular NSAID users, COX-2 expression was proportionally 18% lower. However, the sample sizes in the strata were small and neither association nor the test for interaction

was statistically significant. Among persons who did not take NSAIDs, TGF-α expression was proportionately 57.5% higher in cases (p=0.12), but among those who did take NSAIDs, there was no difference between cases and controls. No other associations appeared to vary by NSAID-use (data not shown).

In a sensitivity analysis adjusting for batch as a random effect, differences between cases and controls for COX-2 were slightly larger in magnitude, although in the same direction, compared to results presented here. When batch was included as a random covariate, COX-2 expression was proportionally 23% greater in cases compared to controls (p=0.25) and appeared to be directly associated with adenoma (OR: 1.32, 95% CI: 0.49-3.54; adjusted for age and sex; data in text only). Among never or rare NSAID-users, COX-2 expression was 29% higher in cases than controls (p=0.16), but there was no apparent difference between cases and controls among NSAID-users (p=0.89; data in text only). Adjustment for batch as a fixed covariate in COX-2 analyses and different batch-adjustment methods for TGF-α and TGF-β_1 produced results comparable in magnitude and direction to those presented here (data not shown).

When investigating the potential relationship between diet, COX-2, TGF-α, and TGF-β_1, we found statistically significant differences in biomarker expression across inflammation-related risk factors for colorectal cancer (data presented in text only). COX-2 expression was directly associated with intake of total and individual polyunsaturated fatty acids (PUFA) ($p<0.05$), regardless of batch-adjustment method. TGF-β_1 was also directly associated with the ratio of total omega-6 to marine omega-3 fatty acid intake ($p<0.05$). COX-2 was lower and TGF-β_1 was higher among persons with

high total calcium and low-fat dairy intake ($p<0.10$). The ratio of TGF-α to TGF-β_1 was higher among obese (BMI$\geq$30) persons and the ratio of TGF-β_1 to COX-2 was higher among non-drinkers ($p<0.10$).

DISCUSSION

Upregulation of COX-2 expression is a key oncogenic event in human colon neoplasia, typifying 85% of colon cancers and 50% of colon adenomas (180), and interventions to block COX action have been effective in preventing adenoma recurrence (28-30). However, in the MAP II study, expression of COX-2 did not differ substantially in the normal-appearing colorectal mucosa of persons with or without incident sporadic adenoma. However, there was some suggestion that this association may vary by NSAID-use with slightly higher expression in cases vs. controls who did not take NSAIDs. We previously reported that TGF-α expression was more than 50% higher ($p=0.05$) in cases compared to controls, but found no difference among those who took NSAIDs (20). The ratio of TGF-α to TGF-β_1 was directly associated with risk of adenoma, but the ratio of TGF-β_1 to COX-2 was relatively uninformative and suggested that other measures of their relationship in this population may be more appropriate. Our findings of associations of these markers with colorectal cancer risk factors that may modulate inflammatory pathways, such as polyunsaturated fatty acids and calcium (18, 81, 112), support a role for these biomarkers in inflammation-mediated carcinogenesis in humans.

A wide variety of growth regulatory and stimulatory signals may affect COX-2 signaling, including those of the transforming growth factor (TGF) and epidermal growth

factor pathway (59-67). We previously reported that TGF-α was significantly associated with risk of adenoma and NSAID-use in this population (178). TGF-α may serve an important modulatory function in regulating prostaglandin release under inflammatory conditions (69) and aspirin treatment has been shown to significantly reduce TGF-α expression in the colorectal mucosa (60). Conversely, growth regulatory proteins of the transforming growth factor-β family are one of the few classes of endogenous inhibitors of cell growth (41, 47) and also serve an important function as immunoregulatory cytokines (181). In our population, the ratio of TGF-α to TGF-β_1 was associated with higher risk, and expression of TGF-β_1 appeared to be lower in adenoma cases compared to adenoma-free controls.

The influence of cyclooxygenase-2 (COX-2) overexpression on the development of tumors has been well documented, but the underlying mechanism is still not completely understood. An escape of proliferating cells from the regulatory influence of TGF-β has been postulated, as well as a preponderance or prolongation of growth factor stimulation (59). Experiments in cell lines (59, 63) and mice (62) suggested that COX-2 and TGF-β_1 may play opposing roles in colorectal carcinogenesis and during periods of stress or inflammation (181). However, little or no research has been done in the normal-appearing rectal mucosa of free-living humans at varied risk for colorectal neoplasms and the positive correlation between COX-2 and TGF-β_1 expression in MAP II cases warrants further investigation. It is important to note that we specifically measured COX-2 and TGF-β_1 expression within the cells of normal-appearing colorectal crypts and not in transformed cells, stromal cells, or macrophages where expression of COX-2 and TGF-β_1 is also likely to vary. Additionally, NSAID use appears to modulate this

relationship in healthy human colon, potentially stimulating TGF-β_1 through its suppression of COX-2 (54), but we did not have sufficient sample size to definitively evaluate the ratio of TGF-β_1 to COX-2, or individual markers, by strata.

Potential associations with inflammatory risk factors, such as NSAID-use, obesity, calcium, and PUFA intake, support the role of all three of these markers in inflammation-mediated colorectal carcinogenesis. Environmental risk factors for colorectal cancer have been reviewed extensively (17), but few single interventions have been effective, with the exception of calcium (182, 183) and NSAIDs (28-30), whose proposed anti-carcinogenic properties include suppression of growth and inflammation (18). Associations with PUFAs are also biologically plausible, as they feed directly in to prostaglandin synthesis via COX-2, yet findings in epidemiologic studies have been inconsistent (101). Our results suggest that further investigations of COX-2, TGF-β_1, and TGF-α, coupled with the development of functional biomarkers of risk to facilitate observational studies of diet in free-living individuals, may clarify some of these issues.

The role of COX-2, TGF-β_1, and TGF-α in colorectal carcinogenesis and their potential collaboration in the regulation of cell turnover and the response to inflammation, is supported by a growing body of laboratory-based evidence, but more research is needed to clarify their potential in colorectal cancer prevention. Despite study limitations [described in reference (178)] and somewhat restrictive sample size, findings suggest that the expression of these proteins from the normal-appearing rectal mucosa may be useful in determining at-risk phenotypes for sporadic colorectal neoplasms and in the study of diet-colorectal cancer mechanisms. Larger samples, representative of the population at large, will be needed to validate these findings, particularly the potential

interactions between COX-2, TGF-β_1, and TGF-α in the normal-appearing colorectal mucosa, and their relationships with diet and lifestyle.

Table 1. Selected characteristics of participants with (cases) and without (controls) incident sporadic colorectal adenoma among persons with usable biomarker subsamples

	COX-2 sample			TGF-β_1 sample		
Characteristic[1]	**Cases n=33**	**Controls n=32**	***P*[2]**	**Cases n=44**	**Controls n=45**	***P*[2]**
Age at recruitment, mean yrs±SD	57.2	55.7	*0.5*	55.6	56.0	*0.8*
Male, (%)	54.6	46.9	*0.6*	50.0	55.6	*0.7*
Caucasian, (%)	97.0	96.9	*1.0*	95.5	97.8	*0.6*
Current smoker, (%)	21.2	9.4	*0.4*	25.0	4.4	*0.0*
College graduate, (%)	42.4	31.3	*0.2*	36.4	31.1	*0.8*
HRT use among women (%)	80.0	70.6	*0.8*	77.3	65.0	*0.7*
Consumes alcohol currently (%)	66.7	71.9	*0.8*	63.6	60.0	*0.8*
Regular NSAID use (%)	30.3	37.5	*0.7*	36.4	40.0	*0.8*
1st degree relative with history of CRC (%)	6.1	6.3	*0.8*	11.4	17.8	*0.8*
Recreational physical activity, mean METS	26.9	22.9	*0.4*	25.7	24.8	*0.8*
Body mass index (BMI), mean	31.8	31.3	*0.8*	31.2	31.4	*0.9*
Daily intakes[3], mean						
Total energy intake, kcal men/women	1,999.1	1,524.4	*<0.01*	1,921.1	1,620.4	*0.1*
Total calcium intake, mg	866.7	993.5	*0.3*	919.2	960.8	*0.7*
Total vitamin D intake, IU	360.3	415.6	*0.5*	330.7	374.3	*0.5*
Total omega-6 intake	12.5	12.1	*0.8*	12.3	13.5	*0.3*
Total marine omega-3 intake	0.2	0.3	*0.5*	0.2	0.3	*0.4*
Total omega-3 intake	1.4	1.5	*0.8*	1.4	1.7	*0.1*
Whole fruit & vegetable serv/day	3.5	3.6	*0.9*	3.5	3.5	*1.0*
Weekly intakes[3], mean						
Processed meat servings/wk	2.4	2.2	*0.7*	2.6	2.7	*0.8*
Red meat servings/wk	7.3	8.0	*0.8*	6.0	8.2	*0.4*
Low-fat dairy servings/wk	3.4	4.9	*0.3*	4.3	4.5	*0.8*

HRT=hormone replacement therapy, NSAID=nonsteroidal antiinflammatory drug, CRC=colorectal cancer, MET=metabolic equivalents

