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Myfanwy H. Hopkins Date

Circulating Biomarkers of Inflammation and Oxidative Stress: Associations with Risk Factors for Colorectal Neoplasms and Response to Micronutrient-Based Chemopreventive Interventions

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B. S., Emory University, 2006

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An Abstract of

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ABSTRACT

Circulating Biomarkers of Inflammation and Oxidative Stress: Associations with Risk Factors for Colorectal Neoplasms and Response to Micronutrient-Based Chemopreventive Interventions

By Myfanwy H. Hopkins

Colorectal cancer (CRC), the second leading cause of cancer deaths in the U.S., is a disease highly correlated with excess adiposity, smoking, and a Western-style diet. Twenty-fold variations in international CRC rates and migration studies showing high acquired risk within one generation, emphasize the importance of environmental exposures in the etiology of CRC, and thus its preventability. It is now clear that increased inflammation and oxidative stress play a major role in colon carcinogenesis, basic science indicates antioxidants may reduce oxidative stress, and dietary antioxidants, calcium and vitamin D may play important roles in reducing inflammation in the colon. In addition to preventative strategies, treatable biomarkers of risk analogous to cholesterol in relation to hearth disease are needed to quickly and accurately assess the effectiveness of preventative strategies. This dissertation investigates associations of biomarkers of inflammation and oxidative stress with risk factors for CRC, and modulation of the markers by micronutrient-based chemopreventive interventions in patients at higher risk for developing CRC.

In a randomized, double-blind, placebo-controlled clinical trial of antioxidant micronutrient supplementation in colorectal adenoma patients, significant reductions in oxidative stress and inflammation biomarkers were found in non-smokers, but were found to increase in smokers. In a randomized, double-blind, placebo-controlled trial investigating the effects of calcium and/or vitamin D_3 on biomarkers of inflammation in colorectal adenoma patients, a combined inflammation z-score was reduced with vitamin D_3 or calcium supplementation. In addition, significant associations were found between several biomarkers of inflammation and increased levels of adiposity in colorectal adenoma patients.

The results of this dissertation indicate that in those at risk for developing CRC, 1) an antioxidant micronutrient cocktail can reduce biomarkers of inflammation and oxidative stress in non-smokers, 2) vitamin D or calcium can modulate biomarkers of inflammation, and 3) these biomarkers of inflammation are associated with increased general and central adiposity. The results of this dissertation warrant further investigation of these circulating inflammation and oxidative stress biomarkers for use as pre-neoplastic biomarkers of risk for CRC, and for use in assessing the efficacy, safety, and optimum dose range for chemopreventive interventions.

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TABLE OF CONTENTS

ILLUSTRATIONS AND TABLES

LIST OF ABBREVIATIONS

AA	Arachidonic acid
APC	Adenomatous polyposis coli
ATBC	Alpha-Tocopherol and Beta-Carotene Cancer Prevention Study
BMI	Body mass index
CARET	Carotene and Retinol Efficacy Trial
CaSR	Calcium sensing receptor
COX-2	Cyclooxygenase-2
CRP	C-reactive protein
Cys	Cysteine
CySS	Cystine
EGF	Epidermal growth factor
FAP	Familial adenomatous polyposis
F2-iP	F_2-isoprostane
GSH	Glutathione
GSSG	Glutathione disulfide
HNPCC	Hereditary non-polyposis colon cancer
HPLC	High performance liquid chromatography
NHANES	National Health and Nutrition Examination Survey
NSAID	Non-steroidal anti-inflammatory drugs
IBD	Inflammatory bowel disease
IGF-1	Insulin-like growth factor-1
IL-6	Interleukin-6
IL-1β	Interleukin-1β

IL-8	Interleukin-8
IL-10	Interleukin-10
IOM	Institute of Medicine
LCA	Lithocholic acid
MMR	Mismatch repair pathway
NF-κB	Nuclear factor-κ B
RDA	Recommended daily allowance
RONS	Reactive oxygen and nitrogen species
SELECT	Selenium and Vitamin E Cancer Prevention Trial
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor-α
VDR	Vitamin D receptor
WHR	Waist-to-hip ratio

CHAPTER 1. BACKGROUND

Colorectal Cancer Epidemiology

Colorectal cancer is the second leading cause of cancer deaths in the U.S. in both men and women combined, and now accounts for more deaths than heart disease in people under the age of 85 (1-3). Incidence rates of colorectal cancer have declined modestly over the past two decades, attributable to screening by colonoscopy and removal of colorectal polyps. However, in people under the age of 50 at average risk, for whom screening is not recommended, incidence rates continue to increase.

World-wide, colon cancer incidence rates vary 20-fold, with the highest rates observed in the U.S., Europe, Australia, and New Zealand, and the lowest rates observed in parts of Asia, South America, and Africa (4). Migration studies show that when moving from an area of low risk to high (Western) risk, incidence rates increase within one generation (3). Also, while incidence rates have remained steady or slightly decreased in the US over the last 30 years, they have moderately increased in Europe and greatly in Asia (5). This indicates the importance of environmental exposures such as dietary habits and lifestyle in the etiology of colorectal cancer.

Colorectal cancer is described by three specific patterns: sporadic, inherited, and familial. Sporadic colorectal cancer accounts for about 70% of cases, occurring in patients with no family history of the disease (6). Familial cases account for approximately 30% of colorectal cancer cases, and have some family history of the disease. In 3% of cases a germline mutation in the DNA mismatch repair pathway results in hereditary non-polyposis colon cancer (HNPCC), and <1% of cases arise from a

germline mutation in the *APC* tumor suppressor gene that results in familial adenomatous polyposis (FAP) (7).

In addition to genetic predisposition, there are other several risk factors for colorectal cancer that are unavoidable, such as increasing age, personal history of the disease, and inflammatory conditions such as Crohn's disease and ulcerative colitis (1, 8, 9). Incidence rates increase sharply after age 50, regardless of sex or ethnicity (10). In North America, Australia, and other areas of high incidence rates, rates of colon cancer are slightly higher in men than women (3, 11). In the U.S., colon cancer rates also tend to differ by race, with higher rates of incidence and mortality in black men and women than in whites, and lower rates are Asian and Native Americans, with the lowest rate in Hispanics (10).

There are also several risk factors for colorectal cancer that can be modified. Colorectal cancer risk is associated with smoking (12), increased adiposity (13), high intake of red meat and sugar (14), and high total energy intake (15). Decreased colorectal cancer risk is associated with physical activity (14), NSAID use (16), high intake of fruits and vegetables (17), high intake of calcium (18), and high serum vitamin D (19).

The only reliable method for diagnosing and removing colon adenomas, or polyps (early pre-cancerous lesions) (Figure 1), is colonoscopy. Removal of adenomas can prevent cancer, but these patients have a 40-500% increased risk of developing colorectal cancer in the future (20).

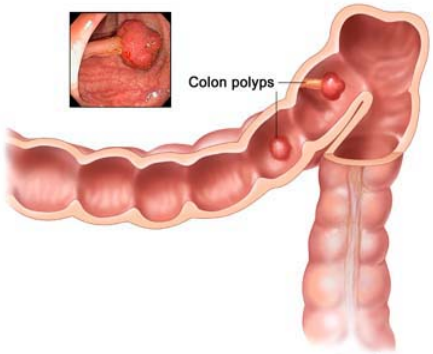

Figure 1. Colon polyp Morphology. From the National Cancer Institute at the National Institute of Health, 2011.

Previous colorectal adenoma remains the only reliable biomarker of risk for colorectal cancer. However, colonoscopy is an expensive, invasive, and poorly tolerated procedure. More precise, better tolerated screening and detection methods are needed. Particularly, treatable biomarkers of risk, akin to treating high blood pressure to reduce heart disease risk, would greatly improve the ability to assess the effectiveness of preventative interventions.

Colon Morphology

The colon epithelium is comprised of deep invaginations called colon crypts, making up the main morphologic units of the colorectal mucosa. At the base of the crypt, colonic stem cells proliferate and the daughter cells differentiate as they migrate upwards towards the luminal surface epithelium (21). The stem cells regenerate a new layer of surface epithelium cells every 4-8 days. At the top of the crypt, differentiated cells or damaged stem cells, undergo a tightly regulated apoptotic mechanism.

The colon epithelium is under a constant barrage of foreign material, and is an important barrier between the damaging bile acids, antigens, and foodstuffs traversing the colon (22). Tight regulation of proliferation, differentiation, and apoptosis are exceedingly important in this environment. The colon also provides an important barrier between the microflora inhabiting the gut, and the immune system. A certain degree of immuno-tolerance is required to avoid a constant reaction to benign food or beneficial micro-organism antigens. A delicate balance is required to avoid chronic inflammation, inflammatory bowel disease, or neoplasia (23).

Molecular Mechanisms of Colon Carcinogenesis

Colon carcinogenesis is driven by two major pathways, which may operate separately or in conjunction. The first involves tumor suppressor *APC*, known as the "APC Pathway" and accounts for nearly 80% of sporadic colorectal cancer cases, as well as all familial adenomatous polyposis (FAP) cases (24-27). Patients with FAP are born with one inactivated or mutated copy of *APC*, and must only acquire one mutation in the second allele for the development of colorectal neoplasm. In sporadic cases, however, two inactivating mutations, either somatic or epigenetic, must be obtained for colorectal neoplasm development.

The second main pathway for colorectal carcinogenesis involves the mismatch repair pathway (MMR), predominately *MSH2* and *MLH1* (28). In hereditary non-polyposis colon cancer (HNPCC) the affected person is born with an inactivating mutation in one of the MMR genes. In about 15% of sporadic colon cancer, the affected person will acquire an inactivating mutation in either *MSH2* or *MLH1*, by somatic mutation or epigenetic silencing, predominantly the latter.

The protein product of *APC* normally activates the degradation of β-catenin and prevents its translocation to the nucleus (29). APC also regulates E-cadherin, a calcium-dependent cell adhesion transmembrane protein necessary for proper colon cell crypt structure and function. E-cadherin plays a significant role in regulating adhesion dependent growth inhibition, and regulation of cell differentiation (30). In the absence of APC, β-catenin is allowed to translocate to the nucleus and aid in up-regulation of c-myc and cyclin D, promoting entry of colonocytes into the proliferation stage of the cell cycle,

while decreasing differentiation and apoptosis (31). The loss of adhesion dependent cell inhibition, alterations in cell proliferation patterns, and decreasing differentiation and apoptosis are hallmarks of the progression from the normal colon crypt to colon carcinogenesis.

Although inactivation of these major pathway genes, *APC* or MMR, will lead to aberrant growth and the development of polyps, other genetic alterations are necessary for the progression to carcinogenesis. Inactivation of tumor suppressor genes, *p53* and *p21*, is necessary to avoid differentiation or apoptosis, while activation of c-myc, and K-ras, or some other tumor promoting, pro-growth genes are necessary for sustained cell proliferation (32). Apoptosis is further avoided by increased apoptosis inhibitor (bcl-2) expression, while decreasing apoptosis promoter (bax) expression.

Several lines of research evidence indicate that colorectal neoplasia is mediated at least in part through chronic inflammation. A defect in the epithelial colon barrier (*e.g.*, induced by bacteria, an autoimmune process, or bile acids) may result in local irritation that produces a focal inflammatory response that activates cyclooxygenase-2 (COX-2) and generates prostaglandins from arachidonic acid (AA). Products of COX-2 stimulate epithelial cell proliferation and generate oxidative stress that is in turn mutagenic and mitogenic leading to the promotion of carcinogenesis (33, 34).

Inflammation regulation plays an important role in colon carcinogenesis prevention, in particular, suppressing COX-2 activity has been consistently linked to reduced colorectal neoplasia (35-39). Non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit COX-2, reduced the occurrence and caused the regression or disappearance of adenomas in FAP patients (35-37), and selective COX-2 inhibitors (celecoxib and

rofecoxib) reduced sporadic adenoma recurrence in three large trials (40, 41). While the use of NSAIDs have shown preventative efficacy, increased risk of cardiovascular events makes the widespread use of these drugs inadvisable (42, 43).

Oxidative stress is commonly defined as "a disturbance in the pro-oxidant/antioxidant balance in favor of the former"(44). According to the prevailing view, oxidative stress occurs as a result of damage induced by free radicals or reactive oxygen and nitrogen species (RONS) (45-50). Recently, the theory of oxidative stress was refined to account for an alternative mechanism—a disruption of thiol-redox circuits, which leads to aberrant cell signaling and dysfunctional redox control without invoking RONS-induced macromolecular damage (51, 52). This distinction between free radical (RONS) chemistry in purified systems and in biological systems may be critical in understanding oxidative stress because biological systems may generate more non-radical oxidants than free radicals. Damage caused by RONS to lipid molecules can be indicated by measuring F_2-isoprostane (F2-iP), a prostaglandin-like compound synthesized from arachidonic acid through free-radical catalyzation in a process called peroxidation (53, 54). While F2-iP provides useful information about RONS-mediated oxidative stress, it may not capture the whole story. Plasma redox status, measurable by the ratios of glutathione to glutathione disulfide (GSH/GSSG) and cysteine to cystine (Cys/CySS), gives an indication of non-RON induced damage and disruption of thiol-redox circuits (55).

A key molecular link between inflammation and tumor development is nuclear factor-kappa B (NF-κB). NF-κB DNA binding is regulated by intracellular thiol/disulfide balance, and is activated by many pro-inflammatory cytokines, including

interleukin-1-beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α) (56). NF-κB has an oxidatively regulated DNA binding domain, and induces the transcription of inflammatory cytokines, anti-apoptotic proteins, and oxidative stress responsive enzymes that together promote cellular transformation and tumor formation (57).

The molecular mechanisms involved in colorectal carcinogenesis provide targets for interventions, as well as potential treatable biomarkers of risk. Understanding the "usual suspects" and primary pathways for neoplastic transformation allows for better detection of adenoma risk. Using validated, treatable biomarkers of risk could lead to a quick, effective way to evaluate chemoprevention strategies, giving data on patient response even before the development of adenoma.

Inflammation, Oxidative Stress, and Colorectal Cancer

A major risk factor for colorectal cancer is inflammatory bowel disease (IBD), such as Crohn's disease or ulcerative colitis (8, 9). There is currently no known genetic basis for this association: increased risk is associated with longer duration of IBD, greater extent of the inflamed area, and the presence of other inflammatory conditions. Further strengthening the association between inflammation and colorectal cancer, is the reduction in risk observed with treatment by non-steroidal anti-inflammatory drugs (NSAIDs) (35-37, 40, 41, 58-60).

Several studies have linked inflammatory cytokines and oxidative stress with colorectal adenomas, colorectal cancer, and other risk factors associated with both.

Chronic inflammation and oxidative stress are thought to play a crucial role in carcinogenesis by promoting DNA mutation and modification, promoting cell proliferation, and inhibiting cell differentiation and apoptosis (61, 62). Also, activated immune cells release RONS, which induce genomic mutations, and enhance DNA methylation (62). This dissertation focuses on the inflammatory cytokines most strongly supported as risk factors for colorectal cancer, as well as two oxidative stress biomarkers indicative of oxidative imbalance. This includes: C-reactive protein (CRP), an acute phase reactant during inflammation (63); tumor necrosis factor alpha (TNF-α), a pivotal pro-inflammatory cytokine closely linked to carcinogenesis and inflammatory bowel disease (61, 64); interleukin-6 (IL-6), a pro-inflammatory cytokine involved with chronic inflammation and colon carcinogenesis (62); interleukin-1β (IL-1β), a ubiquitous pro-inflammatory cytokine with multifactorial actions (65); interleukin-8 (IL-8), an anti-inflammatory cytokine with increased expression in colon cancer (66, 67); interleukin-10 (IL-10), an anti-inflammatory cytokine linked to inflammatory bowel disease (62); CySS, a cysteine disulfide, the most abundant plasma aminothiol; and F2-iP, a marker of lipid peroxidation.