[1] Derived from questionnaire data

[2] Fishers exact for categorical variables; Pdiff (ANOVA) for continuous variables with adjustment for age

[3] Total nutrient intake (diet + supplements); all nutrients adjusted for total energy intake

Table 2. Correlations[1] among markers in incident, sporadic colorectal adenoma cases and adenoma-free controls[2], MAP II study

	CASES			CONTROLS		
	TGF-α	**TGF-β**	**COX-2**	**TGF-α**	**TGF-β**	**COX-2**
TGF-α	1.00	0.17	0.23	1.00	0.28	0.35
p		0.36	0.30		0.17	0.13
n	*31*	*31*	*22*	*29*	*26*	*21*
TGF-β	0.17	1.00	0.69	0.28	1.00	0.11
p	0.36		0.0001	0.17		0.57
n	*31*	*44*	*30*	*26*	*45*	*29*
COX-2	0.23	0.69	1.00	0.35	0.11	1.00
p	0.30	0.0001		0.13	0.57	
n	*22*	*30*	*33*	*21*	*29*	*32*

[1] Pearson correlation coefficients and corresponding p-values

[2] Case-control status diagnosed at study colonoscopy

Table 3. Marker expression (staining optical density[1]) in the normal-appearing rectal mucosa of incident, sporadic colorectal adenoma cases and controls, MAP II

Marker	N[2]	Marker Expression in Cases[3]	Controls[3]	Proportional Difference %[4]	P_{diff}[5]	Odds Ratio[6] (95% Confidence Interval)
COX-2	33, 32	1.01 (0.11)	0.98 (0.11)	3.1	0.85	1.08 (0.50-2.39)
TGF-β	44, 45	0.85 (0.23)	1.22 (0.24)	-30.3	0.28	0.83 (0.57-1.20)
TGF-α/TGFβ	31, 26	3.08 (0.61)	1.17 (0.66)	163.2	0.04	1.40 (0.96-2.06)
TGFβ/COX-2	30, 29	1.52 (0.47)	1.16 (0.48)	31.0	0.59	1.07 (0.85-1.34)

[1] Batch-standardized as individual value divided by batch mean; all estimates adjusted for age and sex

[2] Number of cases, controls in subsample

[3] Mean staining densities and standard errors

[4] Difference between means (cases - controls) divided by mean in controls x 100%

[5] Difference p-value for comparison of means (ANCOVA)

[6] Association between marker expression (as continous variable) and adenoma (relative odds of being a case); adjusted for age and sex

MAP II Study

APPENDIX TABLES

Table 1A. Selected characteristics of persons with (cases) and without (controls) incident sporadic colorectal adenoma, all MAP II participants

Characteristic[1]	Cases n=49	Controls n=149	*P*[2]
Age at recruitment, mean yrs	56.2	55.5	*0.6*
Current smoker (%)	22.5	14.1	*0.4*
Male (%)	44.9	50.3	*0.5*
HRT use among women (%)	77.3	62.7	*0.2*
Caucasian (%)	95.9	95.3	*0.8*
College graduate or higher (%)	36.7	34.2	*0.8*
Consumes alcohol currently (%)	65.3	59.1	*0.4*
NSAID use > once/week (%)	32.7	36.2	*0.6*
Aspirin use > once/week (%)	38.8	39.6	*0.7*
Family history of CRC (%)	14.3	19.5	*0.7*
Recreational physical activity, mean METs	24.8	24.9	*0.9*
Body mass index (BMI), mean kg/m^2	30.8	29.8	*0.4*
Waist to hip ratio (WHR), mean	0.9	0.9	*0.2*
Total energy intake, mean kcal	1,902.9	1,622.0	*0.0*
Total[3] calcium intake, mean mg	896.3	904.2	*0.9*
Total[3] vitamin D intake, mean IU	332.4	348.8	*0.7*
Total polyunsaturated fat, mean g	13.8	14.2	*0.7*
Total omega-6, mean g	12.4	12.6	*0.8*
Total[3] marine omega-3, mean g	0.2	0.2	*0.6*
Total[3] omega-3, mean g	1.4	1.5	*0.7*
Processed meat servings/wk, mean	2.7	2.4	*0.4*
Red meat servings/wk, mean	5.8	6.0	*0.9*
Low-fat dairy servings/wk, mean	4.3	4.0	*0.7*
Whole fruit & vegetable serv/day, mean	3.6	3.3	*0.3*

Abbreviations: HRT=hormone replacement therapy, NSAID=nonsteroidal antiinflammatory drug, CRC=colorectal cancer, MET=metabolic equivalents,

[1] Derived from questionnaire data

[2] Fishers exact for categorical variables; Pdiff (ANOVA) for continuous variables adjusted for age and total energy intake, as appropiate

[3] Total nutrient intake (diet + supplements) adjusted for total energy intake

Table 2A. Marker expression (staining optical density[1]) in the normal-appearing rectal mucosa of incident sporadic colorectal adenoma cases and controls stratified by NSAID-use, MAPII

Marker, NSAID-use	N[2]	Marker Expression in Cases[3]	Marker Expression in Controls[3]	Prop Diff %[4]	P_{diff}[5]	Odds Ratio[6] (95% CI)
TGF-α, no	21, 13	1.37 (0.19)	0.87 (0.24)	57.5	0.12	2.59 (0.81-8.26)
TGF-α, yes	10, 16	0.73 (0.17)	0.74 (0.14)	0.0	0.98	0.98 (0.20-4.89)
COX-2, no	22, 20	1.11 (0.14)	0.96 (0.15)	15.6	0.47	1.46 (0.54-3.93)
COX-2, yes	10, 12	0.82 (0.20)	1.00 (0.18)	-18.0	0.51	0.56 (0.11-2.89)

[1] Batch-standardized as individual value divided by batch mean; all estimates adjusted for age and sex

[2] Number of cases, controls in subsample

[3] Mean staining densities and standard errors

[4] Difference between means (cases - controls) divided by mean in controls x 100%

[5] Difference p-value for comparison of means (ANCOVA)

[6] Association between marker expression (as continous variable) and adenoma (relative odds of being a case); adjusted for age and sex

Dietary intake of omega-6 and omega-3 fatty acids and risk of colorectal cancer in a prospective cohort of U.S. men and women

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ABSTRACT

Background: Omega-6 and omega-3 fatty acid intakes may play opposing roles in inflammation-driven colorectal carcinogenesis, but results from previous epidemiologic studies have been inconsistent. We examined the relationship of these fatty acids and the ratio of their intake with colorectal cancer risk in a large U.S. prospective cohort. We additionally examined potential effect modification by nonsteroidal anti-inflammatory drug (NSAID) use.

Design: Participants in the Cancer Prevention Study (CPS) II Nutrition Cohort completed a detailed questionnaire on diet, medical history and lifestyle in 1999. Between 1999 and 2005, 778 incident colorectal cancer (CRC) cases (406 in men and 372 in women) were identified among 96,152 participants (41,364 men and 54,788 women). Multivariate-adjusted rate ratios (RR) were calculated using Cox Proportional Hazards models.

Results: The ratio of total omega-6 to total omega-3 fatty acids was not associated with CRC risk in either sex. However, among women, marine omega-3 intake, above the lowest quartile, was associated with lower risk [RRs: 0.70, 0.80, 0.81, respectively], particularly in women who did not use NSAIDs regularly (P interaction=0.14). Contrary to our initial hypothesis, total omega-6 intake was inversely related to CRC risk in men (RR and 95% Confidence Interval (CI) for highest to lowest quartile: 0.79; 95% CI: 0.59-1.06; P trend=0.07) and alpha-linolenic acid, the primary contributor to total omega-3 intake, was associated with increased risk in women (RR: 1.47; 95% CI: 1.07-2.01; P trend=0.10).

Conclusions: The ratio of omega-6 to omega-3 intake was not related to CRC risk in this cohort; however marine omega-3 and total omega-6 intake were related to lower risk of CRC in women and men, respectively. Differential associations by sex and NSAID use warrants further investigation.

INTRODUCTION

Colorectal cancer, the third most common cancer in U.S. men and women, is highly correlated with Western-style diet characterized by a constellation of dietary components including lower intakes of fruit and vegetables and higher intakes of red and processed meats, refined grains, sugars, fats, and a higher ratio of omega-6 to omega-3 fatty acids (2, 19-21). While collective epidemiologic research (17) does not corroborate the relationship between total fat intake and colorectal cancer suggested by ecologic comparisons, polyunsaturated fatty acids (PUFAs) are of particular interest due to their potential role in inflammation-driven colorectal carcinogenesis. Experimental studies report anti-inflammatory and anti-carcinogenic effects in the colon for omega-3 PUFAs [eicosapentaenoic (EPA), docosahexaenoic (DHA), and alpha-linolenic (ALA) acid] highest in fish and seed oils, and adverse effects for omega-6 PUFAs, [linoleic (LA) and arachidonic (AA) acid] found in commercially popular oils and animal products (106-117). Despite evidence supporting the omega-6 to omega-3 ratio as a biologically plausible target (37, 105, 110, 115, 117, 118), observational studies have generally not found an association with colorectal cancer (101) and prospective data for the ratio is sparse (119, 120).

Omega-3 fatty acids are hypothesized to reduce the risk of colorectal cancer by inhibiting the production of pro-inflammatory, omega-6 derived eicosanoids via the cycolooxgenase-2 (COX-2) enzyme (37, 104-106). Prospective studies and relatively short-term clinical trials have shown that omega-3 fatty acids, particularly the long-chain or marine fatty acids (DHA and EPA), decrease both biomarkers of inflammation (96, 121, 122), and rectal cell proliferation (108, 111-114). Such evidence, coupled with the efficacy of nonsteroidal anti-inflammatory drug (NSAIDs), strong COX inhibitors, to reduce risk of colorectal neoplasia (28-30, 123) supports the promise for omega-3 PUFAs in the prevention of colorectal cancer through modulation of similar mechanisms. An international review of prospective cohort studies investigating fish, omega-3 intake and colorectal cancer risk suggests sex and population-specific differences (130). Inconsistent findings and differential associations by sex for both omega-6 and omega-3 fatty acids are apparent across both biomarker- (131-133) and dietary assessment- (119, 134-138) based prospective studies.

With promising experimental evidence but current observational data largely inconclusive, we examined the association between colorectal cancer incidence and dietary intake of omega-6 and omega-3 fatty acids, both separately and in relation to one another with a ratio, among men and women from a large U.S. prospective cohort. To our knowledge, no large U.S. prospective cohort composed of both men and women has comprehensively evaluated these relationships. We evaluated potential effect modification by NSAID use as such drugs may circumvent the upstream effects of fatty acids in the inflammatory pathway. We hypothesized *a priori* that a higher ratio of omega-6 to omega-3 intake would be associated with increased risk of colorectal cancer

and we expected associations to be stronger in persons who did not take NSAIDs regularly.

MATERIALS & METHODS

Study cohort: Men and women in this analysis were drawn from participants in the Cancer Prevention Study II (CPS-II) Nutrition cohort, a prospective study of cancer incidence and mortality in the United States established in 1992 and described in detail elsewhere (184). At enrollment, participants completed a mailed self-administered questionnaire including information on demographic, medical, diet, and lifestyle factors. Follow-up questionnaires to update exposure information and to ascertain newly diagnosed cancers were sent in 1997, 1999, 2001, 2003, and 2005. Reported cancers were verified through medical records, registry linkage, or death certificates. The response rate for each follow-up questionnaire was at least 88%. The Emory University Institutional Review Board approves all aspects of the CPS-II Nutrition Cohort.

Follow-up for this analysis began on the date of completion of the 1999 follow-up questionnaire. The 152-item Food Frequency Questionnaire (FFQ) first administered in 1999 provided a more comprehensive assessment of the exposures of interest than the 68-item FFQ administered at enrollment in 1992 (184). A 90% response rate was achieved for overall follow-up in 1999. Of these (151,349) persons, 87% (58,555 men and 73,643 women) returned the full-length FFQ, while the remaining 13% completed a shorter follow-up questionnaire with no dietary information and were therefore excluded. Of those who returned the FFQ, we excluded participants who were lost to follow-up (4,805 men and 5,302 women), who had a history of colorectal cancer (1,599 men and 1,273

women) or cancer other than non melanoma skin cancer (9,605 men, 11,309 women) or who provided incomplete or improbable FFQ data (1,182 men and 971 women) as indicated by implausibly high or low total energy intake (800<men<4200; 600<women<3500). A total of 41,364 men and 54,788 women remained for analysis.

Follow-up for each subject began on the date of the returned 1999 survey and continued until the date of colorectal cancer diagnosis, the date of censoring due to loss to follow-up, death, report of a different cancer, or June 30, 2005, whichever came first. Individuals who self-reported colorectal cancer that could not be verified were censored at the last cancer-free survey.

Incident Colorectal Cancer: We identified and verified a total of 778 incident cases of colorectal cancer (406 in men, 372 in women) in the analytic cohort. Of these, 348 male cases and 304 female cases were initially identified by self-report (185) and subsequently verified by obtaining medical records (288 M, 254 F) or through linkage with state registries (60 M, 50 F) when complete medical records could not be obtained (184). An additional 110 cases (51 M, 59 F) were identified as primary or contributory deaths ascertained through computerized linkage with the National Death Index. The remaining cases (7 M, 9 F) were identified as a colorectal cancer case during verification of another reported cancer.

Of the 778 total cases, we identified 614 (311 male, 303 female) incident cancers of the colon (*International Classification of Diseases Oncology* codes: C18.0, C18.2-C18.9). Of these, 318 (151 M, 167 F) were identified as proximal (cecum to splenic flexure), 144 (74 M, 70 F) as distal (descending to sigmoid colon), 147 (83 M, 64 F) as unspecified colon, and 5 (3 M, 2 F) as overlapping lesions of the colon. We identified

156 (91 M, 65 F) cancers of the rectosigmoid junction (C19.9) or rectum (C20.9). Eight (4 M, 4 F) additional colorectal cases were of unknown subsite. Overlapping cases (11 M, 6 F) were counted only once in total cases.

Dietary Assessment: Diet was assessed in 1999 using a 152-item food frequency questionnaire (FFQ) (155). Similar versions of the FFQ have been validated for specific fatty acid values collected in comparable populations using biomarker and food-based comparison methods (186, 187). No information was collected on flax or fish oil supplements; thus only dietary intake was considered. Queries regarding the frequency of seafood intake included canned tuna fish, dark-meat fish (mackerel, salmon, sardines, bluefish, swordfish), other fish (cod, haddock, halibut), shellfish (shrimp, lobster, scallops, clams), and breaded fish (fish sticks, cakes, or pieces). The FFQ queried about fats usually used for frying or sautéing and baking, as well as type and brand of cooking oil and margarine/spread usually used at home with one open-ended question for each. Of those who indicated ever eating fried or sautéed food prepared at home, approximately one-third had invalid or missing information for cooking oil brand or type. Canola oil, the most commonly reported oil, was assigned as the default for missing values. Quartile classifications with and without individuals with unspecified cooking oil were nearly equivalent.

All individual fatty acids and other nutrient values were energy adjusted according to the residual regression method (159). Total omega-6 fatty acid intake is the sum of LA, AA, gamma-linolenic acid (GLA), and other minor omega-6's. Marine omega-3 fatty acid intake is the sum of values for DHA, EPA, and docosapentaenoic acid (DPA). Total omega-3 intake includes ALA, EPA, DHA, and DPA. We computed ratios

of omega-6 to omega-3 fatty acid intake for both total and marine omega-3 fatty acids, and categorized each variable into sex-specific quartiles.

Statistical Analysis: We conducted analyses separately for men and women, as there was a statistically significant interaction between most fatty acid exposures and gender in relation to colorectal cancer risk [p interaction 0.01-0.05, except marine omega-3 ($p=0.15$) and ratio of total omega-6 to total omega-3 ($p=0.40$)]. We used Cox proportional hazards models to estimate incidence rate ratios (RR) and 95% confidence intervals (CI) for colorectal cancer in relation to each main exposure and adjusted for age using the stratified Cox procedure with 1-year age strata. To test for any violation of the Cox proportional hazards assumption we examined the data both graphically and by creating interaction terms between all main effect variables and time. Statistical interaction and the Cox Proportional Hazards assumption were assessed using the likelihood ratio test (188). P values for linear trend were estimated by creating a continuous variable using the median value within quartiles. If results appeared to strongly diverge from a linear pattern, we further examined the possible non-linear relationship with restricted cubic splines (189). Tests for non-linearity used the likelihood-ratio test, comparing the model with only the linear term to the model with the linear and cubic spline terms. Each of these tests lacked statistical significance; therefore only the linear trend is presented. Results from two-sided chi square tests were considered statistically significant at $p \leq 0.05$. All analyses were conducted in SAS version 9.1 (SAS Institute, Cary, NC).

For each main exposure variable we constructed sex-specific quartiles and examined three models. Model 1 included age and total energy intake. Model 2 included

age, energy, and non-dietary risk factors for CRC. Model 3 included age, energy, non-dietary and dietary risk factors for CRC. We considered as potential confounders those factors identified *a priori* as established risk factors based on literature review and which were also associated with fatty acid intake in the analytic cohort. Non-dietary covariates presented in the final multivariate models included colorectal screening history (never or ever colonoscopy or sigmoidoscopy by 1999), body mass index (BMI; <25, 25-30, ≥30), hormone replacement therapy among women [HRT; never use, current estrogen replacement therapy (ERT), current estrogen/progesterone combined replacement therapy (CHRT), former use], recreational (moderate to vigorous) physical activity in metabolic equivalents (METs; in quartiles), and anti-inflammatory drug use (total of aspirin, NSAIDs and/or baby aspirin counted as ¼ of a pill; never, 1-14, 15-29, 30+ pills per month). Dietary covariates presented in the final multivariate models included red and processed meats, low-fat dairy, whole fruit and vegetable servings per day or per week (in sex-specific quartiles). Addition or substitution of other covariates, including alcohol, education, smoking history, multivitamin or supplement use, history of cholesterol-lowering drug use, history of diabetes, intakes (total or diet only) of calcium, vitamin D, fiber or folate, and fatty acids mutually adjusted for each other did not influence results.