Numerous studies have linked these cytokines to IBD, colon cancer, or the carcinogenic process or pathways. Serum levels of CRP, IL-6, and IL-8 were found to be elevated in colorectal cancer cases when compared to healthy controls, and increased with increasing tumor stage by TNM classification (68, 69). Another study of colorectal cancer patients done in Greece, also found significantly higher serum CRP, IL-6 and TNF-α in colorectal cancer patients in comparison to controls (70). In a case-control study, serum CRP, TNF-α, and IL-6 were found to be associated with colorectal

adenomas, as well as with risk factors for colorectal adenomas such as age, smoking and BMI (71). Two separate clinical trials found serum TNF-α levels to be correlated with a poorer prognosis, with significantly higher levels in colorectal cancer cases than controls (69, 72). TNF-α was also found to increase oxidative stress and nitric oxide production, directly contributing to the neoplastic transformation of tumor cells (72). Chung et al. found that IL-6 serum levels were significantly higher in colorectal cancer patients than controls (73). IL-6 was found to play a crucial role in the initiation and maintenance of inflammation in a mouse model of ulcerative colitis (74). Also, promoter polymorphisms leading to gene up-regulation in IL-8, IL-6, and TNF-α were linked to poor prognosis in colorectal cancer patients (75). Promoter polymorphisms in IL-1β leading to increased expression are linked to squamous cell carcinoma of the pharynx, gastric cancer, and ROS production and cell proliferation (76-78). In addition, IL-1β is involved in COX-2 activation and activates the Wnt cell cycle activation pathway, the primary pathway of colon cell proliferation (79). Kaler et al. found that IL-1β-induced WNT signaling could be moderated by vitamin D_3. IL-10 is closely linked with IBD; mice lacking this cytokine quickly develop IDB, but supplementation with vitamin D_3 ameliorated symptoms and blocked the progression of the disease (80).

CySS, the oxidized disulfide of the amino acid cysteine (Cys), plays a crucial role in intra- and extracellular redox homeostasis (52). A shift in the pool of available CySS indicates an increase in oxidation, while higher Cys indicates a more reduced state (81). High plasma CySS has been associated with several risk factors and comorbidities for colorectal cancer, such as obesity, older age, cardiovascular disease, diabetes, rheumatoid arthritis, and other inflammatory diseases (82).

As discussed previously, F2-iP is a marker of lipid peroxidation. It is hypothesized that colorectal neoplasia is induced, in part, through oxidative stress caused by high concentrations of bile acids and iron, which catalyze the formation of reactive oxygen species in the colon (83). If this mechanism is correct, than F2-iP may be a suitable biomarker of risk for colonic neoplasms.

Antioxidants and colon cancer

It has been suggested that about 70% of all colon cancer deaths in the United States are potentially avoidable by dietary modifications (84). Diets high in fruits, vegetables, and antioxidants have been associated with a lower risk of cancer (85), especially colorectal cancer (33, 86, 87). As recently reviewed (84), most, but not all, of the 22 cohort studies published to date found decreased risk of cancer with increased intake of fruit. Modern "Western" diets as compared to "healthy" dietary patterns are shown to increase circulating biomarkers of inflammation, such as CRP, TNF-α, and interleukins (88-92). In an animal model, several oxidative stress regulatory pathways, including the glutathione redox pathway, are disrupted with a "Western" style diet (93). "Western" style diets are characterized by high animal fat, high red meat, high sugar, low fiber, low fruit and vegetables, and low mineral and micronutrient content. In contrast, a "healthy" diet is characterized by lower amounts of sugar; higher amounts of vegetable fat, fiber, and fruits and vegetables; and adequate levels of minerals and micronutrients. "Western" diets are thought to contribute to oxidative stress, disruption of thiol-redox

balances through lipid peroxidation, and production of RONS, which can induce oxidative DNA and lipid damage (94).

While observational studies tend to support inverse associations of high fruit and vegetable diets (major sources of antioxidant micronutrients) with colorectal cancer, supplementation trials with individual or limited combinations of antioxidants have yielded mixed results. Colorectal epithelial cell proliferation, a potential biomarker of risk for colorectal cancer, was reduced by several antioxidant micronutrients, such as vitamins A, C, E and β-carotene (95-97). Antioxidants reduced circulating TNF-α levels in two randomized, placebo-controlled, clinical trials, one (n = 26) with lycopene, α-tocopherol, and β-carotene, and another (n = 50) with zinc (98, 99). In a long-term placebo-controlled clinical trial (n = 87), plasma cystine (CySS, an oxidative stress biomarker) concentration was decreased with vitamin C, vitamin E, and β-carotene treatment, as well as by zinc (100, 101). In a randomized, placebo-controlled clinical trial (n = 385), vitamin C and E treatment reduced plasma isoprostanes in obese patients with high baseline levels of isoprostanes (102). Conversely, in a large, randomized clinical trial, supplementation with β-carotene and vitamins C and E found no effect on adenoma recurrence (103), and in other large randomized clinical trials, high dose β-carotene supplements actually increased lung cancer incidence in smokers (104-106). This was found in the Alpha-Tocopherol and Beta-Carotene Cancer Prevention Study (ATBC) and the Carotene and Retinol Efficacy Trial (CARET), in which smokers were given a supraphysiological dose of β-carotene daily (20 mg/day plus α-tocopherol 50 mg/day in ATBC, 30 mg/day plus retinyl palmitate 25,000 IU/day in CARET) (104-106). These studies found that high doses of β-carotene daily increased lung cancer incidence

in the participants. In addition, in a large national study on vitamin E and/or selenium for the prevention of prostate cancer (SELECT), the incidence of prostate cancer was significantly higher in the group supplemented with vitamin E after 7-12 years of follow-up (107).

The protective and/or harmful effects of antioxidant supplementation may depend heavily on lifestyle factors, such as smoking, antioxidant combination, dose, form, or specific outcome of interest (*i.e.*, heart disease protection vs. cancer prevention). The abundance of negative outcomes from antioxidant supplementation limits future research in this area, particularly of very high dose β-carotene supplementation. Our study on colorectal adenoma patients provided a unique opportunity to investigate possible mechanisms for these unexpected outcomes of antioxidant supplementation by measuring the effects on biomarkers of inflammation and oxidative stress in regards to smoking status.

Vitamin D and Colon Cancer

Vitamin D (including vitamin D_2 and vitamin D_3) is a unique vitamin synthesized in the skin from 7-dehydrocholesterol with exposure to UV-B sunlight. Synthesized through exposure to sunlight, or obtained through the diet from fish, mushrooms, or supplements, pre-vitamin D, or 25-OH-vitamin D, is stable and can be measured in the blood (108, 109). 25-OH-vitamin D is currently the only reliable marker for vitamin D status, because the metabolically active form of vitamin D, 1α,25-$(OH)_2$-vitamin D, is tightly regulated and a poor indicator of vitamin D exposure (110). While studies of

dietary intake of vitamin D have been inconsistent, serum vitamin D has been consistently linked to lower risk for developing colorectal adenoma in a growing number of observational studies (111-113). While a consistent inverse association is seen with serum vitamin D level and adenoma risk, there is still uncertainty about what constitutes a "sufficient' serum level for this vitamin. The 2011 IOM report on vitamin D requirements states that brief sun exposure or supplementation with 6-800 IU of vitamin D per day is sufficient to reach an adequate serum concentration of 25-OH-Vitamin D of 20ng/ml (114). However, others hypothesize that optimal vitamin D status is only achieved at a serum concentration above 32ng/ml (115-117).

Vitamin D is most commonly known for its classical function related to calcium homeostasis, but vitamin D also acts a major autocrine/paracrine signal transduction mediator though the nuclear vitamin D receptor (VDR), as well as a few less-understood membrane receptors. The VDR is expressed in many human tissues, including the colon, and modulates more than 200 responsive genes with a wide array of functions including regulating cell proliferation, differentiation, and apoptosis; growth factor signaling; protection against oxidative stress; bile acid and xenobiotic metabolism; immunomodulation; cell adhesion; DNA repair; and angiogenesis (111, 117-119). The level of available, active 1α,25-$(OH)_2$-vitamin D, and hence, VDR activity, is tightly regulated by CYP27B1 and CYP24 enzymes, which, respectively, synthesize and degrade vitamin D (80, 120).

Several distinct mechanisms have been proposed to explain the protective effects of vitamin D for colorectal cancer, although these pathways may not be acting

independently, including, a) bile acid catabolism, b) direct effects on the cell cycle, c) growth factor signaling, and d) immunomodulation (121).

Bile acid hypothesis:

Vitamin D activation of the vitamin D receptor (VDR) induces expression of CYP3A4, a cytochrome P450 enzyme that detoxifies the bile acid lithocholic acid (LCA) in the intestine and liver (122). This decrease in bile acid concentration may reduce colonocyte DNA mutation, cell damage, and inflammation.

Direct effect on cell cycle hypothesis:

Vitamin D has been shown to influence cell proliferation, differentiation, and apoptosis, through both VDR dependent and independent mechanisms. 1α,25-$(OH)_2$-vitamin D binding of the VDR induces the transcription of the cyclin dependent kinase inhibitors, p21 and p27, which leads to reduced cell proliferation (123). VDR activation also leads to repression of pro-apoptotic protein BCL-2, and promotion of anti-apoptotic protein, Bax (124). Palmer et al. have shown that vitamin D promotes E-cadherin transcription, which aids in sequestering β-catenin in the cytoplasm and inhibiting β -catenin transcription of growth-promoting factors (123, 125). Vitamin D also can reduce expression of *c-myc*, *c-fos*, and *c-jun* oncogenes, and suppress telomerase (126-129).

Growth factor signaling hypothesis:

Vitamin D signaling is involved in many growth factor pathways related to colon carcinogenesis, including insulin-like growth factor (IGF-1), TGF-β, E-cadherin, and β-catenin. Vitamin D interferes with epidermal growth factor (EGF) signaling (126, 128, 129), reduces expression of insulin-like growth factor-1 (IGF-1) receptor (126),

and inhibits IGF-1 signaling generally (130). *In vitro* vitamin D increased the amount of active TGF-β. Also, 1α,25-$(OH)_2$-vitamin D_3 sensitized colon cancer cell lines to TGF-β growth inhibition, increased IGF-IIR expression, which increased activation of latent TGF-β, and, in combination with TGF-β, compared to 1α,25-$(OH)_2$-vitamin D_3 alone, reduced cell proliferation.

Immunomodulation hypothesis:

Vitamin D appears to have important effects on immunity and control of inflammation (120, 131-133) that may be relevant to colon carcinogenesis and prevention. The colon is a reservoir for microbes, and inflammation is an established risk factor for colorectal cancer. Various cell types involved in immunologic reactions express VDR and CYP27B1. Local 1α,25-$(OH)_2$-vitamin D synthesis in immune cells is considered critically important for regulating and controlling immune responses. The growing list of vitamin D responsive inflammation control genes includes multiple interleukins and TNFα.

Calcium and Colon Cancer

In addition to bone structure, calcium is an essential part of cell signaling as a second messenger, and plays an important role in cell proliferation and differentiation through the calcium sensing receptor (CaSR). Only 30% of dietary calcium intake is absorbed through active transcellular, and passive paracellular pathways (134). The remaining 70% binds to free bile acids and fatty acids in the colon, precipitating and expelling them from the colon (135).

There are several proposed mechanisms by which calcium may help protect against colorectal cancer. Proposed mechanisms include: protection of colonocytes from the damaging effects of bile acids and free fatty acids (136), promotion of colonocyte differentiation (134), and modulation of E-cadherin and β -catenin expression through the CaSR (30). It has been proposed that a calcium gradient along the length of the colon crypt may play a role in differentiation of colonocytes, possibly by modulating E-cadherin binding affinity (137). Another hypothesis is that calcium acts as an oxidative stress and inflammation reducing agent in the colon. Bile acids can cause oxidative damage to epithelial cell membranes, invoking an inflammatory response (138). Free calcium in the colon binds to these bile acids, rendering them inert. Further investigation is needed to understand the role of calcium in colon carcinogenesis.

Regardless of mechanism, epidemiological studies consistently indicate that higher calcium intakes may modestly lower risk for colorectal adenomas. Thirteen out of fifteen observational epidemiological studies found inverse associations of calcium and colorectal adenoma [two cohort studies (139, 140), seven case-control studies (141-147), four case-control/cohort studies nested in randomized clinical trials (148-151), and two cross-sectional studies (152, 153)]. However, only three were statistically significant (142, 149, 152). Two clinical trials investigated the effects of calcium supplementation, and one investigated calcium and antioxidant supplementation (18, 154, 155). All three found a reduction in colorectal adenoma recurrence, although only one (the largest) was statistically significant.

In summary, calcium has been consistently inversely associated with colorectal adenoma incidence in observational studies, and calcium supplementation reduced

adenoma recurrence in randomized controlled trials. However, additional epidemiological and mechanistic confirmation is needed to confirm this association.

HYPOTHESES

1. I hypothesize that an antioxidant micronutrient combination will reduce circulating biomarkers of inflammation and oxidative stress in colorectal adenoma patients. Also, I hypothesize that smoking status may modify the effects that this antioxidant combination has on these biomarkers.
2. I hypothesize that circulating pro-inflammatory cytokines, as well as a summary z-score of these cytokines, will be associated with risk factors for colorectal cancer, particularly adiposity. I hypothesize that the combined inflammation z-score will be more strongly associated with several risk factors of colorectal cancer than any one cytokine.
3. I hypothesize that vitamin D and calcium, alone or in combination, will reduce circulating pro-inflammatory cytokines, and increase an anti-inflammatory cytokine, IL-10. Moreover, I hypothesize that the effects of calcium and/or vitamin D supplementation may be absent in those taking NSAIDs regularly, and that the effects of supplementation may differ by sex.

SPECIFIC AIMS

Aim 1: Investigate the effects of an antioxidant micronutrient cocktail on biomarkers of inflammation and oxidative stress using data and blood samples from a randomized, double-blind, placebo-controlled trial in patients with a history of sporadic colorectal adenoma (n = 47).

a) Estimate the effects of an antioxidant combination (*dl*-α-tocopherol acetate 800 mg, β-carotene 24 mg, vitamin C 1.0 g, *1*-selenomethionine 200 μg, riboflavin 7.2 mg, niacin 80 mg, zinc 60 mg, manganese 5 mg) given over four months on plasma TNF-α, IL-6, CySS, and F2-isoprostane levels.

b) Investigate whether the effects of this antioxidant combination may differ according to smoking status.

Aim 2: Using data from a pilot, randomized, double-blind, placebo-controlled 2x2 factorial chemoprevention trial at baseline, investigate a potential links between risk factors for CRC and biomarkers of inflammation, individually and in combination, with potential colorectal cancer risk factors in a population of colorectal adenoma patients (n=92).

a) Estimate the associations between circulating levels of CRP, TNF-α, IL-6, IL-8, IL-1β, as well as a combined z-score of these cytokines and risk factors for colorectal cancer (sex, age, body mass index, waist-to-

hip ratio, NSAID use, serum 25-OH-vitamin D, physical activity, and total energy and calcium intakes) in colorectal adenoma patients.