We examined whether the association between fatty acid intake and colorectal cancer incidence varied by anti-inflammatory drug-use (defined as ≤ 15 pills per month vs. ≥ 30 pills per month). We also considered effect modification by body mass index (defined at BMI above or below median, 27.5), by colorectal screening history (defined as never vs. ever colonoscopy or sigmoidoscopy), and HRT use (defined as never vs. ever

use of ERT or CHRT). In further subanalyses, we examined associations between fatty acids and colorectal cancer after excluding individuals with missing cooking oil brand.

RESULTS

Table 1 shows the mean intakes and correlation coefficients between energy-adjusted values of the different polyunsaturated fatty acids. LA, the primary nutrient contributor to total omega-6 intake, was correlated with ALA, the primary nutrient contributor to total omega-3 intake (r^2=0.76). Marine-omega-3 intake was not correlated with total omega-6 intake (r^2=-0.05) or ALA intake (r^2=0.02). The top food contributors to omega-3 and omega-6 PUFA intake, i.e., contributing >5% of total intake from a single food item, are presented in Table 2. Salad dressing was the primary food contributor to total omega-6, ALA, and therefore total omega-3 fatty acid intake. Dark fish and canned tuna were the primary food contributors to marine omega-3 fatty acid intake. Lesser food sources differed for men and women.

The distributions of baseline characteristics and other dietary factors according to quartile of total omega-6 and quartile of marine omega-3 fatty acids are provided in Table 3. The mean age at baseline was 70 years in men [standard deviation (SD), 6] and 68 years in women (SD, 6). In both men and women, the range of total omega-6 intake varied 2-fold and marine omega-3 intake varied 4-fold between medians of extreme quartiles of intake (Table 3). In general, men and women with higher total omega-6, or lower marine omega-3 intake, were more likely to be overweight or obese and have a history of diabetes and less likely to be screened for colorectal cancer, college-educated, physically active, or to be taking multivitamins or cholesterol-lowering drugs. They also

generally had lower intakes of calcium, vitamin D, folate, fiber, fish, low-fat dairy, fruits and vegetables. Heavy alcohol consumption was lower among individuals with higher intakes of total omega-6 fatty acids. Total omega-3 intake, which we expected to be comparable to marine omega-3 intake, followed a less marked trend across risk factors with little difference by body size, multivitamin use, and nutrient intakes of folate, fiber, and vitamin D (not shown).

The associations between fatty acid intake and risk of colorectal cancer among women and men are provided in Tables 4 and 5, respectively. In both women and men, there was no association between the ratio of total omega-6 to total omega-3 intake and colorectal cancer risk. In women, higher total omega-6 intake and a higher ratio of total omega-6 to marine omega-3 intake was associated with an increased risk of colorectal cancer, evident primarily in the top quartile (age and energy-adjusted RR: 1.44; 95% CI: 1.09-1.91; P for trend = 0.004, RR: 1.36; 95% CI: 1.03-1.79; P for trend = 0.004, respectively), but statistically significant associations did not hold in models adjusted for dietary factors (P trend=0.10 and 0.12, respectively). In women, total omega-3, driven entirely by ALA intake, showed a statistically significant positive association with colorectal cancer for all intakes above the lowest quartile, which was only slightly attenuated following adjustment for dietary factors. Marine omega-3 intake was associated with an apparent non-linear reduction in colorectal cancer risk, beginning in the second quartile. However, neither the linear test for trend nor spline test for curvature (i.e., non-linear relationship) (189) was statistically significant and relative risks were somewhat attenuated with multivariate adjustment. Likewise, dark or fatty fish (including canned tuna) intake, above the lowest quartile, was associated with a

moderately lower risk of colorectal cancer in women [multivariate RRs (95% CI): 0.80 (0.59-1.09), 0.77 (0.58-1.03), 0.82 (0.59-1.12), once to thrice per month, once per week, more than once per week, respectively; data in text only].

Among men, no inverse associations between omega-3 intake and colorectal cancer risk were observed (Table 5). In contrast to our findings in women, the highest quartile of total omega-6 intake and the ratio of total omega-6 to marine omega-3 appeared to be inversely associated (RR: 0.79; 95% CI 0.59-1.06; P for trend=0.07, RR: 0.78; 95% CI 0.58-1.05; P for trend=0.09, respectively). Differential associations for omega-6 fatty acids in men and women were only apparent in the highest quartile of intake. These associations persisted despite adjustment for omega-3 intake (not shown).

We detected no statistically significant interactions with NSAID use among women or men. However, among women, there appeared to be some evidence of effect modification between marine omega-3 intake and NSAID use (p=0.14). In women who did not take NSAIDs regularly (<15 pills per month), marine omega-3 fatty acid intake was associated with a 30 to 40% reduction in risk for intakes above the lowest quartile [multivariate RRs (95% CI): 0.57 (0.40-0.80), 0.70 (0.50-0.96), 0.73 (0.52-1.01); P for trend=0.57; data in text only]. In women taking NSAIDs regularly (>30 pills per month), there was no apparent association with marine omega-3 fatty acid intake. Among men not taking NSAIDs regularly, the inverse association with total omega-6 was also stronger (multivariate RR: 0.67; 95% CI 0.46-0.97; P trend=0.04), whereas among men taking NSAIDs regularly, there was no association (P interaction=0.18; data in text only). No effect modification by body mass index, colorectal screening history, or HRT use was observed.

We repeated analyses excluding men and women with missing cooking oil brand or type. In both men and women, results were weaker and not statistically significant, but in the same direction, as one would expect with a reduction in sample size (data not shown). Results were also comparable in sensitivity analysis excluding potential influential or extreme intakes outside of two interquartile ranges.

DISCUSSION

In this large prospective cohort, we observed no association between the ratio of total omega-6 to total omega-3 intake and colorectal cancer risk, which may be due, in part, to unexpected findings for total omega-6 and total omega-3 intake and their differential associations in men and women. Intakes of even relatively low amounts of marine omega-3 intake were associated with a 30% lower risk of colorectal cancer among women not taking NSAIDs regularly. In contrast, ALA, the primary contributor to total omega-3 intake, was associated with increased risk in women. Total omega-6 intake appeared to be directly related to risk in women, but counter to our initial hypothesis was inversely related to risk in men. We cannot rule out chance as an explanation for our findings due to multiple associations examined and because associations were not strongly linearly related to risk.

Our findings suggesting marine omega-3 intake may lower colorectal risk in women are consistent with strongly supportive experimental data (108, 111-114), although we did not see this relationship in men. Findings from a recent meta-analysis of prospective cohort studies also found a moderate protective association (pooled RR: 0.78; 95% CI: 0.58-1.06) for fish intake in women, but a decidedly null association for men

(130). Of the seven prospective studies deriving marine omega-3 intake from food frequency questionnaires, two showed an inverse association (134, 136) while five found no association with colorectal cancer (120, 132, 135, 138) or adenoma (119). Two prospective studies measuring blood levels of marine omega-3 fatty acids found an inverse association with colorectal cancer in both sexes (131, 133). The small amount of data available from chemoprevention trials showed that fish oil supplementation decreases cell proliferation within the colorectal mucosa (108, 112-114), a potential marker of colorectal carcinogenesis and progression.

Despite evidence for COX-2 as a key target in colorectal cancer prevention and promising findings in NSAID intervention trials (28-30, 123, 177), anti-inflammatory drugs are not currently recommended to the general population for the prevention of colorectal cancer due to the potential risk of other adverse health effects (190). However, omega-3 fatty acids may share a potential mechanism with NSAIDs to lower the risk of colorectal cancer by inhibiting the production of pro-inflammatory cytokines through COX. Thus, we expected to see stronger associations for PUFA exposures among persons who did not use NSAIDs regularly and associations potentially masked in regular users. Our findings suggest that dietary intake of marine omega-3 fatty acids may potentially reduce the risk of colorectal cancer among women not taking NSAIDs regularly. Few other studies (119, 133, 134) tested the interaction between anti-inflammatory drug-use, marine omega-3 intake, and colorectal cancer risk and only one found that blood levels of marine omega-3 fatty acids were associated with lower risk of colorectal cancer in men not taking aspirin (P interaction = 0.04) (133).

Unexpectedly, we found total omega-3, driven by ALA intake, to be associated with increased risk of colorectal cancer among women and no association in men. While some experimental data (96, 105, 118) have supported the protective effects of both short and long chain omega-3 fatty acids, most observational studies have also found differential associations (101, 131, 139). The potentially inefficient metabolic conversion of nutritionally essential ALA to long chain omega-3's (103) coupled with the absence of "healthier" food sources rich in ALA, like flax, on the FFQ may make it more difficult to evaluate the potential benefits of ALA. Shared primary food sources, such as salad dressing and mayonnaise, and correlated nutrient intakes for ALA and LA have also been reported in other U.S. cohorts (147, 191). We encourage more research on this topic, particularly since ALA is currently publicized in popular media as a healthy source of omega-3 fatty acids to reduce risk of heart disease and some cancers.

We also found opposite associations in men and women for total omega-6 intake, primarily in the highest intake quartile. Among women, total omega-6 intake appeared to be directly related to colorectal cancer risk as hypothesized based on inflammatory mechanisms, but intake was inversely related to risk in men. A nested case-control study from the PHS cohort (133) and a prospective study among Japanese men (131) also observed non-significant inverse trends for blood levels of omega-6 fatty acids with colorectal cancer risk, while prospective cohorts using intake from dietary questionnaires generally found no association in men (120, 137, 138). Although eicosanoids derived from omega-6 fatty acids are generally pro-inflammatory, some derivatives of LA have been shown to have anticarcinogenic effects (192, 193).

Sex-specific differences in results raise important questions about the effect of estrogen from either endogenous or exogenous sources on fat metabolism and vice versa. Estrogen levels may lead to alterations in normal fatty acid metabolism through changes in fatty acid utilization and oxidation (92, 128, 145). In our female population, 98% were post-menopausal at baseline and 60% reported taking hormone replacement therapy (HRT) at some point in their lives. Estrogen synthesis in fat tissue also contributes considerably to circulating estrogen levels in post-menopausal women (194). In other studies, HRT has been shown to improve fatty acid profiles in postmenopausal women and, to some extent, attenuate the effects of diet on health outcomes (92, 128, 146). Intake of marine omega-3 fatty acids relative to omega-6 fatty acids may also affect endogenous estrogen production through mechanisms related to prostaglandin production and aromatase activity (106). Despite these hypotheses, we did not detect effect modification by HRT use or BMI.

We hypothesized that the ratio of intake of omega-6 to omega-3 fatty acids would be more relevant than independent measures of these fatty acid intakes given their competing roles in inflammatory pathways. When intake of omega-3 fatty acids is sufficiently high, they are preferentially metabolized by shared metabolic and COX enzymes leading to "competitive inhibition" with omega-6 metabolism. Similarly, high intakes of omega-6's, which are far more common in U.S. diets, can depress metabolism of omega-3 fatty acids, leading to an influx of the pro-inflammatory class of eicosanoids (21, 104-106). Five (115, 118, 139-141) of nine (119, 120, 142, 143) previous observational studies reviewed supported direct associations between a ratio of omega-6 to omega-3 fatty acids and colorectal cancer, but tended to use retrospective designs or

measured outcomes related to earlier phases of colorectal carcinogenesis, such as colorectal adenoma (140) or rectal cell proliferation (115, 118). Our lack of an association for the total ratio, and only weak associations for the omega-6 to marine omega-3 ratio, may be due to an insufficient range of exposure. In our U.S. population, marine omega-3 intake, the more bioavailable source of omega-3 fatty acids (103), was very low relative to intakes of omega-6 and ALA (short chain omega-3) fatty acids. The ideal omega-6 to omega-3 ratio to reduce risk of cardiovascular disease and cancer is unclear (109, 145-147). The typical Western diet contains 10-20 times more omega-6 than omega-3 polyunsaturated fatty acids. In contrast, human beings evolved on a diet with a ratio of ~1, and current recommendations extend from an "optimal range" of 1-4:1 to an "acceptable range" of 1-8:1 (21, 109). In our study, we were able to compare those with a ratio of 12:1 to those with a ratio of 7:1, a difference that may not be meaningful compared to the ratio limited to marine omega-3 only, which varied more than 5-fold in our population.

We attempted to address limitations of previous epidemiological studies of PUFAs and colorectal cancer. We were able to prospectively evaluate differential associations by sex suggested in the literature, as well as consider plausible confounders, effect modifiers, and the relative contributions of omega-6 and short (ALA) vs. long chain (marine) omega-3 fatty acids. Limitations of our study include the six year follow-up time and somewhat limited power in subanalyses. With the exception of marine omega-3, correlated nutrient intakes due to shared food sources, may have limited our ability to assess independent associations. Although the FFQ captures brands and has been validated for fatty acids using biomarkers (186, 187), polyunsaturated fatty acids are

derived from both endogenous and exogenous sources, suggesting a combination of dietary assessment and adipose tissue or blood levels may be optimal to address measurement error and risk of misclassification (195, 196).

Although the omega-6 to omega-3 ratio was not related to colorectal cancer risk in this cohort, we observed an inverse association between marine omega-3 intake and colorectal cancer risk in women, which was stronger among non-NSAID users. These findings seem promising, but it is unclear whether ALA, and therefore total omega-3 intake, has similar effects on risk. Furthermore, opposing associations for omega-6 fatty acids in men and women raise important questions regarding sex-specific differences in fatty acid metabolism and potential confounding by diet patterns and lifestyle choices. Differential findings by sex and NSAID use warrant further investigation.

Table 1. Mean intakes and correlations between polyunsaturated fatty acids in the CPSII Nutrition Cohort, 1999

Variable[1]	**total PUFA**	**total ω-6**	LA	AA	GLA	**total ω-3**	ALA	**marine ω-3**	DHA	EPA	DPA
Mean intakes (g/day)	*11.50*	*11.12*	*9.91*	*0.09*	*0.01*	*1.20*	*1.06*	*0.19*	*0.12*	*0.06*	*0.02*
Correlation[2]											
total PUFA	1.00	0.98	0.99	0.15	0.09	0.74	0.79	-0.004	0.003	-0.01	-0.02
total ω-6	0.98	1.00	0.98	0.15	0.09	0.69	0.76	-0.05	-0.04	-0.06	-0.04
LA	0.99	0.98	1.00	0.12	0.07	0.69	0.76	-0.05	-0.05	-0.06	-0.06
AA	0.15	0.16	0.12	1.00	0.38	0.16	0.06	0.37	0.40	0.30	0.42
GLA	0.09	0.09	0.07	0.37	1.00	0.13	0.06	0.23	0.23	0.22	0.32
total ω-3	0.74	0.69	0.69	0.16	0.13	1.00	0.94	0.35	0.35	0.35	0.27
ALA	0.79	0.76	0.76	0.06	0.06	0.94	1.00	0.02	0.02	0.010	0
marine ω-3[3]	-0.004	-0.05	-0.05	0.37	0.23	0.35	0.02	1.00	0.99	0.98	0.84
DHA	0.003	-0.04	-0.05	0.40	0.23	0.36	0.02	0.99	1.00	0.94	0.82
EPA	-0.01	-0.06	-0.06	0.30	0.22	0.34	0.01	0.98	0.94	1.00	0.83
DPA	-0.02	-0.04	-0.06	0.42	0.32	0.27	0	0.84	0.82	0.83	1.00

[1] PUFA, polyunsaturated fatty acids; LA, linoleic acid; AA, arachidonic acid; GLA, gamma linolenic acid; ALA, alpha-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; DPA, docasapentaenoic acid

[2] Pearson correlation coefficients; all correlations are significant ($P<0.01$) with the exception of total marine ω-3 & DHA with total PUFA, ALA with DPA

Table 2. Top food item contributors (>5%) to nutrient value for daily intake in the CPSII Nutrition Cohort, 1999

	WOMEN		MEN	
Nutrient	Food	%	Food	%
Total ω-3	salad dressing	10.87	salad dressing	11.04
	walnuts	8.28	mayo, regular	6.86
	mayo, regular	7.23	dark fish	6.09
	dark fish	6.19	walnuts	5.87
	margarine	5.32	margarine	5.66
Short chain ω-3 (ALA)	salad dressing	12.62	salad dressing	12.88
	walnuts	9.61	mayo, regular	7.99
	mayo, regular	8.38	walnuts	6.85
	margarine	6.07	margarine	6.48
	cabbage/coleslaw	5.24		
Long chain, marine ω-3	dark fish	39.22	dark fish	38.34
	canned tuna	20.1	canned tuna	20.08
	other fish, light	17.52	other fish, light	17.39
	chicken/turkey	6.22	shellfish	5.11
	shellfish	5.20		
Total ω-6	salad dressing	10.57	salad dressing	9.77
	margarine	5.32	peanut butter	5.38
			margarine	5.19

Table 3. Baseline characteristics[1] of CPSII women and men by extreme quartile (Q) of dietary intakes of omega-6 and marine omega-3 fatty acids, 1999