Aim 3: Investigate the effects of vitamin D and/or calcium supplementation on blood markers of inflammation in patients at risk for developing colorectal cancer, using data and blood samples from a pilot, randomized, double-blind, placebo-controlled 2x2 factorial chemoprevention trial of calcium and vitamin D_3 supplementation, alone or in combination, over six months (n=92).

a) Estimate the effects of 2 g/day calcium and/or 800 IU/day vitamin D_3 supplementation vs. placebo on a panel of circulating pro- (CRP, TNF-α, IL-6, IL-8, IL-1β) and anti-inflammatory (IL-10) markers in patients with a history of sporadic colorectal adenoma.

b) Estimate the effects of 2 g/day calcium and/or 800 IU/day vitamin D_3 supplementation vs. placebo on a novel combined inflammation z-score in patients with a history of sporadic colorectal adenoma.

c) Examine the differences in treatment effects on the above markers according to sex and regular NSAID use.

METHODS

To address aim 1, I used data from a pilot, randomized, double-blind, placebo-controlled clinical trial (*n*=47) of an antioxidant combination (*dl*-α-tocopherol acetate 800 mg, β-carotene 24 mg, vitamin C 1.0 g, *1*-selenomethionine 200 μg, riboflavin 7.2 mg, niacin 80 mg, zinc 60 mg, manganese 5 mg) given daily over four months in patients with a history of colorectal adenoma.

Study participants were recruited from the patient population attending a large GI practice in Winston-Salem, NC. Eligibility included patients between the ages of 30 and 74, in good general health, capable of informed consent, and with at least one pathology-confirmed sporadic colon or rectal adenoma in the past five years. Of 115 potential participants who met chart screening eligibility criteria, 48 (41.7%) were enrolled and consented to participate, and of these 47 (97.9%) successfully completed the placebo run-in trial and were randomized. Exclusions included contraindications to antioxidant micronutrient supplementation or rectal biopsy procedures, and medical conditions, habits, medication usage, intake of intervention supplements greater than the RDA, or any other conditions that would otherwise interfere with the study. Participants (n = 47) were randomly assigned (stratified by sex and current smoking status) to the following two treatments for four months: placebo (n = 23), or an antioxidant micronutrient cocktail (n = 24) delivering *dl*-α tocopherol acetate (vitamin E) 800 mg, β-carotene 24 mg, vitamin C 1,000 mg, *l*-selenomethionine 200 μg, riboflavin 7.2 mg, niacin 80 mg, zinc 60 mg, and manganese 5 mg, in two divided doses given twice daily with meals. One participant from the antioxidant treatment group was lost to follow-up due to

unwillingness to continue participation. The following data were collected at baseline and 4 month follow-up for each participant: dietary variables from a Willett FFQ, medical history, medications and supplements, and inflammation and oxidative stress biomarkers TNF-α, IL-6, CySS, and F2-isprostanes (see appendix for detailed description of laboratory procedure for CySS quantification), measured from participant's plasma. A more detailed description of the clinical trial protocol and laboratory methods for TNF-α, IL-6, and F2-isprostanes measurement are described in the methods section of Chapter 2.

To address aims 2 and 3, I used data from a pilot, randomized, double-blind, placebo-controlled, 2 x 2 factorial chemoprevention trial (n = 92) of 2 g/day calcium and/or 800 IU/day vitamin D_3 supplementation vs. placebo over six months in patients with sporadic colorectal adenomatous polyps. Participants in this study were recruited from the patient population attending the Digestive Diseases Clinic of the Emory Clinic, at Emory University. After initial chart screening for eligibility, 224 of 522 were contacted, and 105 were eligible and consented to participate. After a placebo run-in trial, participants (n = 92) were randomly assigned to the following four treatment groups: a placebo control group (n = 23), a 2.0 g elemental calcium (as calcium carbonate in equal doses twice daily) supplementation group (n = 23), an 800 IU vitamin D_3 supplement group (400 IU twice daily) (n = 23), and a calcium plus vitamin D supplement group taking 2.0 g elemental calcium plus 800 IU of vitamin D_3 daily (n = 23). Seven people (8%) were lost to follow-up due to: perceived drug intolerance (n = 2), unwillingness to continue participation (n = 3), physician's advice (n = 1), and cardiovascular death (n = 1). Dropouts included one person from the vitamin D supplementation group, and two persons from each of other three groups. The following

data were collected at baseline and 6-months follow-up for each participant in this chemoprevention trial: dietary variables from a Willett FFQ, medical history, medications and supplements, lifestyle/behavior variables, plasma 25-(OH)-vitamin D level, and plasma concentrations of pro-inflammatory markers (CRP, TNF-α, IL-6, IL-1β, and IL-8) and an anti-inflammatory marker (IL-10). For aim 2, the data used from this study was limited to those collected at baseline. The detailed clinical trial protocol and laboratory methods are described in the methods sections of Chapters 4 and 5.

CHAPTER 2. ANTIOXIDANT MICRONUTRIENTS AND BIOMARKERS OF OXIDATIVE STRESS AND INFLAMMATION IN COLORECTAL ADENOMA PATIENTS: RESULTS FROM A RANDOMIZED, CONTROLLED CLINICAL TRIAL

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Abstract

Background: Previous observational and experimental studies that investigated the potential of antioxidant micronutrients to modulate cancer risk produced inconsistent results. Accordingly, we investigated the effects of an antioxidant micronutrient cocktail on biomarkers of inflammation and oxidative stress in a chemoprevention trial in patients at risk for colorectal cancer.

Methods: We conducted a pilot, randomized, double-blind, placebo-controlled clinical trial (*n*=47) of an antioxidant combination (*dl*-α-tocopherol acetate 800 mg, β-carotene 24 mg, vitamin C 1.0 g, *1*-selenomethionine 200 μg, riboflavin 7.2 mg, niacin 80 mg, zinc 60 mg, manganese 5 mg) given daily over four months in patients with a history of colorectal adenoma. Plasma tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and F2-isoprostane concentrations were measured using enzyme-linked immuno-assays, and cystine (CySS) was measured using high performance liquid chromatography.

Results: Plasma TNF-α concentration decreased in the active treatment group 37% relative to the placebo group (p=0.002), and cystine (CySS) decreased 19% (p=0.03); however, IL-6 and F2-isoprostane concentrations decreased in antioxidant treated nonsmokers, but increased in smokers, although these findings were not statistically significant. The decreases of TNF-α and CySS were more pronounced in non-smokers.

Conclusions: These data suggest that an antioxidant micronutrient cocktail can modulate biomarkers of oxidative stress and inflammation in humans, and that these effects may differ according to smoking status.

Impact: This study supports 1) further investigation of biomarkers of inflammation and oxidative stress as potential treatable biomarkers of risk for colorectal cancer, and 2) a larger trial with a modified antioxidant micronutrient cocktail and/or a factorial design.

Introduction

Colorectal cancer is the second leading cause of cancer mortality in the United States, with 90% of cases presenting first as an adenomatous polyp, a benign intestinal tumor that is the only accepted biomarker of risk for colorectal cancer (156, 157). Easily treatable, pre-neoplastic biomarkers of risk could aid in preventing colorectal cancer morbidity and mortality. Chronic inflammation, such as in inflammatory bowel disease, is associated with increased risk of colorectal cancer, and specific pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), increase with colorectal cancer progression (73, 158-160). Oxidative stress may also be a significant factor in the etiology of colorectal cancer, as evidenced by increased risk in smokers, and the abundance of oxidative DNA lesions in transformed colorectal epithelial cells (161-163). Antioxidants have been frequently proposed as potential preventive interventions against colorectal neoplasms. However, studies that investigated a potential link between antioxidants and cancer produced variable results, especially in smokers (85, 96, 104, 164-169). Cancer is a complex disease, and variable responses to preventive interventions are likely. Using biomarkers of risk, individual preventive

treatment response could be predicted, and treatment enhanced or stopped where appropriate.

TNF-α, IL-6, and markers of oxidative stress, cystine (CySS) and F2-isoprostanes, were chosen in this study as potential biomarkers because they have been associated with colorectal cancer, and because antioxidants were found to modulate these molecules. In an observational case-control study, risk factors for colorectal adenomas, such as old age, smoking, and adiposity, were found to be associated with higher levels of inflammatory cytokines, specifically TNF-α and IL-6 (158). Higher circulating levels of TNF-α and IL-6 were found in patients with colorectal cancer than in disease free controls, and higher levels of oxidative stress were found in colon adenocarcinoma than in normal appearing rectal mucosa (158, 163). Antioxidants reduced TNF-α production in blood in two randomized, placebo-controlled, clinical trials, one (n = 26) with lycopene, α-tocopherol, and β-carotene, and another (n = 50) with zinc (98, 99). In a long-term placebo-controlled clinical trial (n = 87), plasma CySS concentration was decreased with vitamin C, vitamin E, and β-carotene treatment, and also by zinc (100, 101). In a randomized, placebo-controlled clinical trial (n = 385), vitamin C and E treatment reduced plasma isoprostanes in obese patients with high baseline levels of isoprostanes (102). Conversely, in some large, randomized clinical trials, high dose β-carotene supplements increased lung cancer incidence in smokers (104-106). Therefore, TNF-α, IL-6, CySS, and F2-isoprostanes may serve as barometers of potential good as well as potential harm from antioxidant treatment.

The aim of the randomized, double-blind, placebo-controlled trial reported here was to assess the effects of an antioxidant micronutrient cocktail on plasma TNF-α, IL-6,

CySS, and F2-isoprostane levels in patients with a history of sporadic colorectal adenoma.

Materials and Methods

The original study was approved by the Institutional Review Board of Wake Forest University. Informed consent was obtained from each study participant. This subsequent laboratory and data analysis project using de-identified data was approved by the Institutional Review Board of Emory University.

Study population

Study participants were recruited from the patient population attending a large GI practice in Winston-Salem, NC. Eligibility included age 30 to 74 years, in good general health, capable of informed consent, and at least one pathology-confirmed sporadic colon or rectal adenoma in the past five years. Of 115 potential participants who met chart screening eligibility criteria, 48 (41.7%) were enrolled and consented to participate, and of these 47 (97.9%) successfully completed the placebo run-in trial and were randomized. Exclusions included contraindications to antioxidant micronutrient supplementation or rectal biopsy procedures, and medical conditions, habits, or medication usage, or intake of intervention supplements greater than RDA, that would otherwise interfere with the study.

Clinical trial protocol

In 1993, all age-eligible practice patients diagnosed with at least one pathology-confirmed adenomatous colonic or rectal polyp within the previous five years were identified as potential study participants. After an initial medical chart screening,

potential eligible patients were sent an introductory letter followed up by a telephone interview during which willingness to participate and further eligibility was assessed, and, if appropriate, an in-person eligibility visit was scheduled. During their first eligibility visit, potential participants were interviewed and signed a consent form, their medication and nutritional supplement bottles were reviewed, and they completed questionnaires (on medical history, medication and nutrition supplement use, lifestyle, family history, and others) and provided a blood sample. Diet was assessed with a semi-quantitative food frequency questionnaire (170). Medical and pathology records were reviewed. Those still eligible and willing to participate then entered a four week placebo run-in trial. Only participants without perceived side effects and who took at least 80% of their capsules during the run-in trial were randomized. Eligible participants then underwent a blood draw and were randomized to treatment groups.

Participants (n = 47) were randomly assigned (stratified by sex and current smoking status) to the following two treatments for four months: placebo (n = 23), or an antioxidant micronutrient cocktail (n = 24) delivering *dl*-α tocopherol acetate (vitamin E) 800 mg, β-carotene 24 mg, vitamin C 1,000 mg, *l*-selenomethionine 200 μg, riboflavin 7.2 mg, niacin 80 mg, zinc 60 mg, and manganese 5 mg, in two divided doses given twice daily with meals. The corresponding supplement and placebo capsules were identical in size, appearance, and taste. All study participants were asked to remain on their usual diet and not take any nutritional supplements not in use on entry into the study.

At the time this study was originally planned (1993), the criteria for the antioxidant micronutrient cocktail included all commercially available antioxidant related micronutrients for which there were established Recommended Daily Allowances. Doses

for the lipid soluble (vitamin E, and β-carotene) and the water soluble (vitamin C) direct antioxidants were chosen to be as high as possible without causing side effects and were generally regarded as safe. Doses for the other agents, which are essential components of various antioxidant enzymes, were chosen to ensure no insufficiency of these agents. The amount of vitamin E given in this study was 80 times the RDA; β-carotene was 40 times the RDA; vitamin C was 20 times the RDA; selenium, riboflavin, niacin, and zinc were four times the RDA; and manganese was one times the RDA. This clinical trial was conducted prior to reports from the ATBC and CARET clinical trial findings of increased lung cancer risk in male smokers randomized to take high dose β-carotene (104, 106). The duration of the intervention in this trial was chosen to ensure that the steady-state levels of the lipid soluble antioxidants would be achieved.

Over the four month treatment period, participants attended follow-up visits at two and four months after randomization and were called for a phone interview at one and three months after randomization. At follow-up visits, pill-taking adherence was assessed by questionnaire, interview, and pill count. At the four month follow-up participants were interviewed and completed questionnaires. At all visits the participants underwent venipuncture. Before all visits participants were required to fast and refrain from smoking after midnight, and all visits were conducted in the morning. Factors hypothesized to be related to inflammation and oxidative stress were assessed at the baseline and final follow-up visits. Participant visit adherence was 98%, and pill taking adherence was 97%. There were no adverse events or toxicities reported during the study.

Peripheral venous blood samples were taken by study staff from participants after the subject sat upright with legs uncrossed for five minutes. Duplicate blinded samples were drawn at 10% of all study visits. Blood was drawn into red-coated, pre-chilled vacutainer tubes for whole blood, plasma (red top EDTA tubes), and serum, and then immediately placed on ice and shielded from light. After centrifugation in a refrigerated centrifuge, BHT and salicylic acid as lipid and aqueous antioxidants, respectively, were added to the blood fractions to be used for oxidative stress measurements. Blood fractions were aliquotted, placed in amber-colored cryopreservation tubes, the air was displaced with nitrogen gas, the tubes were sealed with O-ring screw caps, and then the tubes were immediately placed in a -80º C freezer until analysis. In 1993, plasma α tocopherol and β-carotene were measured by high performance liquid chromatography (HPLC). Measurement reliability assessed by intra-class correlation coefficients were 0.99 and 0.96 for α tocopherol and β-carotene, respectively.

Inflammation and oxidative stress biomarker analyses

Prior to analysis, the samples were thawed only twice to remove aliquots, then frozen again at -80º C. All samples during handling were blinded and were treated identically. In 2009, plasma samples were analyzed by HPLC for CySS and quantified relative to an internal standard, γ-glutamyl glutamate, 10 μM (171). The average intra-assay coefficient of variation for CySS was 7.5%. Multiplex enzyme linked immunoassays (ELISAs) (R&D systems, Minneapolis, MN) were used to measure IL-6 and TNF-α, in duplicate, according to the manufacturer's protocol. The average intra-assay coefficient of variation for IL-6 was 10.6%, and for TNF-α, 6.7%. Plasma levels of F2-isoprostanes were measured by hydrolyzing the plasma samples with NaOH, then

neutralizing them with HCl before performing the direct 8-iso-prostaglandin $F_{2\alpha}$ Enzyme Immunoassay (Assay Designs, Ann Arbor, MI), in duplicate, according to the manufacturer's protocol. The average intra-assay coefficient of variation for F2-isoprostanes was 11.6%. The actual numbers of blood samples available with biomarker values obtained above the limits of assay detection were 45 for CySS, 44 for IL-6 and TNF-α, and 43 for F2-isoprostane.