	WOMEN				MEN			
	total ω-6		**marine ω-3**		**total ω-6**		**marine ω-3**	
	Q1	Q4	Q1	Q4	Q1	Q4	Q1	Q4
Median intake (g/day) =	*7.4*	*13.9*	*0.07*	*0.31*	*8.5*	*15.7*	*0.08*	*0.33*
Age at 1999 interview, mean years	68.5	68.4	68.9	67.9	70.2	70.0	70.3	69.8
Caucasian (%)	97.9	97.5	98.2	96.8	98.3	97.7	98.2	97.3
College education or higher (%)	36.7	30.3	25.3	43.6	55.3	48.3	39.3	64.3
Family history of colon or rectal cancer (%)	13.1	14.0	13.6	13.6	11.5	12.9	12.0	11.9
Overweight, 30>BMI ≥25 (%)	30.2	31.8	32.6	29.5	43.1	45.2	45.4	43.9
Obese, BMI ≥30 (%)	12.8	18.8	16.7	13.9	12.6	15.6	15.6	12.0
Recreational physical activity, mean METs	16.0	13.9	13.1	17.3	19.3	17.1	16.1	20.3
Never use HRT (%)	37.0	35.5	39.3	33.1	-	-	-	-
Current ERT/CHRT use (%)	44.1	45.7	42.1	47.5	-	-	-	-
Colonoscopy/sigmoidoscopy screening (%)	61.4	57.8	55.0	64.1	68.4	65.2	61.5	72.0
NSAID use <15 pills/mo. (%)	66.0	65.7	65.8	66.5	61.3	63.4	63.1	62.7
NSAID use ≥30 pills/mo. (%)	24.8	25.3	25.5	23.7	30.3	28.0	29.6	27.9
Cholesterol-lowering drug use (%)	20.8	18.5	18.4	20.7	30.5	24.9	24.1	32.7
History of diabetes (%)	5.2	8.6	6.9	6.5	8.0	13.7	10.5	10.9
Current smoker (%)	3.8	5.3	5.7	3.1	3.6	4.7	5.5	2.3
Alcohol consumption ≥2 drinks/day (%)	9.3	3.8	4.9	6.5	22.6	8.9	14.1	14.8
Multivitamin use (%)	60.9	54.9	53.4	63.4	53.3	48.8	45.4	56.0

[1] All values (except age) were standardized to the age distribution of the study population. All nutrients were adjusted for total energy intake.

[2] Abbreviations: BMI, body mass index; MET, metabolic equivalents; HRT, ERT, CHRT, hormone (estrogen or combination) replacement therapy; NSAID, nonsteroidal anti-inflammatory drug; total refers to intake from diet + supplements

Table 3. Continued

	WOMEN				MEN			
	total ω-6		**marine ω-3**		**total ω-6**		**marine ω-3**	
	Q1	Q4	Q1	Q4	Q1	Q4	Q1	Q4
Mean intakes of:								
total energy intake (kcal/day)	1,583.6	1,621.9	1,619.0	1,573.9	1,874.0	1,894.5	1,909.5	1,856.3
total vitamin D (IU/day)	453.2	364.6	350.2	494.1	429.9	356.6	331.5	478.8
total calcium (mg/day)	1,422.5	1,125.1	1,200.7	1,349.2	1,011.4	829.6	888.6	949.8
total folate (mcg/day)	674.8	581.8	583.0	678.1	662.2	572.9	564.2	671.5
total fiber - AOAC (g/day)	19.1	17.2	17.3	19.4	19.4	18.0	17.7	20.2
red & processed meat (servings/wk)	4.5	6.5	6.1	4.7	6.3	8.8	8.5	6.6
dark/fatty fish (servings/wk)	0.9	0.8	0.3	1.8	0.9	0.8	0.2	1.8
low-fat dairy (servings/wk)	11.1	5.9	8.3	8.1	9.7	5.9	7.8	7.5
fruits, not including juice (servings/day)	1.9	1.4	1.6	1.8	1.9	1.4	1.5	1.8
vegetables, not including potatos (servings/day)	3.3	3.2	2.8	3.8	3.0	2.9	2.5	3.5
Cooking oil usually used at home (%):								
olive oil	18.6	10.8	9.9	21.0	19.9	10.2	9.9	20.3
canola oil	30.9	29.9	28.6	31.4	26.7	26.3	25.3	26.5
corn oil	10.1	13.9	13.7	9.3	11.2	16.2	15.2	12.0
vegetable/soybean oil	9.0	13.7	14.3	8.4	7.9	12.7	12.2	7.0
missing/unclassifiable	31.5	31.7	33.5	29.9	34.3	34.7	37.4	34.2
Mean % of total energy intake from PUFA	4.2	8.4	6.1	6.0	4.4	8.4	6.3	6.1

[1] All values (except age) were standardized to the age distribution of the study population. All nutrients were adjusted for total energy intake.

[2] Abbreviations: BMI, body mass index; MET, metabolic equivalents; HRT, ERT, CHRT, hormone (estrogen or combination) replacement therapy; NSAID, nonsteroidal anti-inflammatory drug; total refers to intake from diet + supplements

Table 4. Age-, energy-, and multivariate-adjusted RRs and 95% CIs for incident colorectal cancer associated with omega-6 and omega-3 fatty acid intake, CPSII Nutrition Cohort Women (1999-2005)

Nutrient	*cases*	RR[1]	95%CI	P_{trend}[4]	RR[2]	95%CI	P_{trend}[4]	RR[3]	95%CI	P_{trend}[4]
Total ω-6 (g/day)										
Q1 (<8.4)	*82*	1.00			1.00			1.00		
Q2 (8.4-<10.0)	*81*	1.00	0.74-4.37		1.00	0.74-1.36		0.95	0.69-1.29	
Q3 (10.0-<12.1)	*92*	1.15	0.85-1.54		1.14	0.85-1.54		1.03	0.76-1.40	
Q4 (≥12.1)	*117*	1.44	1.09-1.91	*0.004*	1.41	1.06-1.87	*0.007*	1.22	0.91-1.64	*0.10*
Total ω-3 (g/day)										
Q1 (<0.93)	*71*	1.00			1.00			1.00		
Q2 (0.93-<1.13)	*96*	1.35	0.99-1.83		1.37	1.01-1.87		1.36	1.00-1.86	
Q3 (1.13-<1.38)	*102*	1.43	1.06-1.94		1.47	1.09-1.99		1.43	1.05-1.95	
Q4 (≥1.38)	*103*	1.43	1.05-1.93	*0.04*	1.46	1.08-1.97	*0.03*	1.40	1.03-1.91	*0.08*
α-linolenic (g/day)										
Q1 (<0.78)	*66*	1.00			1.00			1.00		
Q2 (0.78-<0.95)	*103*	1.56	1.14-2.12		1.57	1.15-2.14		1.54	1.13-2.10	
Q3 (0.95-<1.19)	*96*	1.43	1.05-1.96		1.44	1.05-1.97		1.38	1.01-1.90	
Q4 (≥1.19)	*107*	1.57	1.16-2.14	*0.03*	1.57	1.16-2.14	*0.03*	1.47	1.07-2.01	*0.10*
Marine ω-3 (g/day)										
Q1 (<0.10)	*117*	1.00			1.00			1.00		
Q2 (0.10-<0.14)	*87*	0.67	0.50-0.89		0.69	0.52-0.93		0.70	0.52-0.94	
Q3 (0.14-<0.23)	*85*	0.73	0.55-0.96		0.78	0.59-1.04		0.80	0.60-1.06	
Q4 (≥0.23)	*83*	0.69	0.52-0.91	*0.16*	0.76	0.57-1.02	*0.46*	0.81	0.60-1.09	*0.76*
Total ω-6/total ω-3										
Q1 (<7.6)	*83*	1.00			1.00			1.00		
Q2 (7.6-<8.7)	*100*	1.22	0.91-1.63		1.20	0.90-1.61		1.13	0.84-1.52	
Q3 (8.7-<10.0)	*93*	1.16	0.86-1.55		1.12	0.83-1.51		1.01	0.74-1.38	
Q4 (≥10.0)	*96*	1.21	0.90-1.62	*0.30*	1.15	0.85-1.54	*0.52*	1.00	0.73-1.37	*0.77*
Total ω-6/marine ω-3										
Q1 (<42.1)	*86*	1.00			1.00			1.00		
Q2 (42.1-<70.4)	*77*	0.89	0.65-1.21		0.87	0.64-1.18		0.83	0.60-1.13	
Q3 (70.4-<115.0)	*89*	1.02	0.76-1.37		0.97	0.72-1.31		0.89	0.66-1.21	
Q4 (≥115.0)	*120*	1.36	1.03-1.79	*0.004*	1.26	0.95-1.66	*0.02*	1.12	0.84-1.50	*0.12*

[1] Age- and energy-adjusted model

[2] Multivariate-adjusted model controlling for age, energy, HRT (in women only), recreational physical activity, NSAID use, colorectal screening, BMI

[3] Multivariate-adjusted model controlling for age, energy, HRT (in women only), recreational physical activity, NSAID use, colorectal screening, BMI, red & processed meat, low-fat dairy, fruit & vegetable intake

[4] P for trend assessed by chi-square test for linear trend

Table 5. Age-, energy-, and multivariate-adjusted RRs and 95% CIs for incident colorectal cancer associated with omega-6 and omega-3 fatty acid intake, CPSII Nutrition Cohort Men (1999-2005)