Statistical analyses

Treatment groups were assessed for comparability of characteristics at baseline and final follow-up by t-test or Wilcoxon test for continuous variables, and χ^2-test for categorical variables. Variables that were not normally distributed were transformed, as appropriate, before statistical testing.

Primary analyses were based on assigned treatment at the time of randomization regardless of adherence (intent-to-treat analysis). Biomarker levels below the limits of detection were treated as missing values. Mean biomarker concentrations were calculated for each treatment group for the baseline and four-month follow-up visits. Treatment effects were evaluated by assessing the differences in biomarker concentrations from baseline to four months follow-up between the active treatment group and the placebo group by a repeated measures linear MIXED effects model, as implemented using the Proc Mixed procedure of the Statistical Analysis System (SAS). The model included the intercept, treatment group, visit, and a treatment x visit interaction term. Absolute treatment effects were calculated as the absolute change from baseline in the active treatment group minus the absolute change from baseline in the placebo group. Since concentrations of the measured biomarkers in plasma are not

widely familiar, to provide perspective on the magnitude of treatment effects, relative effects were calculated, defined as (treatment group follow-up /treatment group baseline)/(placebo follow-up /placebo baseline). The relative effect provides an estimate of the proportional change in the treatment group relative to that in the placebo group. The interpretation of the relative effect is somewhat analogous to that of an odds ratio (e.g., a relative effect of 2.0 means that the relative proportional change in the treatment group was twice as great as that in the placebo group). Stratified analyses were conducted to investigate potential differential treatment effects by smoking status and sex. The correlation of age with baseline biomarker concentrations was calculated using the Pearson correlation coefficient.

Statistical analyses were performed using SAS software, version 9.2 (Copyright© 2002-2008 by SAS Institute Inc., Cary, NC, USA). A cutoff level of $P \leq 0.05$ (two sided) was used for assessing statistical significance.

Results

Study participants

Selected baseline characteristics of the study participants are shown in Table 1.1. The mean age of participants was 60 years, 49% were men, 42% were current smokers, 17% took a non-aspirin nonsteroidal anti-inflammatory drug (NSAID) once a week or more, and 24% had a family history of colorectal cancer in a first-degree relative. The treatment groups were not significantly different on risk factors for colorectal neoplasms or factors that may be related to inflammation or oxidative stress, except for total vitamin

C intake. However, the amount of vitamin C given in the active treatment supplement was 20 times the amount by which the treatment groups differed at baseline.

Baseline and four-month follow-up plasma levels of α tocopherol were 9.4 and 7.9 μg/ml, respectively, in the placebo group, and 9.1 and 23.0 μg/ml, respectively, in the active treatment group ($p < 0.01$; data not shown). The corresponding values for β-carotene were 0.09 and 0.06 μg/ml, respectively, in the placebo group, and 0.08 and 0.95 μg/ml, respectively, in the active treatment group ($p < 0.001$; data not shown).

Effects of antioxidants on TNF-α, CySS, IL-6, and F2-isoprostane concentrations

Table 1.2 shows the effects of the antioxidant micronutrient combination on TNF-α, CySS, IL-6, and F2-isoprostane plasma concentrations relative to placebo. After four months of treatment, mean TNF-α plasma concentration decreased by 36% in the active treatment group relative to the placebo group ($p = 0.001$), CySS decreased by 39% ($p = 0.02$), and IL-6 decreased 15% ($p = 0.75$). Overall, F2-isoprostane concentration did not change in the antioxidant treatment group relative to the placebo group.

The effects of the antioxidant micronutrient combination on TNF-α, CySS, IL-6, and F2-isoprostane concentrations relative to placebo, by current smoking status, are shown in Table 1.3. The estimated treatment effects on TNF-α and CySS were more pronounced among non-smokers, but for F2-isoprostanes and IL-6, they differed in the direction of response by current smoking status. In the antioxidant treatment group relative to placebo, TNF-α plasma concentration decreased 50% in nonsmokers ($p<0.0001$), and 11% in smokers ($p=0.61$). Similarly, CySS plasma concentration decreased 53% in nonsmokers ($p = 0.007$), and 12% in smokers ($p=0.88$). However, IL-6 concentration decreased by 62% in nonsmokers ($p=0.10$), but increased

148% among smokers (p=0.27). Similarly, F2-isoprostane concentration decreased by 20% in nonsmokers (p=0.44), but increased by 40% in smokers (p=0.18).

In analyses stratified by sex, we found no evidence of differential treatment effects by sex (data not shown). Although CySS levels were previously found to increase with age (82), we found no correlation of age with CySS or the other potential biomarkers (all $r < 0.04$).

Discussion

The findings from this pilot, randomized, double-blind, placebo-controlled clinical trial indicate that an antioxidant micronutrient combination supplement can substantially decrease circulating levels of the inflammatory marker TNF-α and the oxidative stress marker CySS in sporadic colorectal adenoma patients, and suggest that these treatment effects may be more pronounced in non-smokers. Our findings also suggest that an antioxidant micronutrient cocktail containing high dose β-carotene may also reduce circulating levels of the inflammatory cytokine IL-6 and the oxidative stress marker F2-isoprostane in non-smokers, but increase them in smokers; however, these findings were not statistically significant in this small pilot study.

Diets high in fruits, vegetables, and antioxidants have been associated with a lower risk of cancer (85), especially colorectal cancer (33, 86). In addition, colorectal epithelial cell proliferation, a potential biomarker of risk for colorectal cancer, was reduced by several antioxidant micronutrients, such as vitamins A, C, E and β-carotene (27, 95-97). Our findings that an antioxidant micronutrient combination can decrease TNF-α and CySS are consistent with hypotheses that fruits, vegetables, and antioxidant

micronutrients reduce colorectal epithelial cell proliferation and risk of colorectal cancer by reducing inflammation and oxidative stress.

TNF-α, a pro-inflammatory cytokine, is linked to both oxidative stress and colorectal cancer (172) and increases oxidative DNA lesions and chromosomal instability in cell culture (173). Pro-inflammatory cytokines, such as TNF-α and IL-6, when released from macrophages or other cells, can promote proliferation, angiogenesis, and metastasis (174). These pro-carcinogenic actions of TNF-α are consistent with mouse experimental models that show that high levels of TNF-α promote colon tumor growth (175). A phase II clinical trial of patients with treatment resistant renal cell carcinomas found partial clinical response and stable disease (assessed according to the Response Evaluation Criteria in Solid Tumors) when the patients were treated with TNF-α monoclonal antibodies (176). These findings suggest that lowering TNF-α levels with antioxidants before clinical disease presentation could prevent tumors, and slow tumor growth and cancer progression.

Antioxidants, such as N-acetyl-l-cysteine (NAC), suppressed TNF-α-induced NF-κB activity in cultured human synovial cells (177). NF-κB, a major regulator of inflammation and response to oxidative stress, is a downstream target of TNF-α (178). NF-κB has an oxidatively regulated DNA binding domain, and induces the transcription of inflammatory cytokines, anti-apoptotic proteins, and oxidative stress responsive enzymes that together promote cellular transformation and tumor formation (179). Reducing NF-κB and TNF-α levels by NAC or other antioxidants can inhibit this transformation-promoting pathway.

CySS, the most abundant amino-thiol in plasma, is associated with both oxidative stress and inflammation. CySS is the oxidized disulfide of the amino acid cysteine (Cys) and, therefore, a product of NAC. CySS and Cys participate in intra- and extra-cellular reduction/oxidation (redox) reactions (52). A shift in the pool of available CySS indicates an increase in oxidation, and higher Cys indicates a more reduced state (81). In our study, unfortunately, we were unable to measure Cys, and thus the Cys/CySS ratio, because the samples were not preserved in a way that would allow valid measurement. Increased CySS levels have been directly associated with older age, cardiovascular disease, diabetes, rheumatoid arthritis, and other inflammatory diseases (82). In addition, inflammatory cytokines, such as TNF-α and IL-1β, are directly correlated with high CySS levels (180). Decreasing CySS levels with antioxidants may decrease oxidative stress and inflammation, and therefore may reduce risk of colorectal cancer.

F2-isoprostanes are formed by a non-enzymatic free radical oxidation of esterified arachidonic acid. Phospholipases then cleave and release free isoprostanes into the circulation. For this reason, F2-isoprostane levels are widely used as a blood biomarker of oxidative stress (54). The colon is under a high degree of oxidative stress caused by high concentrations of bile acids and iron, which catalyze the formation of reactive oxygen species (ROS) (83). Increased ROS can cause oxidative lesions in DNA, which are associated with colorectal cancer progression (163).

Although certain antioxidants, specifically β-carotene and vitamins C and E, are free radical scavengers, and can, therefore, decrease oxidative stress by removing free radicals, giving β-carotene in supraphysiological doses to smokers may be detrimental. This was suggested by the Alpha-Tocopherol and Beta-Carotene Cancer Prevention

Study (ATBC) and the Carotene and Retinol Efficacy Trial (CARET), in which smokers were given a supraphysiological dose of β-carotene daily (20 mg/day plus α-tocopherol 50 mg/day in ATBC, 30 mg/day plus retinyl palmitate 25,000 IU/day in CARET) (104-106). These studies found that high doses of β-carotene daily increased lung cancer incidence in the participants. In our study, in which we used a dose of β-carotene (24 mg/day) intermediate to those used in the ATBC and CARET trials and was conducted prior to the publication of the CARET and ATBC results, in the antioxidant treatment group, F2-isoprostane and IL-6 levels decreased in non-smokers but increased in smokers, although these findings were not statistically significant. Biological plausibility for these findings is suggested by two studies in mice. In one, β-carotene was found to act as a pro-oxidant, inducing lipid peroxidation (for which F2-isprostane is a biomarker) in the presence of cigarette tar in lung tissues exposed to oxygen (181). In the second study, cigarette smoke-induced lung epithelial cell DNA damage increased IL-6 expression (182). These studies, taken together, suggest that high β-carotene supplementation in smokers may induce oxidative stress and lung epithelial cell DNA damage, thereby increasing circulating levels of F2-isoprostanes and IL-6.

Our study has several limitations and strengths. First, the sample size was small, especially for stratified analyses. In addition, the blood biomarker analyses were done about 16 years after the completion of the clinical trial, and although the samples were stored at -80° C, storage duration could have impacted the levels of oxidative stress and inflammation markers. The large dose of β-carotene used in this study was a strength and a limitation. While the large dose may have masked potential benefits of other antioxidants on biomarkers of inflammation and oxidative stress in smokers, this unique

study design allowed for an analysis of the potential mechanism behind increased lung cancer incidence seen in the ATBC and CARET studies. Another limitation of this study was that only one antioxidant micronutrient combination was investigated, and the effect of different doses and combinations could have different effects. On the other hand, this is the first study, to our knowledge, to investigate this particular antioxidant micronutrient combination on blood biomarkers of both inflammation and oxidative stress. Finally, this study was done on sporadic colorectal adenoma patients, and therefore has limited generalizability to the whole population.

Strengths of this study included the randomized, double-blind, placebo-controlled clinical trial design, the opportunity to explore potential treatment effects by current smoking status, the very high protocol adherence, and the balance in the treatment groups on many potential confounding risk factors for colorectal cancer, oxidative stress, and inflammation.

In summary, we found that an antioxidant micronutrient cocktail can substantially decrease circulating biomarkers of inflammation (TNF-α) and oxidative stress (CySS) in sporadic colorectal adenoma patients, but, although antioxidants may also decrease circulating levels of IL-6 and F2-isoprostanes in non-smokers, they may increase these biomarkers in smokers. Also, taken together with previous literature, this study *a*) suggests that the effect of an antioxidant micronutrient cocktail supplement may differ in magnitude and direction according to current smoking status, *b*) supports the further investigation of these biomarkers of inflammation and oxidative stress as potential treatable biomarkers of risk for colorectal cancer, *c*) provides a potential explanation for adverse effects of β-carotene in large doses in smokers, and *d*) supports a larger trial with

a reformulated antioxidant cocktail (with eliminated or reduced β-carotene, or a factorial design) and measurement of biomarkers in both tissue and surrogate fluids (blood, urine), and multiple follow-ups.

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Notes

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Tables

Table 1.1 Selected baseline characteristics of participants (n=47).

Characteristics[1]	Placebo (n=23)	Antioxidants (n=24)	P Value[2]
Demographics			
Age (yrs.), median (range)	59 (36-71)	61 (41-73)	0.55
Sex (% males)	52.2	50.0	0.88
Race (% white)	91.3	87.5	>0.99
Education (% attended college)	21.7	26.1	>0.99
Medical history			
First degree relative with CRC (%)	21.7	26.1	>0.99
Take NSAID regularly[3] (%)	13.0	20.8	0.70
Currently smoke (%)	45.0	39.0	0.76
Alcohol intake (g/day), median (range)	0 (0-31.5)	0.25 (0-42)	0.46
Postmenopausal (n=23) (%)	45.5	50.0	>0.99
Anthropometrics			
BMI (kg/m^2), median (range) Men	25.1 (19.0-30.2)	27.2 (23.1-30.7)	0.11
Women	29.5 (16.6-41.0)	26.7 (18.5-65.8)	0.16
Waist-to-hip ratio Men	0.99 (0.10)	1.01 (0.05)	0.55
Women	0.84 (0.11)	0.85 (0.07)	0.96
Colorectal adenoma history			
No. of adenomas removed, median (range)	2 (1-6)	2 (1-11)	0.65
Size of largest adenoma removed (cm), median (range)	0.5 (0.2-3.0)	0.65 (0.2-2.5)	0.29
Villous/tubulovillus adenoma (%)	8.7	12.5	>0.99
Mean dietary intakes			
Total energy intake (kcal/day) Males	2,133 (193)	1,685 (193)	0.11
Females	1,351 (152)	1,750 (145)	0.07
Total[4] α-tocopherol (mg/day)	7.9 (0.7)	7.4 (0.7)	0.60
Total[4] vitamin C (mg/day)	90 (14)	141 (13)	0.01
Total[4] β-carotene (μg/day)	3,418 (405)	3,813 (388)	0.49
Total[4] riboflavin (mg/day)	1.6 (0.1)	1.7 (0.1)	0.53
Total[4] niacin (mg/day)	22 (1.6)	20 (1.5)	0.39
Total[4] zinc (mg/day)	11 (0.9)	10 (0.9)	0.59
Total[4] manganese (mg/day)	2.7 (0.3)	2.7 (0.3)	0.93
Total[4] iron (mg/day)	12.2 (1.3)	11.9 (1.3)	0.88
Red meat (servings/week)	4.7 (3.3)	3.1 (2.7)	0.09
Total fruits/vegetables (servings/week)	36 (16.4)	40.5 (19.1)	0.41

Table 1.1 (continued)

Characteristics[1]	**Placebo (n=23)**	**Antioxidants (n=24)**	**P Value**[2]
Plasma antioxidant levels			
β-carotene (μg/ml), median (range)	0.09 (0.01-0.17)	0.08 (0.02-0.51)	>0.99
α tocopherol (μg/ml), median (range)	9.4 (6.0-13.7)	9.1 (5.7-15.0)	0.67

Abbreviations: CRC, colorectal cancer; NSAID, nonsteroidal anti-inflammatory drug; BMI, body mass index.
[1] Data are given as means (SD) unless otherwise specified.
[2] P values based on χ^2 test for categorical variables, t-test of means, or Wilcoxon test for medians.
[3] At least once a week.
[4] Total = dietary + supplemental.

Table 1.2 Effects of antioxidant micronutrients on inflammatory cytokines and oxidative stress markers in plasma

Biomarkers	n[1]	Placebo Mean (SD)	Antioxidants[1] Mean (SD)	Absolute Treatment Effects[2] Mean (95% CI)	Relative Treatment Effects[3]	P [4]
IL-6 (pg/ml) [5]	22/22					
Baseline		1.31 (1.33)	1.18 (1.32)			
4 mo		2.14 (1.32)	1.64 (1.31)	-0.16 (-1. 15, 0.83)	0.85	0.75
TNF-α (pg/ml)	21/23					
Baseline		2.51 (0.37)	3.01 (0.35)			
4 mo		3.60 (0.37)	2.77 (0.35)	-1.32 (-2.08, -0.56)	0.64	0.001
F2-isoprostane (pg/ml)	21/23					
Baseline		1,970 (249)	1,930 (237)			
4 mo		2,205 (249)	2,137 (237)	-28 (-912, 856)	0.99	0.95
CySS (μM/ml)	22/23					
Baseline		36.8 (3.9)	37.1 (3.8)			
4 mo		41.8 (3.9)	25.8 (3.8)	-16.4 (-30.1, -2.7)	0.61	0.02

Abbreviations: IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; CySS, cystine.

[1] Placebo/Antioxidants: The antioxidant cocktail consists of *dl*-α-tocopherol acetate 800 mg, β-carotene 24 mg, vitamin C 1.0 g, *1*-selenomethionine 200 μg, riboflavin 7.2 mg, niacin 80 mg, zinc 60 mg, and manganese 5 mg, given daily over four months.

[2] Absolute treatment effect is the absolute change from baseline to follow-up in the treatment group minus the absolute change from baseline to follow-up in the placebo group from mixed model.

[3] Relative treatment effect is defined as: (treatment group follow-up /treatment group baseline)/(placebo follow-up /placebo baseline). The interpretation of the relative effect is similar to that of an odds ratio (e.g., a relative effect of 2.0 would mean that the relative proportional change in the treatment group was twice as great as that in the placebo group).

[4] P values for baseline to follow-up differences between placebo group and active treatment group from mixed model.

[5] Geometric means with standard errors are reported, calculated by exponentiating the mean of the log transformed values.

Table 1.3 Effects of antioxidant micronutrients on inflammatory cytokines and oxidative stress markers in plasma by current smoking status

Biomarkers	n[1]	Placebo Mean (SE)	Antioxidants [2] Mean (SE)	Absolute Treatment Effects[3] Mean (95% CI)	Relative Effects[4]	P Value[5]
Non-smokers						
IL-6 (pg/ml) [6]	13/13					
Baseline		1.26 (1.42)	2.04 (1.40)			
4 mo		2.46 (1.42)	1.51 (1.38)	-0.97 (-2.13, 0.19)	0.38	0.10
TNF-α (pg/ml)	12/14					
Baseline		2.29 (0.46)	2.69 (0.43)			
4 mo		3.85 (0.46)	2.22 (0.43)	-2.02 (-2.87, -1.17)	0.50	<0.0001
F2-isoprostane (pg/ml)	13/14					
Baseline		2,047 (788)	2,090 (2,157)			
4 mo		2,253 (807)	1,835 (620)	-461 (-1,678, 755)	0.80	0.44
CySS (μM/ml)	12/14					
Baseline		32.6 (5.2)	40.8 (4.8)			
4 mo		41.5 (5.2)	24.5 (4.8)	-25.1 (-43.1, -7.2)	0.47	0.008
Current smokers						
IL-6 (pg/ml) [5]	9/9					
Baseline		1.20 (1.61)	0.52 (1.61)			
4 mo		1.74 (1.61)	1.87 (1.61)	0.94 (-0.82, 2.70)	2.48	0.27
TNF-α (pg/ml)	9/9					
Baseline		2.80 (0.58)	3.49 (0.58)			
4 mo		3.28 (0.58)	3.63 (0.58)	-0.33 (-1.72, 1.05)	0.89	0.61
F2-isoprostane (pg/ml)	8/8					
Baseline		1,855 (535)	1,650 (552)			
4 mo		2,133 (755)	2,665 (997)	736 (-377, 1,850)	1.40	0.18
CySS (μM/ml)	10/9					
Baseline		41.8 (6.0)	31.4 (6.3)			
4 mo		42.3 (6.0)	27.8 (6.3)	-4.0 (-26.7, 18.6)	0.88	0.71

Abbreviations: IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; CySS, cystine.

[1] Placebo/Antioxidants.

[2] The antioxidant cocktail consists of *dl*-α-tocopherol acetate 800 mg, β-carotene 24 mg, vitamin C 1.0 g, *1*-selenomethionine 200 μg, riboflavin 7.2 mg, niacin 80 mg, zinc 60 mg, and manganese 5 mg, given daily over four months.

[3] Absolute treatment effect is the absolute change from baseline to follow-up in the treatment group minus the absolute change from baseline to follow-up in the placebo group from mixed model.

[4] Relative treatment effect is defined as: (treatment group follow-up /treatment group baseline)/(placebo follow-up /placebo baseline). The interpretation of the relative effect is similar to that of an odds ratio (e.g., a relative effect of 2.0 would mean that the relative proportional change in the treatment group was twice as great as that in the placebo group).

[5] P values for baseline to follow-up differences between placebo group and active treatment group from mixed model.

[6] Geometric means with standard errors are reported, calculated by exponentiating the mean of the log transformed values.

CHAPTER 3. ASSOCIATIONS OF CIRCULATING INFLAMMATORY BIOMARKERS WITH RISK FACTORS FOR COLORECTAL CANCER IN COLORECTAL ADENOMA PATIENTS

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Abstract

Background: Obesity and central adiposity are associated with colorectal cancer risk, and have been linked to inflammation. Inflammation is a complex, interactive response that may most accurately be summarized through multiple, simultaneously measured cytokines.

Methods: In this cross-sectional analysis, we investigated associations of circulating plasma levels of C-reactive protein (CRP), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-1β (IL-1β), and a summary inflammation z-score with risk factors for colorectal cancer in colorectal adenoma patients (n=92). Multivariable logistic regression was used to investigate associations between cytokine levels and known risk factors for colorectal neoplasms.

Results: Mean cytokine levels tended to increase with increasing body mass index (BMI), with statistically significant trends in relation to CRP, IL-6, and the combined inflammation z-score (p for trend <0.001, 0.02, and <0.001, respectively). The odds ratios for associations of the inflammation z-score with being overweight (BMI 25-29.9 kg/m^2), obese (BMI ≥30 kg/m^2), or having an above the study population median weight-to-hip ratio were 4.33 (95% CI [confidence interval] 1.04, 18.00), 5.54 (95% CI 1.37, 22.42), and 4.09 (95% CI 1.67, 9.98), respectively.

Conclusions: Our findings support 1) associations of inflammation with increased general and central adiposity, and 2) investigation of a combined inflammation score as a risk factor for colorectal neoplasms.

Impact: Better ways to evaluate inflammation, a factor known to play a role in carcinogenesis, and identification of modifiable factors that may be associated with inflammation, may lead to improved cancer prevention efforts.

Introduction

Colorectal cancer (CRC) is the second leading cause of cancer mortality in the United States with 70% of cases having no family history of the disease, pointing to a large environmental component, and hence its preventability (1). Among the potentially modifiable risk factors for CRC, physical activity, non-steroidal anti-inflammatory drug (NSAID) use, and calcium intake have consistently been associated with decreased risk, while adiposity is associated with increased risk (13, 16, 18). Other factors previously examined and fairly well supported to modulate risk for CRC are smoking, serum 25-OH-vitamin D levels, and energy intake (15, 19, 183). Established, non-modifiable risk factors for CRC are old age, family or personal history of CRC, and inflammatory bowel disease (184).

There is increasing evidence for a link between inflammation and colorectal cancer; NSAID use reduced sporadic colorectal adenoma recurrence in clinical trials, and specific pro-inflammatory markers, such as C-reactive protein (CRP), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6), are elevated in inflammatory bowel disease and colorectal adenoma patients (16, 73, 87, 184, 185). CRP, TNF-α, and IL-6 also have been directly associated with adenomas, higher tumor grade, and increased risk of mortality in colorectal cancer patients (184, 186, 187). High levels of these inflammatory

markers were also found to be associated with risk factors for colorectal adenomas, such as old age, smoking, and adiposity in a case-control study (71).

To further investigate potential links between risk factors for CRC and biomarkers of inflammation, individually and in combination, we examined the associations between circulating levels of CRP, TNF-α, IL-6, IL-8, IL-1β, as well as a previously reported combined z-score (188), with potential colorectal cancer risk factors (sex, age, body mass index [BMI], waist-to-hip ratio [WHR], NSAID use, serum 25-OH-vitamin D, physical activity, and total energy and calcium intakes) in a population of colorectal adenoma patients.

Materials and Methods

This study was approved by the Emory University Institutional Review Board. Written informed consent was obtained from each study participant.

Study population

This cross-sectional study was conducted using stored blood samples collected at baseline from participants in a randomized, controlled chemoprevention trial that administered calcium and/or vitamin D to sporadic colorectal adenoma patients. The detailed protocol of study recruitment and procedures was published previously (189). Briefly, study participants were recruited from the patient population attending the Digestive Diseases Clinic of Emory University. Eligibility included age 30 to 75 years, in good general health, capable of informed consent, and at least one pathology-confirmed sporadic colorectal adenoma in the past 36 months. Exclusions included the

use of calcium or vitamin D supplements, or any medical conditions, habits, or medication usage that would otherwise interfere with the study (189).

Clinical trial protocol

Between April 2005 and January 2006, 92 eligible participants were randomized into the trial. Participants underwent a blood draw and answered questionnaires, including a Willett Food Frequency questionnaire. Participants were asked to abstain from aspirin (but not other NSAID) use for 7 days before the blood draw/rectal biopsy visit.

Inflammation biomarker analyses

A single enzyme linked immunoassay (ELISA) (R&D systems, Minneapolis, MN) was used to measure CRP, and a High Sensitivity Multiplex ELISA (R&D systems, Minneapolis, MN) was used to measure TNF-α, IL-6, IL-1β, and IL-8 in duplicate, according to the manufacturer's protocol. The average intra-assay coefficient of variation (CV) for CRP was 6.6%, for TNF-α 11.5%, IL-6 11.7%, IL-1β 10.6%, and IL-8 7.9%. Cytokine levels below the limits of detection were assigned a value equal to the lower limit of detection for that cytokine.

Statistical analysis

Risk factors for CRC evaluated included sex, regular NSAID use ($\geq$ once per week), adiposity (indicated by BMI and WHR), age, physical activity (in METs/d), total energy and calcium intakes, and serum 25-OH-vitamin D level. All continuous variables except BMI were dichotomized as "Low" for values below the 50th percentile and "High" if they were above; WHR was dichotomized at the 50^{th} percentile of the sex-specific distributions. BMI was categorized based on WHO definitions (1997 WHO

Consultation on Obesity) as normal or underweight (< 25 kg/m^2), overweight (25-29.9 kg/m^2), or obese (≥30 kg/m^2). Mean cytokine values were calculated for each risk factor, and evaluated by *t* test for dichotomized risk factors, and by a general linear model for BMI. Associations between CRC risk factors and high levels of inflammatory cytokines were evaluated using multivariable logistic regression. Inflammatory cytokines and a summary z-score were classified as high if a subject's level was above the 50^{th} percentile of the population distribution.

Due to the strong associations between BMI and our panel of inflammatory cytokines in our study as well as previous literature, BMI was treated as a confounder in all regression models. Also, physical activity was adjusted for total energy intake, and vice versa.

To assess the associations of CRC risk factors with a summary score of the inflammatory cytokines combined, a summary inflammation z-score was calculated. This score was calculated as follows: first, a normalized z-score for each individual cytokine value, with a mean of zero and standard deviation of 1.0, was calculated as $z = (x - \mu)/\sigma$, where x is a participant's cytokine value, and μ and σ are the study population mean and standard deviation, respectively; and then the combined inflammation z-score for each participant was created by summing the z-scores of each inflammatory marker.

Results

Study participants

Selected characteristics of the study participants are shown in Table 3.1. The mean age of participants was 61 years, 70% were men, 71% were white, and 20% had a

family history of colorectal cancer in a first-degree relative. Only three participants smoked, therefore we did not evaluate this risk factor.

Although men had statistically significant 77% lower mean CRP levels than did women, the odds ratio was not statistically significant (Table 3.2). None of the other inflammatory cytokines or the inflammation z-score differed by sex. CRP levels in obese participants were significantly higher (138%, p for trend <0.001) than in those with a normal BMI (odds ratio [OR] 5.20; 95% CI [confidence interval] 1.42-19.04). Although there was a trend of higher inflammatory marker levels with increasing BMI, the trends were statistically significant only for CRP, IL-6, and the inflammation z-score (p for trend <0.001, 0.02, >0.001, respectively). Mirroring the results for BMI, with a higher WHR CRP, IL-6, and the inflammation z-score were statistically significantly higher by 77%, 98%, and 173%, respectively. The inflammation z-score was strongly associated with being overweight or obese (ORs 4.33 [95% CI 1.04, 18.00] and 5.54 [95% CI 1.37, 22.42], respectively) and having a higher WHR (OR 4.09 [95% CI 1.67, 9.98]). High TNF-α and IL-8 were associated with a high WHR, although their mean levels were not statistically significantly different. Among those who took NSAIDs regularly, TNF-α was statistically significantly higher by 90%, and higher CRP levels were found in participants who were less physically active (107%); however, the ORs for the associations included 1.0. There were no statistically significant findings in relation to age, serum 25-OH-vitamin D levels, or total energy or calcium intakes.

Discussion

The results of this study support a direct association of inflammatory cytokines with adiposity-related risk factors for colorectal cancer. Our findings indicate that associations of risk factors for colorectal neoplasms with inflammation may be more strongly reflected by the use of a combined inflammation z-score than by any single cytokine that reflects only a small aspect of inflammation/immunomodulation.

Previous studies suggested that there is a direct association between colorectal cancer risk and the inflammatory cytokines assessed in this study. Several CRC case-control studies found blood levels of CRP, TNF-α, IL-6, and IL-8 to be higher in cases (69, 184, 185). Expression-enhancing polymorphisms in the genes for IL-6, TNF-α, IL-1β, and IL-8 were associated with increased adenoma risk in two case-control studies (75, 190). IL-1β is involved in COX-2 activation and activates the Wnt cell cycle activation pathway, the primary pathway of colon cell proliferation (191). While these inflammatory cytokines are increasingly supported as risk factors for CRC, more research is needed to examine their usefulness as biomarkers of risk for colorectal adenomas.

Obesity was previously linked to inflammation, and is proposed to be an inflammatory condition (192). CRP was associated with BMI in obese or overweight young adults in a large, cross-sectional study (NHANES III) (193). IL-6, which stimulates CRP release from the liver, is positively correlated with CRP levels in the adipose tissue of obese individuals (192). Obesity, insulin resistance, and atherosclerosis are associated with higher levels of TNF-α, a strong mediator of inflammation and reactive oxygen/nitrogen species (192). We found statistically significant higher levels of CRP, IL-6, and the inflammation z-score in our obese participants, as well as in those

with a higher WHR, further supporting that higher levels of inflammatory cytokines are associated with general and central adiposity in patients at risk for colorectal cancer.

We found no statistically significant associations of sex, age, serum 25-OH-vitamin D levels, NSAID use, or total energy or calcium intakes with inflammatory cytokines or the inflammation z-score. This may have been due to our small sample size and the relatively homogenous population. We found that CRP was statistically significantly higher in women, and TNF-α was statistically significantly higher in NSAID users; however, the ORs for the associations were not statistically significant, possibly due to the small number of women and NSAID users. Larger studies are needed to more adequately assess these possible associations.