Nutrient	*cases*	RR[1]	95%CI	P_{trend}[4]	RR[2]	95%CI	P_{trend}[4]	RR[3]	95%CI	P_{trend}[4]
Total ω-6 (g/day)										
Q1 (<9.7)	*99*	1.00			1.00			1.00		
Q2 (9.7-<11.5)	*113*	1.14	0.87-1.50		1.12	0.85-1.46		1.09	0.83-1.43	
Q3 (11.5-<13.8)	*106*	1.07	0.81-1.41		1.04	0.79-1.36		0.99	0.75-1.31	
Q4 (≥13.8)	*88*	0.89	0.67-1.18	*0.30*	0.84	0.63-1.13	*0.16*	0.79	0.59-1.06	*0.07*
Total ω-3 (g/day)										
Q1 (<0.99)	*103*	1.00			1.00			1.00		
Q2 (0.99-<1.20)	*116*	1.12	0.86-1.46		1.13	0.87-1.48		1.12	0.86-1.46	
Q3 (1.20-<1.47)	*92*	0.88	0.67-1.17		0.89	0.67-1.18		0.88	0.66-1.17	
Q4 (≥1.47)	*95*	0.91	0.69-1.20	*0.25*	0.91	0.69-1.21	*0.26*	0.89	0.67-1.18	*0.19*
α-linolenic (g/day)										
Q1 (<0.82)	*97*	1.00			1.00			1.00		
Q2 (0.82-<1.00)	*118*	1.21	0.82-1.58		1.20	0.92-1.57		1.19	0.91-1.56	
Q3 (1.00-<1.25)	*94*	0.96	0.72-1.27		0.94	0.71-1.25		0.92	0.69-1.23	
Q4 (≥1.25)	*97*	0.98	0.74-1.30	*0.46*	0.95	0.72-1.26	*0.34*	0.92	0.69-1.23	*0.25*
Marine ω-3 (g/day)										
Q1 (<0.10)	*95*	1.00			1.00			1.00		
Q2 (0.10-<0.16)	*103*	1.18	0.90-1.57		1.23	0.93-1.62		1.23	0.93-1.63	
Q3 (0.16-<0.25)	*120*	1.17	0.89-1.53		1.26	0.96-1.65		1.27	0.97-1.67	
Q4 (≥0.25)	*88*	0.98	0.73-1.31	*0.66*	1.10	0.82-1.48	*0.99*	1.12	0.83-1.51	*0.82*
Total ω-6/total ω-3										
Q1 (<8.2)	*90*	1.00			1.00			1.00		
Q2 (8.2-<9.4)	*101*	1.13	0.85-1.50		1.10	0.82-1.46		1.08	0.81-1.43	
Q3 (9.4-<11.0)	*122*	1.37	1.05-1.80		1.31	0.99-1.72		1.27	0.96-1.69	
Q4 (≥11.0)	*93*	1.05	0.79-1.40	*0.70*	0.99	0.74-1.32	*0.98*	0.96	0.71-1.30	*0.76*
Total ω-6/marine ω-3										
Q1 (<44.5)	*99*	1.00			1.00			1.00		
Q2 (44.5-<72.7)	*100*	1.01	0.77-1.33		0.96	0.73-1.27		0.94	0.71-1.24	
Q3 (72.7-<116.7)	*115*	1.16	0.89-1.52		1.06	0.81-1.40		1.03	0.78-1.36	
Q4 (≥116.7)	*92*	0.92	0.69-1.22	*0.55*	0.81	0.61-1.08	*0.14*	0.78	0.58-1.05	*0.09*

[1] Age- and energy-adjusted model

[2] Multivariate-adjusted model controlling for age, energy, recreational physical activity, NSAID use, colorectal screening, BMI

[3] Multivariate-adjusted model controlling for age, energy, recreational physical activity, NSAID use, colorectal screening, BMI, red & processed meat, low-fat dairy, fruit & vegetable intake

[4] P for trend assessed by chi-square test for linear trend

CHAPTER 3

CONCLUSIONS AND FUTURE DIRECTIONS

The long, latent nature of colorectal cancer presents many challenges for prospective and clinical investigations attempting to address the role of diet and lifestyle in risk and prevention. Identifying at-risk molecular phenotypes is critical to advance the understanding of how colorectal cancer develops and how to target risk at a reversible stage. Early proliferative and inflammatory changes in the normal colorectal mucosal crypt epithelium appear to precede or at least accompany the development of polyps or cancer and, thus, may have value as a predictive or diagnostic marker (161-166). However, there are relatively few human studies attempting to address these issues, particularly the role of diet and lifestyle in these changes. Environmental risk factors for colorectal cancer have been reviewed extensively and many of the most salient are related to growth and inflammatory mechanisms (14, 67, 194).

In the MAP II study, expression of TGF-α, an autocrine/paracrine growth factor of the EGF family (33, 38, 148, 149), was statistically significantly higher in normal-appearing rectal tissue from adenoma cases compared to adenoma-free controls, and strong, direct associations with adenoma persisted despite adjustment for potential confounders. Expression of COX-2, a potential marker of inflammation, did not differ significantly in the normal-appearing colorectal mucosa of persons with or without incident sporadic adenoma. However, there was some suggestion that any possible association of COX-2 expression in normal colon mucosa with adenomas may vary by NSAID use. Although the literature suggested that COX-2 and TGF-β_1 may act as promoters and inhibitors of inflammation, respectively, their expression was positively

correlated in MAP II cases. However, little or no research has been done in the normal-appearing human rectal mucosa and associations suggested by the literature may only be translatable to stressed or inflamed tissue during progressive or advanced stages of colorectal carcinogenesis. In this population where we investigated early, at-risk phenotypes for adenoma, the ratio of TGF-β_1 to COX-2 was relatively uninformative and suggested that other measures of their relationship may be more appropriate, but more research in normal colon is needed. The ratio of TGF-α to TGF-β_1, early promoters and inhibitors of growth, respectively, was directly associated with risk of adenoma, although not as strongly associated with risk as TGF-α alone. Associations of these markers with colorectal cancer risk factors that may modulate inflammatory pathways, such as NSAID use, polyunsaturated fatty acids and calcium (18, 81, 112), further support their role in inflammation-mediated carcinogenesis.

While our findings are preliminary and require further validation, they are the most supportive for TGF-α as a modifiable biomarker of risk for colorectal cancer. Results suggest that notably higher TGF-α expression within the normal-appearing colorectal mucosa may indicate an at-risk phenotype, which may also be modulated by risk factors or behaviors believed to be important for the prevention of colorectal neoplasms. The literature also supports a role for TGF-α in early inflammatory changes in the colon mediated by the action of COX-2 (9, 11, 60, 61, 65, 66). However, COX-2 expression from the same population did not differ significantly in cases versus controls, although both markers appeared to be modulated in some manner by NSAID use. Additionally, the colonoscopy-based design of our study may have attenuated true differences, as both cases and controls were derived from persons referred for

colonoscopy, and thus more likely to be similar, at higher risk, and more knowledgeable of the postulated associations with diet and lifestyle factors, such as NSAID-use. However, our sample size was not large enough to adequately address a potential interaction with NSAID-use or multiple confounding effects of diet and lifestyle.

Our results further suggest the possibility that TGF-α signaling may be involved in the progression of normal colorectal mucosa to adenoma, but this was not directly addressable by our study design and requires further confirmation. Associations of TGF-α with adenoma and diet and lifestyle seem promising and informative of the potential mechanisms involved and our study is one of the few, if not the only, study to investigate and report on some of these biologically plausible relationships in free-living humans. Preliminary findings will require validation in larger samples and we encourage further research to clarify whether TGF-α expression and the expression of other markers in colorectal crypts is predictive of relevant precancerous changes leading to the appearance of colorectal neoplasms.

Given the complexity of colorectal carcinogenesis, it is unlikely that any single biomarker will stand alone to progress colorectal cancer research and prevention. The role of COX-2, TGF-β_1, and TGF-α in colorectal carcinogenesis and their potential collaboration in the regulation of cell turnover and the response to inflammation is supported by a growing body of laboratory-based evidence, but more research in humans is needed to establish their potential in colorectal cancer prevention. Specifically, larger samples, representative of the general population, will be needed to validate our findings, particularly the potential interactions among COX-2, TGF-β_1, and TGF-α in the normal colon, and their relationships with diet and lifestyle.

Ecologic comparisons and experimental evidence suggest that a high omega-6 to omega-3 ratio, a component of the Western-style diet, is associated with increased risk of colorectal neoplasms via pro-inflammatory and pro-carcinogenic mechanisms (21, 109), yet very few large U.S. studies, particularly of prospective design, have investigated this exposure in relation to colorectal cancer (101). In our analysis from a large, U.S. prospective cohort, we observed no association between the ratio of omega-6 to omega-3 intake and colorectal cancer risk. This may be due, in part, to unexpected findings for total omega-6 and total omega-3 intake and differential associations in men and women. In addition, stronger associations may have been masked in both studies by regular NSAID use in 25-30% of this population. Intakes of even relatively low amounts of marine omega-3 intake were associated with lower risk of colorectal cancer in women, particularly among irregular or rare NSAID users. In contrast, total omega-3 intake, driven entirely by ALA, was associated with increased risk in women. Total omega-6 intake appeared to be directly related to risk in women, but counter to our initial hypothesis was inversely related to risk in men. These findings seem promising for marine omega-3 intake, but it is unclear whether ALA, and therefore total omega-3 intake, is similarly associated with risk. Furthermore, opposing associations for omega-6 fatty acids in men and women raise important questions regarding sex-specific differences in fatty acid metabolism and potential confounding by diet patterns and lifestyle choices.