CRP stimulates the release of IL-1β, Il-6, IL-8, and TNF-α from mononuclear phagocytes, which supports a collective analysis of these cytokines, such as by using a combined z-score (194). Previously, we reported the effects of vitamin D_3 supplementation on a combined inflammation z-score (including CRP, IL-6, TNF-α, IL-1β, and IL-8) in a randomized, placebo-controlled clinical trial of colorectal adenoma patients (188). We found that, although vitamin D_3 did not significantly reduce each individual cytokine, it statistically significantly reduced the inflammation z-score in this study population by 77% ($p=0.003$) relative to placebo. In this study, it was found that the combined inflammation score was more strongly associated with measures of adiposity than were any of the individual cytokines. In light of these data, we propose further investigation of an inflammation z-score as a biomarker of risk for colorectal adenomas or cancer.

This small cross-sectional study has several limitations. First, the small sample size, especially for important subpopulations (i.e., smokers and NSAID users), limited the analyses and conclusions. Also, the cross-sectional study design does not address temporality and the results may not be generalizable beyond a limited colorectal adenoma patient population. Finally, about half of the study population was obese, with greater representation of overweight individuals than normal weight individuals. While this limits the comparison of obese to normal weight individuals, the high number of obese and overweight individuals allowed for an important comparison between these groups.

Strengths of this study included that it used standardized methods of collecting blood and questionnaire data and assessing vitamin D and cytokine levels. All of the participants had a colorectal adenoma removed in the previous 36 months, and therefore were unlikely to have current colorectal cancer, which could alter cytokine levels. Other strengths of this study included the array of cytokines investigated, which are well supported and linked to colorectal cancer, and the use of a summary z-score to analyze the cytokines collectively.

In summary, our findings in this small cross-sectional study support direct associations of CRP, IL-6 and a combined z-score of inflammatory cytokines with adiposity in colorectal adenoma patients. This study shows that the use of a combined inflammation z-score may have promise in revealing stronger associations of inflammation with colorectal cancer risk than can be discerned by measuring and analyzing only individual aspects of inflammation, a large, complex system. Larger case-control and prospective studies are needed, however, to further assess the validity and

usefulness of these cytokines and the combined inflammation z-score in relation to risk for colorectal neoplasms or other chronic diseases.

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Notes

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Tables

Table 2.1 Selected baseline characteristics of the clinical trial participants

Characteristics	**N=92**
Demographics	
Age (y)	60.7 (8.0)
Men (%)	70.3
White (%)	70.3
College graduate (%)	57.1
Medical history	
History of colorectal cancer in 1° relative (%)	19.8
Take NSAID regularly[1] (%)	17.6
If woman (n = 5), taking estrogens (%)	5.4
Habits	
Current smoker (%)	3.3
Take multivitamin (%)	31.9
Physical activity (METs/d)	18.3 (14.0)
Mean dietary intakes	
Total energy intake (kcal/d)	
Men	1,824 (617)
Women	1,683 (851)
Total fat (g/d)	71.2 (30.9)
Total[2] calcium (mg/d)	761.9 (497.2)
Dietary fiber (g/d)	16.8 (8.9)
Alcohol (g/d)	11.0 (16.9)
Anthropometrics	
Body mass index (kg/m^2)	30.2 (6.1)
Waist-to-hip ratio	0.9 (0.1)
Serum 25-OH-vitamin D (ng/mL)	22.0 (8.5)

NOTE: Data are given as means (SD) unless otherwise specified.
Abbreviations: NSAID, non-steroidal anti-inflammatory drug.
[1] At least once a week.
[2] Diet plus supplements.

Table 2.2 Biomarkers of inflammation according to possible colorectal cancer risk factors in colon adenoma patients

CRC risk factors		Inflammatory biomarkers					
	n	CRP [1]		TNF-α		IL-6	
		Mean (SD)	P[3]	Mean (SD)	P	Mean (SD)	P
Sex							
Male	64	2.38 (2.92)		3.96 (1.72)		2.48 (3.56)	
Female	27	4.23 (4.72)	0.02	4.77 (7.12)	0.39	2.69 (3.77)	0.78
OR (95% CI)[4]		*1.25 (0.45, 3.46)*		*1.25 (0.44, 3.55)*		*1.79 (0.66, 4.83)*	
BMI (kg/m^2)							
1 >25	16	1.80 (3.80)		3.74 (1.76)		1.33 (1.91)	
2 25-29.9	34	2.02 (2.70)		3.82 (1.43)		2.79 (4.44)	
3 ≥30	41	4.30 (4.11)	<0.001	4.69 (5.88)	0.24	2.78 (3.30)	0.02
OR (95% CI) 2 vs.1		*1.86 (0.49, 7.00)*		*1.29 (0.39, 4.25)*		*2.10 (0.56, 7.88)*	
OR (95% CI) 3 vs. 1		*5.20 (1.42, 19.04)*		*1.35 (0.42, 4.32)*		*4.69 (1.29, 17.10)*	
WHR							
Low[5]	42	2.09 (3.14)		4.05 (5.71)		1.65 (2.02)	
High	49	3.72 (4.04)	0.04	4.33 (1.90)	0.75	3.28 (4.43)	0.03
OR (95% CI)		*2.91 (1.02, 8.32)*		*3.59 (1.18, 10.87)*		*1.88 (0.70, 5.00)*	
Age (yrs.)							
≤ 59	45	3.44 (3.20)		4.74 (7.23)		3.22 (4.31)	
> 59	46	2.46 (3.52)	0.22	3.43 (1.67)	0.98	1.85 (2.63)	0.07
OR (95% CI)		*0.41 (0.16, 1.04)*		*1.24 (0.54, 7.83)*		*0.81 (0.33, 1.97)*	
Serum 25-OH-vitamin D (ng/ml)							
≤ 22.3	47	2.69 (3.07)		4.51 (5.44)		2.31 (3.13)	
> 22.3	44	3.28 (4.37)	0.47	3.87 (1.84)	0.46	2.76 (4.08)	0.55
OR (95% CI)		*1.09 (0.42, 2.85)*		*1.58 (0.59, 4.23)*		*1.23 (0.48, 3.16)*	
NSAID[6]							
Non-users	75	2.33 (3.52)		3.62 (1.71)		2.59 (3.82)	
Users	16	3.69 (4.67)	0.08	6.88 (8.79)	0.02	2.25 (2.46)	0.73
OR (95% CI)		*1.06 (0.30, 3.68)*		*2.63 (0.81, 8.51)*		*1.44 (0.44, 4.70)*	
Physical activity (METs/d)							
Low	38	4.64 (4.88)		4.86 (6.03)		2.18 (2.02)	
High	39	2.24 (2.53)	0.02	3.78 (1.78)	0.46	3.59 (4.89)	0.07
OR (95% CI)		*0.59 (0.22, 1.68)*		*1.29 (0.53, 3.13)*		*1.14 (0.45, 2.87)*	
Total energy intake (kcal/d)							
Low	45	3.00 (3.66)		3.47 (1.94)		2.40 (2.96)	
High	46	2.92 (3.82)	0.81	4.41 (5.47)	0.37	2.64 (4.17)	0.62
OR (95% CI)		*1.04 (0.42, 2.59)*		*1.17 (0.51, 2.72)*		*1.01 (0.42, 2.46)*	
Total calcium intake[7] (kg/d)							
Low	46	3.15 (3.62)		4.62 (5.43)		2.58 (3.57)	
High	45	2.78 (3.85)	0.99	3.76 (1.97)	0.39	2.47 (3.68)	0.88
OR (95% CI)		*0.95 (0.42, 2.17)*		*0.80 (0.35, 1.83)*		*0.47 (0.20, 1.08)*	

Table 2.2 (Continued)

CRC risk factors		Inflammatory biomarkers					
	n	IL-8		IL-1β		Inflammation Z-score [2]	
Sex		Mean (SD)	P	Mean (SD)	P	Mean (SD)	P
Male	64	6.47 (4.17)		0.34 (0.61)		-0.13 (2.60)	
Female	27	5.66 (3.27)	0.37	0.40 (0.66)	0.66	-0.03 (3.43)	0.88
OR (95% CI)[4]		*0.77 (0.28, 2.11)*		*1.77 (0.67, 4.63)*		*1.23(0.45, 3.33)*	
BMI (kg/m^2)							
1 >25	16	4.72 (2.01)		0.15 (0.21)		-1.96 (2.85)	
2 25-29.9	34	6.85 (3.94)		0.32 (0.63)		-0.09 (2.63)	
3 ≥30	41	6.31 (4.36)	0.37	0.46 (0.71)	0.19	0.71 (2.78)	<0.001
OR (95% CI) 2 vs.1		*3.55 (1.01, 12.57)*		*1.96 (0.56, 6.85)*		*4.33 (1.04, 18.00)*	
OR (95% CI) 3 vs. 1		*1.90 (0.56, 6.45)*		*3.11 (0.91, 10.58)*		*5.54 (1.37, 22.42)*	
WHR							
Low[5]	42	5.70 (4.35)		0.41 (0.71)		-1.23 (2.67)	
High	49	6.69 (3.49)	0.23	0.31 (0.52)	0.44	0.90 (2.68)	<0.001
OR (95% CI)		*2.69 (1.03, 7.04)*		*1.24 0.50, 3.11)*		4.09 (1.67, 9.98)	
Age (yrs.)							
≤ 59	45	5.60 (3.29)		0.31 (0.53)		-0.22 (3.20)	
> 59	46	6.85 (4.41)	0.13	0.40 (0.70	0.47	0.04 (2.51)	0.68
OR (95% CI)		*0.23 (0.53, 2.84)*		*1.50 (0.60, 3.74)*		1.37 (0.56, 3.34)	
Serum 25-OH-vitamin D (ng/ml)							
≤ 22.3	47	5.91 (3.64)		0.42 (0.70)		-0.26 (3.16)	
> 22.3	44	6.58 (4.22)	0.42	0.29 (0.52)	0.36	0.05 (2.62)	0.63
OR (95% CI)		*1.22 (0.50, 3.00)*		*0.71 (0.28, 1.78)*		1.20 (0.56, 3.05)	
NSAID[6]							
Non-users	75	6.33 (4.14)		0.36 (0.66)		-0.23 (2.85)	
Users	16	5.78 (2.74)	0.61	0.35 (0.44)	0.95	0.53 (2.96)	0.36
OR (95% CI)		*0.75 (0.22, 2.59)*		*1.65 (0.53, 5.13)*		1.24 (0.38, 4.07)	
Physical activity (METs/d)							
Low	38	6.65 (4.79)		0.39 (0.59)		0.56 (2.76)	
High	39	6.18 (3.59)	0.76	0.34 (0.58)	0.74	0.11 (3.03)	0.78
OR (95% CI)		*0.45 (0.18, 1.11)*		*1.19 (0.48, 3.00)*		1.17 (0.46, 2.97)	
Total energy intake (kcal/d)							
Low	45	6.80 (3.80)		0.34 (0.58)		0.43 (2.46)	
High	46	5.68 (4.00)	0.25	0.37 (0.66)	0.90	-0.67 (3.19)	0.14
OR (95% CI)		*2.24 (0.93, 5.41)*		*0.99 (0.41, 2.40)*		1.38 (0.59, 3.43)	
Total calcium intake[7] **(kg/d)**							
Low	46	6.17 (4.13)		0.49 (0.79)		0.39 (2.91)	
High	45	6.29 (3.73)	0.80	0.21 (0.48)	0.09	-0.56 (2.78)	0.28
OR (95% CI)		*0.96 (0.42, 2.18)*		*0.46 (0.16, 1.34)*		0.67 (0.29, 1.53)	

Abbreviations: C-reactive protein, CRP; tumor necrosis factor alpha, TNF-α; interleukin-6, IL-6; interleukin-8, IL-8; interleukin-1 beta, IL-1β; body mass index, BMI; waist-hip ratio, WHR; non-steroidal anti-inflammatory drug, NSAID.

[1] Categories of biomarkers for odds ratio calculations were set at the median for each cytokine: CRP, 1.40 ug/ml; TNF-α, 3.59 pg/ml; IL-6, 1.23 pg/ml; IL-8, 5.11 pg/ml; IL-1β, 0.20 pg/ml; combined inflammation z-score, -0.31.

[2] Inflammation z-score (CRP, TNF-α, IL-6, IL-8, and IL-1β) was calculated by 1) subtracting the mean and dividing by the standard deviation (thus creating a mean of zero and standard deviation of 1.0) for each participant's individual biomarker value, and then 2) summing the biomarker z-score values for each participant.

[3] P value based on *t* test for comparing two means, except for BMI, which is a P for trend from a linear model.

[4] Odds Ratios adjusted for BMI, except WHR, which is adjusted for sex. Physical activity is adjusted for total energy intake, and vice versa.

[5] Throughout the table "Low," below the 50th percentile distribution, and "High," above the 50th percentile distribution; cutoff points for WHR are sex specific (for men, 0.97, women, 0.84); Physical activity, 16 METs/d; Total energy intake, 1,704 kcal/d; Calcium intake, 629.6 mg/d.

[6] ≥ once per week, not including aspirin.

[7] Supplements plus dietary intake.

CHAPTER 4. EFFECTS OF SUPPLEMENTAL VITAMIN D AND CALCIUM ON BIOMARKERS OF INFLAMMATION IN COLORECTAL ADENOMA PATIENTS: A RANDOMIZED, CONTROLLED CLINICAL TRIAL

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Abstract

Vitamin D and calcium affect several pathways involved in inflammation, tumor growth, and immune surveillance relevant to carcinogenesis. Also, epidemiologic evidence indicates that calcium and vitamin D may reduce risk for colorectal adenomas and cancer. To investigate the effects of calcium and vitamin D on biomarkers of inflammation in colorectal adenoma patients, we conducted a pilot, randomized, double-blind, placebo-controlled, 2x2 factorial clinical trial (n=92), of 2 g/day calcium and/or 800 IU/day vitamin D_3 supplementation vs. placebo over six months. Plasma concentrations of pro-inflammatory markers (CRP, TNF-α, IL-6, IL-1β, and IL-8) and an anti-inflammatory marker (IL-10) were measured using enzyme-linked immunoassays. After six months of treatment, in the vitamin D_3 supplementation group, CRP decreased 32% overall (p=0.11), 37% in men (p=0.05), and 41% among non-NSAID users (p=0.05) relative to placebo. In the vitamin D_3 supplementation group, TNF-α decreased 13%, IL-6 32%, IL-1β 50%, and IL-8 15%; in the calcium supplementation group, IL-6 decreased 37%, IL-8 11%, and IL-1β 27%. Although these changes were not statistically significant, a combined inflammatory markers z-score decreased 77% (p=0.003) in the vitamin D_3 treatment group overall, 83% (p=0.01) among men, and 48% among non-NSAID users (p=0.01). There was no evidence of synergy between vitamin D_3 and calcium or effects on IL-10. These preliminary results are consistent with a pattern of reduction in tumor-promoting inflammation biomarkers with vitamin D_3 or calcium supplementation alone, and support further investigation of vitamin D_3 as a chemopreventive agent against inflammation and colorectal neoplasms.

Introduction

Colorectal cancer (CRC) is the second leading cause of cancer mortality in the United States and is consistently inversely associated with calcium intake and serum vitamin D levels (1, 18, 19, 111, 139, 148, 195-197). Inflammation is intricately linked to the etiology of colorectal cancer, and may also be a key in understanding the mechanisms linking calcium and vitamin D to colorectal cancer risk reduction. Inflammatory conditions such as Crohn's disease and ulcerative colitis are established risk factors for colorectal cancer, nonsteroidal anti-inflammatory drug (NSAID) use reduced both polyposis in FAP patients and sporadic colorectal adenoma recurrence in clinical trials, and specific pro-inflammatory markers, such as C-reactive protein (CRP), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6), are elevated in inflammatory bowel disease patients (71, 73, 87, 184, 185). These inflammatory markers are also associated with neoplastic growth, higher tumor grade, and increased risk of mortality in colorectal cancer patients (61, 69, 75, 184-187). In addition, in a case-control study, risk factors for colorectal adenomas, such as old age, smoking, and adiposity, were found to be associated with higher levels of these inflammatory markers (71).

The mechanisms by which calcium is proposed to reduce risk for colorectal cancer are closely related to inflammation. Calcium binds to free fatty acids and bile acids, precipitating them from solution in the colon, which is hypothesized to reduce oxidative stress and inflammation in the colon (136). Calcium also activates the calcium sensing receptor, which is involved in cell-cycle events and differentiation, and promotes cell-cell and cell-matrix adhesion (135, 198). Vitamin D, along with increasing the absorption of

calcium and regulating calcium homeostasis, also regulates more than 200 genes through the vitamin D receptor (VDR). Activation of the VDR is involved in bile acid degradation, direct transcriptional regulation of several inflammatory cytokines, cell cycle regulation, DNA repair, differentiation, and apoptosis (198, 199).

Despite the basic science evidence, there are no published trials of the effects of vitamin D and/or calcium supplementation on blood markers of inflammation in patients at risk for developing colorectal cancer. To address this, we conducted a pilot, randomized, double-blind, placebo-controlled 2x2 factorial chemoprevention trial of calcium and vitamin D_3 supplementation, alone or in combination, versus placebo over six months to estimate their effects on a panel of circulating pro- and anti-inflammatory markers in patients with a history of sporadic colorectal adenoma. We hypothesized that vitamin D_3 and calcium, alone or in combination, would decrease tumor-promoting pro-inflammatory markers, and increase tumor-inhibiting, anti-inflammatory markers.

Materials and Methods

This study was approved by the Emory University Institutional Review Board. Written informed consent was obtained from each study participant. The study reported herein was based on questionnaire data and biological samples collected in a previously conducted and reported chemoprevention trial (189).

Study population

The detailed protocol of study recruitment and procedures was published previously (189). Briefly, study participants were recruited from the patient population attending the Digestive Diseases Clinic of Emory University. Eligibility included age 30 to 75 years,

in good general health, capable of informed consent, and at least one pathology-confirmed sporadic colon or rectal adenoma in the past 36 months. Exclusions included contraindications to calcium or vitamin D_3 supplementation or rectal biopsy procedures, and medical conditions, habits, or medication usage that would otherwise interfere with the study (189).

Clinical trial protocol

Between April 2005 and January 2006, potential participants attended an eligibility visit during which they were interviewed, signed a consent form, completed questionnaires, provided a blood sample, and were entered into a 30-day placebo run-in trial. Diet was assessed with a semi-quantitative food frequency questionnaire (181). After the 30-day placebo run-in trial, 92 participants without significant perceived side effects who had taken at least 80% of their capsules during the run-in trial were eligible for randomization. Eligible participants then underwent a baseline blood draw and rectal biopsy and were randomly assigned (stratified by sex and nonsteroidal anti-inflammatory drug use) to the following four treatment groups: a placebo control group (n = 23), a 2.0 g elemental calcium (1 g twice daily as calcium carbonate) group (n = 23), an 800 IU vitamin D_3 (400 IU twice daily) group (n = 23), and a 2.0 g elemental calcium plus 800 IU vitamin D_3 group (n = 23).

Study tablets were custom manufactured by Tishcon Corp. (Westbury, New York). The corresponding supplement and placebo pills were identical in size, appearance, and taste. The placebo was free of calcium, magnesium, vitamin D, and chelating agents. Additional details and rationale for the doses and forms of calcium and vitamin D supplementation were described previously (189).

Over the 6-month treatment period, participants attended follow-up visits at 2 and 6 months after randomization and were contacted by telephone at monthly intervals between the second and final follow-up visits. At follow-up visits, pill-taking adherence was assessed by questionnaire, interview, and pill count. Adverse events were monitored by interview at each study visit, interim telephone call, and questionnaires and graded according to NIH Common Toxicity Criteria 2.0 and the likelihood that they were study related. Participants were instructed to remain on their usual diet and not take any nutritional supplements not in use on entry into the study. At each follow-up visit, participants were interviewed and completed questionnaires. At the first and last visits, all participants underwent venipuncture and a rectal biopsy procedure. Participants were asked to abstain from aspirin (but not non-aspirin NSAIDs) use for 7 days before each biopsy/blood draw visit. All visits for a given participant were scheduled at the same time of day to control for possible circadian variability in the outcome measures. Factors hypothesized to be related to inflammatory cytokines (e.g., diet and NSAID use) were assessed at baseline, several were reassessed at the first follow-up visit, and all were reassessed at the final follow-up visit.

Peripheral venous blood samples were taken after the subject sat upright with their legs uncrossed for five minutes. Blood was drawn into red-coated, pre-chilled vacutainer tubes for whole blood, plasma, and serum, and then immediately placed on ice and shielded from light. Blood fractions were aliquotted into amber-colored cryopreservation tubes, the air was displaced with argon gas, and then the aliquots were immediately placed in a -80^{o} C freezer until analysis.

Inflammation biomarker analyses

All samples were blinded to treatment group and treated identically. A single enzyme linked immunoassay (ELISA) (R&D systems, Minneapolis, MN) was used to measure CRP, in duplicate, according to the manufacturer's protocol. The average intra-assay coefficient of variation (CV) for CRP was 6.6%. A High Sensitivity Multiplex enzyme linked immunoassay (R&D systems, Minneapolis, MN) was used to measure TNF-α, IL-6, IL-1β, IL-8, IL-5, IL-4, VEGF, IL-2, IL-10, IL-12, GM-CSF, and IFN-γ, in duplicate, according to the manufacturer's protocol. The average intra-assay coefficient of variation (CV) for TNF-α was 11.5%, for IL-6 11.7%, for IL-1β 10.6%, for IL-8 7.9%, for IL-5 34.1%, for IL-4 39.4%, for VEGF 21.0%, for IL-2 45.0%, for IL-10 11.5%, for IL-12 24.5%, and for GM-CSF 38.5%. Low plasma cytokine concentrations create very high variability, and the results for cytokines with CVs above 15% were considered too variable and inaccurate to be reported.

Statistical analysis

Treatment groups were assessed for comparability of characteristics at baseline and final follow-up by the Fisher's exact test for categorical variables and ANOVA for continuous variables. ELISA reliability was assessed using coefficients of variation.

Primary analyses were based on assigned treatment at the time of randomization regardless of adherence (intent-to-treat analysis). Biomarker levels below the limits of detection were assigned a value equal to the lower limit of detection for that biomarker. Variables not normally distributed were transformed, as appropriate, before statistical testing. Mean biomarker concentrations were calculated for each treatment group for the baseline and six-month follow-up visits. Treatment effects were evaluated by assessing

the differences in biomarker concentrations from baseline to 6-months follow-up between each active treatment group and the placebo group by a repeated-measures linear mixed effects model, as implemented using the Proc MIXED procedure of the Statistical Analysis System (SAS, version 9.2 Copyright© 2002-2008 by SAS Institute Inc., Cary, NC, USA). The model included the intercept, indicators for treatment group and visit (baseline and follow-up), and a treatment by visit interaction term. Study participant was treated as a random effect, and absolute treatment effects were calculated and reported. A cutoff level of $P \leq 0.05$ (two-sided) was used for assessing statistical significance. Since concentrations of the measured biomarkers in plasma are not widely familiar, to provide perspective on the magnitude of treatment effects, relative effects were also calculated, defined as (treatment group follow-up/treatment group baseline)/(placebo follow-up/placebo baseline) (110, 189). The relative effect provides a conservative estimate of the average proportional change in the treatment group relative to that in the placebo group. The interpretation of the relative effect is somewhat analogous to that of an odds ratio (e.g., a relative effect of 2.0 means that the relative proportional change in the treatment group was twice as great as that in the placebo group). Stratified analyses were conducted to investigate potential differential treatment effects by sex, age, BMI, and NSAID use.

To assess the effects of vitamin D_3 and/or calcium supplementation on a summary score of all the pro- and anti-inflammatory markers combined, a summary inflammation z-score was calculated. This score was calculated as follows: first, a normalized z-score for each individual biomarker value, with a mean of zero and standard deviation of 1.0, was calculated as $z = (x - \mu)/\sigma$, where x is a participant's biomarker value at a given visit,

and μ and σ are the study population mean and standard deviation, respectively, at baseline; and then the combined inflammation z-score for each participant at each trial visit was created by summing the z-scores of each inflammatory marker (IL-10 was included with a negative sign, because it has been shown to protect against colonic inflammation (200)). This inflammation z-score was then analyzed as for the individual biomarkers.

Results

Study participants

Treatment groups were quite similar on characteristics measured at baseline (Table 2.1) or at final follow-up (data not shown) and no participants changed their NSAID user status during the trial. The mean age of participants was 61 years, 70% were men, 71% were white, and 20% had a family history of colorectal cancer in a first-degree relative. Adherence to visit attendance averaged 92% and did not differ significantly among the four treatment groups. On average, at least 80% of pills were taken by 93% of participants at the first follow-up visit and 84% at the final follow-up visit. There were no complications attributed to study procedures or treatments. Seven participants (8%) were lost to follow-up due to perceived drug intolerance (n = 2), unwillingness to continue participation (n = 3), physician's advice (n = 1), and death attributed to cardiovascular disease (n = 1). Participant dropouts from the trial included one person from the vitamin D_3 supplementation group and two persons from each of other three groups. During the trial, the only serious adverse event during the trial was one death due

to advanced cardiovascular disease, and there were no differences in monitored symptoms according to treatment group.

At baseline, there were no significant differences between the four study groups in serum 25-OH-vitamin D. By study end, serum 25-OH-vitamin D levels statistically significantly ($p<0.0001$) increased by 60% to 29.5 ng/ml in the vitamin D_3 group and by 56% to 28.5 ng/ml in the calcium plus vitamin D_3 group relative to placebo (189).

Changes in CRP, TNF-α, IL-6, IL-1β, IL-8, and IL-10 plasma concentrations relative to placebo in the calcium, vitamin D_3, or combined supplementation groups are shown in Table 2.2. After six months of treatment, in the vitamin D_3 supplementation group, CRP decreased by 32%, TNF-α by 13%, IL-6 by 32%, IL-1β by 50%, and IL-8 by 15%, relative to placebo, although these changes were not statistically significant. In the calcium supplementation group, relative to placebo, CRP decreased 8%, IL-6 decreased 37%, IL-8 by 11%, and IL-1β by 27%, although these changes were also not statistically significant. In the vitamin D_3 plus calcium supplementation group, IL-6 decreased by 8%, IL-8 by 13%, and IL-1β by 35%, relative to placebo, although these changes were not statistically significant. IL-10 decreased a minor non-significant amount in all active treatment groups.

The effects of vitamin D_3 and/or calcium on the combined "inflammation z-score" of all reported inflammatory markers (CRP, TNF-α, IL-6, IL-8, IL-1β, and IL-10) are summarized in Table 2.3. An individual's inflammation z-score allows for the calculation of an aggregate score of all biomarkers by converting them to a comparable score, a z-score, and then totaling the values for each individual. The overall inflammation z-score significantly dropped 77% ($p=0.003$) in the vitamin D_3 treatment

group, 48% (p=0.18) in the calcium treatment group, and 33% (p=0.40) in the combined treatment group relative to placebo.

Men and women have differences in their biochemical makeup (such as estrogen levels) that could lead to differences in response to vitamin D and calcium supplementation; therefore, we investigated potential differences in response by sex (Table 2.4). In men, CRP decreased 37% (p=0.05) in the vitamin D_3 treatment group relative to placebo, but did not change substantially in women. Similar to the results for CRP, the inflammation z-score statistically significantly dropped in men (83%, p=0.01) but not in women in the vitamin D_3 treatment group relative to placebo. Changes in TNF-α, IL-6, IL-8, IL-1β, and IL-10 did not differ substantially by sex (data not shown).

Since NSAID use may overwhelmingly affect inflammation pathways, we investigated the effects of vitamin D_3 and calcium among study participants who were not currently taking NSAIDs (Table 4). In non-NSAID users, the decrease in CRP (41%; p=0.05) was slightly stronger than in all participants combined (32%; p=0.11) in the vitamin D_3 treatment group relative to placebo. The inflammation z-score also decreased significantly by 58% (p=0.01) among non-NSAID users in the vitamin D_3 treatment group. Changes in TNF-α, IL-6, IL-8, IL-1β, and IL-10 among non-NSAID users did not differ substantially from changes among all participants combined (data not shown).

Discussion

The results from this pilot, randomized, controlled clinical trial suggest that supplementation with vitamin D_3 or calcium alone may decrease tumor-promoting pro-inflammatory markers in the plasma of sporadic colorectal adenoma patients. These

findings are consistent with the hypothesis that higher intakes of vitamin D_3 or calcium may decrease inflammation in the colon, and thus reduce risk for colorectal neoplasms. Consistent with previous findings in this same study on oxidative DNA damage in the normal colorectal mucosa (201), our findings also suggest that vitamin D_3 combined with calcium may have a lesser treatment effect on pro-inflammatory markers than do vitamin D_3 or calcium alone.

Inflammation is intricately linked to the etiology of CRC, as evidenced by inflammatory conditions of the colon, such as Crohn's disease and ulcerative colitis, which are established risk factors for the disease (3). Several inflammatory molecules, including CRP, TNF-α, IL-6, and IL-8, were found to be higher in the blood of CRC patients than in controls (69, 184, 185), and have been associated with other risk factors for CRC, such as age, smoking, and high BMI (71). In addition, CRP, TNF-α, and IL-6 are associated with higher tumor grade and poorer prognosis (69, 186), and higher levels of CRP and IL-6 are associated with increased mortality among colorectal cancer patients (184). In a case-control study, polymorphisms in the genes for IL-6, TNF-α, IL-1β, and IL-8 that are linked to increased expression of their corresponding cytokines were associated with increased adenoma risk (75, 190). IL-1β is involved in COX-2 activation and activates the Wnt cell cycle activation pathway, the primary pathway of colon cell proliferation (191). Vitamin D_3 inhibited this pathway *in vitro* by decreasing IL-1β production by macrophages, thus decreasing colon carcinoma cell proliferation (191).

Calcium and vitamin D have several mechanisms of action relevant to our hypothesis that they may decrease inflammatory markers and risk for CRC. Only about 30% of calcium is absorbed in the GI tract, with the other 70% free to bind with and precipitate

bile acids, which have been shown to cause damage to epithelial cell membranes and produce an inflammatory response in these cells (138, 202). This inflammatory response, in turn, may represent a large source of circulating cytokines. Vitamin D, acting through the vitamin D receptor, also reduces bile acids in the colon by increasing the bile acid catabolizing enzyme CYP3A4 (135, 203). 1-25-$(OH)_2$-vitamin D binding of the vitamin D receptor acts as a transcriptional regulator to enhance IL-10 transcription, and represses several pro-inflammatory cytokines, including IL-6, IL-8, and TNF-α (110, 204). In addition, the vitamin D receptor, when activated by vitamin D, suppresses the transcription of RelB, a component of the global transcriptional regulator NF-κB (205), a key regulator of inflammation and response to oxidative stress and a downstream target of TNF-α (178). NF-κB induces the transcription of inflammatory cytokines and anti-apoptotic proteins that together promote cellular transformation and tumor formation (179). Mice lacking IL-10 quickly develop inflammatory bowel disease, but supplementation with vitamin D_3 ameliorated symptoms and blocked the progression of the disease (200). Combined with this biological evidence, the results of our study support vitamin D_3 and calcium as possible inflammation-reducing agents in humans.