Endogenous turnover of polyunsaturated fatty acids (Figure 2 and Figure 3, Chapter 1), which may or may not be reflected in the colon tissue itself, may challenge observational studies utilizing only the FFQ to assess dietary exposures. Smaller studies,

similar in scale to the MAP II analyses, have attempted to study these exposures in relation to colorectal cancer with multiple biomarker measures. One small study (total n=61) measured and compared PUFA profiles in plasma phospholipids and the colorectal mucosa of patients with colorectal cancer (n=22), sporadic adenoma (n=27), and normal colon (control, n=12). Paired biopsy samples were taken from the normal-appearing and diseased mucosa of patients with polyps or cancer. There were no differences in PUFA profiles from both plasma phospholipids and normal mucosa between adenoma and control patients, but there were considerable differences in PUFA profiles between diseased and paired normal mucosa of adenoma patients. A stepwise reduction of EPA concentrations in diseased mucosa from benign adenoma to the most advanced colon cancer was also found ($p = 0.009$). The results from that study did suggest that changes in tissue marine omega-3 (EPA), and possibly other PUFAs, might participate in premalignant and malignant changes during human colorectal carcinogenesis (118), but these differences were not detectable in normal-appearing mucosa from persons with and without adenoma. In another study, 52 cases and 57 controls completed a FFQ, underwent a full colonic examination, and had a fat biopsy taken from the buttocks. The tissue level ratio of marine omega-3 to AA was inversely associated with adenoma (p-trend = 0.002), but the same ratio and the ratio of total omega-6 to total omega-3 intake as assessed by the FFQ was not. Associations for individual PUFAs, as assessed by both FFQ and levels in adipose tissue, were in the direction expected, although not statistically significant (140). No differences by sex were noted in either study. Biomarkers of intake or exposure appear to be useful, but nearly all options are “short-term” in comparison to the long-latent precancerous state of colorectal cancer, and are more likely to reflect

transient nutrient levels than a dietary pattern followed over the past several years. This may explain why both biomarker- (131-133) and dietary assessment- (119, 134-138) based prospective studies have not found consistent associations for PUFAs.

Observational studies may also not be able to capture a sufficient range of PUFA intakes to modulate inflammatory pathways or risk within the colon, as compared to experimental data. Marine omega-3 fatty acid trials to reduce either generalized inflammation (121) or rectal cell proliferation (108, 111-114) provided study participants with far higher dosages of fish oils than are found in the normal U.S. diet. For example, one study administered 800mg/day of DHA to decrease IL-6 levels in blood (121) and another administered 2 g/day of EPA to significantly increase EPA levels in the colorectal mucosa with a subsequent reduction in proliferation and increased mucosal apoptosis (112). Levels this high are rarely to never seen in the dietary intakes captured by dietary questionnaires in U.S. observational studies. For example, in our study less than 1% of the analytic cohort reported consuming more than one gram of total marine omega-3 fatty acids per day through diet; however, we still saw a moderate decrease in risk across relatively low intakes. Although information on fish oil supplements was not collected, their use was likely to be rare in 1999, particularly given the limited evidence for their use available at the time. Dietary intakes of ALA appear to be much greater than marine omega-3 intakes in the U.S., but ALA conversion to EPA and DHA is relatively inefficient and may not provide an adequate source of bioavailable omega-3 (103). Additionally, many foods that contribute to ALA intake are even higher in omega-6 fatty acids. Omega-6 rich foods and oils are common in U.S. diets and intakes commonly exceed 10 g/day; therefore, omega-6 metabolism and eicosanoid production is likely to

out-compete the relatively low intakes of marine omega-3 fatty acids and ALA in this pathway without heavy omega-3 supplementation and/or a reduction in the intake of omega-6. Perhaps this is why few large observational studies in the U.S. have found strong associations for individual PUFAs or their ratios with colorectal cancer (133, 134, 136) despite supportive experimental data. In contrast to the current Western diet that contains 10-20 times more omega-6 than omega-3 polyunsaturated fatty acids, human beings evolved on a diet with a ratio of ~1. Current recommendations to reduce cardiovascular disease extend from an "optimal range" of 1-4:1 to an "acceptable range" of 1-8:1 (21, 109), but the ratio necessary to reduce colorectal cancer risk remains undecided. In our study, we were able to compare those with a ratio of 12:1 to those with a ratio of 7:1, a difference that may not be meaningful compared to the ratio limited to marine omega-3 only, which varied more than 5-fold in our U.S. population.

Differential findings by sex and/or NSAID use warrant further attention and investigation, as differences in risk documented in ours and other large prospective studies (119, 130-138) do not appear to be an issue in smaller, short-term studies of randomized design evaluating the effects of polyunsaturated fatty acids (108, 111-114). Neither the biomarker case-control study, nor the prospective cohort study, was immune to issues of confounding factors and potential interactions, many of which had not been considered or addressed in previous studies. Correlated nutrients due to shared food sources, multiple interactions (sex, NSAID-use), and potentially confounding diet and lifestyle patterns presented challenges in both the small case-control and larger cohort study. Effects of medications (NSAIDs and HRT) are likely to be stronger modifiers of risk than any single dietary factor and these strong effects may mask the weaker effects

of normal dietary intakes (92, 128, 133, 146). However, trial dosages of NSAIDs or aspirin (81mg per day or more) shown to attenuate COX-2 or TGF-α expression within the colon (60, 177), or to mask effects of marine omega-3 intakes on risk of colorectal cancer (133), are likely to be higher than the doses reported and observed in either study presented here where interactions were not statistically significant. Despite these potentially strong pharmacologic effects on risk, the more "subtle" effects of diet and lifestyle remain very important, as it may not be plausible or safe to recommend use of medications or high-dose supplements to the general population. On the other hand, if biomarkers of risk became clinically applicable, tailored recommendations for individuals may be available in the future. Potential interactions between various recommendations (diet, hormones, and other pharmaceuticals) could be either positive or negative and more research is needed to clarify these issues.

Both studies broach different issues and challenges with temporality. We cannot say in the biomarker study whether expression of any of these markers ultimately will lead to a colorectal neoplasm or colorectal cancer itself. However, knowing whether or not one is at increased risk based on evaluation of normal-appearing tissue is several crucial steps ahead of the status-quo: screening for visible lesions. We also cannot say in the cohort study whether long-term diet assessed in 1999 is representative of the relevant etiological time period for the development of colorectal cancer or of the dietary intakes that lead to clinically detectable colorectal cancer. Large prospective studies are challenged by the lengthy follow-up time needed to adequately assess the exposure-disease relationship, as well as the measurement of long-term diet. The "ideal" prospective cohort design to study polyunsaturated fatty acids and colorectal cancer risk

would be comprised of longer follow-up time beginning with younger persons (less than 50 years of age), repeated dietary assessments, coupled with repeated biomarker measures of fatty acid intakes (in blood and tissue) and blood profiles including markers of inflammation. More temporally practical ways of assessing risk and carcinogenic progression within the colon environment, where the earliest indicators of risk may be detected, are also likely to improve research in this area. Results illuminating potential associations between markers of growth and inflammation in the colon with diet and lifestyle risk factors suggest that larger studies to assess biomarkers of risk may elucidate poorly understood diet-cancer mechanisms. In the future, a validated panel of biomarkers for colorectal cancer could also serve as an end point in relatively short-term prospective studies and chemoprevention trials to investigate promising diet and lifestyle exposures and to make vital progress in colorectal research and prevention.

Despite various limitations, findings and challenges discussed here provide direction for future research and study design and contribute to colorectal cancer research and prevention with 1) a translational investigation of early changes in risk within the colon that potentially reflect inflammation and growth-mediated colorectal carcinogenesis, and 2) further evaluation of a biologically plausible dietary exposure related to these mechanisms in a U.S. cohort followed for colorectal cancer. As other studies have shown, measuring biomarkers of inflammation, rectal cell proliferation, and, perhaps in the future, more specific validated markers of growth and inflammatory activity, such as TGF-α or COX-2 within the colon itself, may help bridge the gap between small-scale laboratory and experimental studies to larger population-based studies investigating multiple diet and lifestyle factors. Integrating traditional dietary

assessment methods with biomarkers of exposure from blood or tissue for nutrients such as polyunsaturated fatty acids, which are derived from both endogenous and exogenous sources, may certainly be more optimal than FFQs alone for addressing measurement error or misclassification (195, 196), and to determine categories of exposure that are biologically meaningful. As a whole our findings support the need for new, integrative methods that may allow researchers to detect risk at its earliest stage and/or improve investigations of long-term, cumulative exposures in the face of other confounding and interacting factors. In the near future, combining biomarker and traditional methods into larger prospective studies of diet and cancer, perhaps within a validation subsample, or in the design of small, short-term chemoprevention trials, may help validate or refute these findings and would continue to be useful and informative in the study of promising, biologically plausible, dietary exposures for colorectal cancer chemoprevention.

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