Contrary to our original hypothesis, and the findings of some epidemiological and clinical studies, we found no evidence for a greater than additive effect of combined supplementation of calcium and vitamin D_3 (201, 206-209). Our estimated treatment effects in the calcium plus vitamin D_3 group tended to be less than those for the individual agents. In this same population, we previously reported that combined calcium and vitamin D_3 supplementation may have lesser effects on colorectal epithelial apoptosis, differentiation, and oxidative DNA damage than do calcium or vitamin D_3

alone (189, 201, 210). These statistically non-significant findings of a smaller treatment effect in the combined treatment group may simply be due to chance because of our small sample size. However, given the consistency of this pattern, it is possible that calcium and vitamin D negatively regulate one another. 1,25-$(OH)_2$-vitamin D_3 regulates calcium absorption, and calcium suppresses 1,25-$(OH)_2$-vitamin D_3 synthesis by 1α-hydroxylase (123). One animal study found that high calcium supplementation led to lower circulating levels of 25-OH-vitamin D (203); however, in humans, risk of adenoma recurrence was only decreased by calcium supplementation in individuals with higher serum 25-OH-vitamin D levels (207). In human colon carcinoma cells, calcium and vitamin D synergistically enhanced the expression of E-cadherin; however, the enhanced expression of p21 and p27 by calcium and vitamin D separately was not changed with a combined treatment (211). Another possible explanation for the less than additive effects of calcium plus vitamin D_3 is that too little vitamin D_3 was given. Although 800 IU daily vitamin D_3 supplementation in this population statistically significantly raised serum 25-OH-vitamin D levels, the mean in all treatment and placebo groups was below 32 ng/ml, the suggested level to be considered sufficient for this vitamin (189, 212). Taken together, the combined effect of calcium and vitamin D_3 on biomarkers of colon carcinogenesis and inflammation in humans is unclear, and requires further clarification through larger studies.

Calcium and vitamin D have several known and likely unknown downstream targets involved in inflammation regulation as discussed above, and, therefore, biological effects of these agents may be best measured using a combined detection method. We developed an inflammation z-score to assess the inflammation status of an individual

more comprehensively, and then analyzed the effects of calcium and/or vitamin D_3 on this inflammation z-score. We hypothesized that vitamin D_3 and/or calcium would affect this inflammation z-score more substantially than any single measure of inflammation. Vitamin D_3, but not calcium or the two combined, significantly reduced the inflammation z-score in this study population by 77% ($p=0.003$) relative to placebo. This finding suggests that vitamin D_3 may reduce inflammation in multi-factorial ways. Inflammatory markers, including CRP, IL-6, and TNF-α, were found to be significantly higher in colorectal cancer patients than in controls (69, 184, 185); however, it is not known whether these individual markers are also elevated in colorectal adenoma patients. We propose the use of this inflammation z-score to measure sub-clinical inflammation or to detect small changes in multiple cytokines that combined may produce clinically important changes in inflammation and risk for disease. Further investigation is needed, however, and this score should be explored in cohort and case-control studies to investigate whether it is associated with risk for colorectal adenomas or cancer, as well as in larger chemoprevention trials to investigate its usefulness as an intervention response marker.

In our analysis stratified by sex, there was a significant reduction in CRP and the inflammation z-score with vitamin D_3 supplementation in men but not in women. There are several possible explanations for this, the most obvious one being chance related to the small sample size, especially in women. Another possible explanation is that most women in this study were post-menopausal and not taking hormone replacement therapy, and, therefore, likely had low estrogen levels. Estrogen supplementation was found to increase 1-25-$(OH)_2$-vitamin D signaling and down-regulate inflammation pathways in

the rectal epithelium of postmenopausal women (213). The findings of our study support the hypothesis that low estrogen levels may interfere with response to vitamin D supplementation, VDR signaling, and inflammatory pathways; however, larger studies are needed to investigate these issues more definitively.

When only non-NSAID users were considered, CRP and the inflammation z-score were found to be statistically significantly reduced with vitamin D_3 supplementation. Other than chance due to the small sample size, a possible explanation is that NSAIDs have powerful effects on inflammation pathways that could mask effects of vitamin D_3 or calcium. NSAIDs largely reduce risk for colorectal cancer by blocking a major colon carcinogenesis and inflammatory pathway enzyme, COX-2 (214, 215). Vitamin D supplementation effects on inflammation may only be detectable and important in individuals not already using NSAIDs, although more investigation is needed to clarify this issue.

Our pilot study has several limitations and strengths. First, the sample size was small, limiting the statistical power for detecting treatment effects. A second potential limitation to the study is that all of the blood biomarker analyses except for CRP were done using a high-sensitivity multiplex ELISA. While the low limit of detection allowed for a higher number of samples with detectable analytes, the measurements may have been less reliable and accurate than would have been found with non-multiplex ELISA. We accounted for this lower accuracy of the ELISA by calculating coefficients of variation (CV), and did not report data for those cytokines with a CV > 20%. Third, with the relatively low dose of vitamin D_3 supplementation, although serum 25-OH-vitamin D levels increased significantly, the average serum levels did not reach the “sufficiency”

range of above 32 ng/ml (189, 212). Therefore, higher doses of supplemental vitamin D_3 may produce more definitive changes in pro-inflammatory markers.

Strengths of this study included the randomized, double-blind, placebo-controlled clinical trial design; the high protocol adherence by the study participants; investigation of both the individual and combined effects of calcium and vitamin D_3; and the balance in the treatment groups on many potential confounding risk factors for colorectal cancer and inflammation.

In summary, our preliminary findings suggest that vitamin D_3 or calcium alone may decrease tumor-promoting pro-inflammatory markers in the plasma of sporadic colorectal adenoma patients. Also, taken together with previous literature, this study supports further investigation of a) vitamin D_3 or calcium supplementation for reducing inflammatory biomarkers in sporadic colorectal adenoma patients, b) our investigated biomarkers of inflammation or a combined inflammation z-score as potential treatable biomarkers of risk for colorectal cancer, and c) a larger trial with higher doses of vitamin D_3 on biomarkers of inflammation and risk for colorectal neoplasms.

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Notes

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Tables

Table 3.1 Selected baseline characteristics of the clinical trial participants

Characteristics	Treatment group				
	Placebo (n = 23)	Calcium (n = 23)	Vitamin D (n = 23)	Calcium + Vit. D (n = 23)	P*
Demographics					
Age (y)	58.5 (7.9)	61.9 (8.0)	60.2 (8.1)	61.7 (7.4)	0.39
Men (%)	70	70	70	70	1.00
White (%)	74	83	65	61	0.39
College graduate (%)	65	61	57	44	0.53
Medical history					
History of colorectal cancer in 1° relative (%)	17	30	17	13	0.60
Take NSAID regularly† (%)	17	13	13	30	0.26
If woman (n = 5), taking estrogens (%)	4	9	4	4	1.00
Habits					
Current smoker (%)	9	4	0	0	0.61
Take multivitamin (%)	30	30	26	39	0.86
Mean dietary intakes					
Total energy intake (kcal/d)	1,596 (528)	1,788 (691)	1,848 (821)	1,845 (752)	0.59
Total calcium‡ (mg/d)	618 (308)	746 (335)	843 (526)	824 (714)	0.41
Total vitamin D‡ (IU/d)	277 (230)	336 (202)	360 (317)	415 (316)	0.40
Total fat (g/d)	67 (32)	72 (35)	70 (32)	74 (28)	0.59
Dietary fiber (g/d)	15 (7)	17 (9)	18 (9)	17 (11)	0.97
Alcohol (g/d)	9 (14)	11 (15)	14 (18)	10 (20)	0.84
Anthropometrics					
Body mass index (kg/m^2)	30.6 (7.2)	29.4 (5.5)	29.0 (5.56)	31.6 (6.0)	0.44
Waist-to-hip ratio	0.9 (0.1)	0.9 (0.1)	0.9 (0.1)	1.0 (0.1)	0.17
25-OH-vitamin D (ng/mL)	20.4 (7.6)	25.7 (7.6)	21.0 (8.3)	20.9 (9.7)	0.12

NOTE: Data are given as means (SD) unless otherwise specified.
Abbreviations: NSAID, nonsteroidal anti-inflammatory drug.
* By Fisher's exact test for categorical variables and by ANOVA for continuous variables.
† At least once a week, at baseline.
‡ Diet plus supplements.

Table 3.2 Changes in biomarkers of inflammation in plasma of colorectal adenoma patients

Biomarkers	Baseline				6-month follow-up				Absolute treatment effect†		Relative treatment effect‡
	n	Mean	SD	P*	n	Mean	SD	P§	Mean (95% CI)	P*	
Pro-inflammatory											
CRP# (μg/ml)											
Placebo	22	1.77	3.80	N/A	21	1.88	4.16	0.77	N/A	N/A	1.00
Calcium	21	1.13	3.50	0.21	21	1.09	4.32	0.85	-0.09 (-0.40, 0.56)	0.71	0.92
Vitamin D	22	1.39	2.79	0.49	22	0.99	1.97	0.05	-0.39 (-0.91, 0.86)	0.11	0.68
Calcium + Vit D	21	1.93	2.94	0.82	21	2.21	3.06	0.42	0.08 (-0.40, 0.57)	0.74	1.09
TNF-α# (pg/ml)											
Placebo	23	4.13	1.92	N/A	21	4.57	2.05	0.09	N/A	N/A	1.00
Calcium	23	3.04	1.94	0.28	21	3.35	1.88	0.67	0.06 (-0.21, 0.33)	0.66	1.06
Vitamin D	22	2.92	1.78	0.09	22	2.73	2.52	0.46	-0.14 (-0.40, 0.13)	0.30	0.87
Calcium + Vit D	23	3.62	1.75	0.50	21	4.00	1.62	0.28	0.03 (-0.23, 0.30)	0.81	1.03
IL-6# (pg/ml)											
Placebo	23	1.13	4.54	N/A	21	1.41	2.67	0.30	N/A	N/A	1.00
Calcium	23	1.09	3.65	0.45	21	0.85	3.29	0.27	-0.46 (-1.07, 0.14)	0.13	0.63
Vitamin D	22	0.78	4.68	0.42	22	0.67	3.76	0.47	-0.38 (-0.98, 0.23)	0.22	0.68
Calcium + Vit D	23	1.39	4.49	0.63	21	1.62	3.25	0.50	-0.08 (-0.69, 0.53)	0.80	0.92
IL-8# (pg/ml)											
Placebo	23	4.74	1.59	N/A	21	5.01	1.78	0.10	N/A	N/A	1.00
Calcium	23	5.97	1.72	0.51	21	5.34	1.52	0.56	-0.12 (-0.49, 0.22)	0.45	0.89
Vitamin D	22	5.60	1.52	0.30	22	5.03	1.79	0.62	-0.15 (-0.62, 0.08)	0.13	0.85
Calcium + Vit D	23	5.61	1.68	0.63	21	5.09	1.54	0.60	-0.14 (-0.50, 0.21)	0.42	0.87
IL-1β# (pg/ml)											
Placebo	23	0.22	2.03	N/A	21	0.23	2.64	0.56	N/A	N/A	1.00
Calcium	23	0.27	3.82	0.56	21	0.22	3.06	0.62	-0.32 (-1.16, 0.51)	0.44	0.73
Vitamin D	22	0.16	2.04	0.30	22	0.13	2.28	0.07	-0.70 (-1.53, 0.12)	0.09	0.50
Calcium + Vit D	23	0.27	2.62	0.59	21	0.23	2.24	0.40	-0.43 (-1.27, 0.41)	0.31	0.65
Anti-inflammatory											
IL-10# (pg/ml)											
Placebo	23	0.54	1.57	N/A	21	0.53	1.96	0.63	N/A	N/A	1.00
Calcium	23	0.58	1.68	0.63	21	0.50	1.56	0.16	-0.07 (-0.29, 0.15)	0.52	0.93
Vitamin D	22	0.48	1.53	0.37	22	0.43	1.38	0.23	-0.05 (-0.27, 0.16)	0.62	0.95
Calcium + Vit D	23	0.26	1.49	0.66	21	0.55	1.55	0.35	-0.04 (-0.26, 0.19)	0.75	0.96

Abbreviations: C-reactive protein, CRP; Tumor necrosis factor alpha, TNF-α; Interleukin-6, IL-6; Interleukin-8, Il-8; Interleukin-1 beta, IL-1β; Interleukin-10, IL-10.

† Absolute treatment effect is the absolute change from baseline to follow-up in the treatment group minus the absolute change from baseline to follow-up in the placebo group from mixed model.

‡ Relative treatment effect is defined as: (treatment group follow-up /treatment group baseline)/(placebo follow-up /placebo baseline). The interpretation of the relative effect

is similar to that of an odds ratio (e.g., a relative effect of 2.0 would mean that the relative proportional change in the treatment group was twice as great as that in the placebo group).

* P values for difference between each treatment group and placebo group from mixed model.

[§] P values for difference between follow-up visit and baseline visit from mixed model.

[#] Geometric means with standard errors are reported, calculated by exponentiating the mean of the log transformed values.

Table 3.3 Changes in inflammation Z-score in plasma of colorectal adenoma patients

		Baseline		6-month follow-up		Absolute treatment effect[†]		Relative treatment effect[‡]
Inflammation z-score[§]	n	Mean	SD	Mean	SD	Mean (95% CI)	P[*]	
Placebo	23	0.20	2.96	0.69	3.48	N/A	N/A	1.00
Calcium	23	-0.35	2.79	-0.50	2.94	-0.65 (-1.62, 0.31)	0.18	0.52
Vitamin D	22	-0.81	2.66	-1.78	2.74	-1.47 (-2.42, -0.51)	0.003	0.23
Calcium + Vit D	23	0.60	3.05	0.69	2.99	-0.41 (-1.37, 0.55)	0.40	0.67

[†] Absolute treatment effect is the absolute change from baseline to follow-up in the treatment group minus the absolute change from baseline to follow-up in the placebo group from mixed model.

[‡] Relative treatment effect is defined as: (treatment group follow-up /treatment group baseline)/(placebo follow-up /placebo baseline).

[§] Inflammation z-score: Z-score of pro- and anti-inflammatory markers (CRP, IL6, IL-1β, TNF-α, IL-8 and IL-10) calculated by 1) subtracting the mean and dividing by the standard deviation (thus creating a mean of zero and standard deviation of 1.0) for each participants individual biomarker value at each visit, and then 2) summing the biomarker z-score values for each participant at each visit (IL-10 was included with a negative sign).

* P values for difference between each treatment group and the placebo group from mixed model.

Table 3.4 Changes in plasma CRP and inflammation z-score levels stratified by sex and NSAID use in colorectal adenoma patients

		Baseline		6-month follow-up		Absolute treatment effect[†]		Relative treatment effect[‡]
CRP (μg/ml)	n	Mean	SD	Mean	SD	Mean (95% CI)	P[*]	
Women								
Placebo	7	3.55	3.50	2.99	2.85	N/A	N/A	1.00
Calcium	7	1.78	4.51	2.07	5.66	0.32 (-0.91, 1.55)	0.60	1.38
Vitamin D	6	1.52	3.32	0.98	1.89	-0.27 (-1.55, 1.00)	0.66	0.76
Calcium + Vit D	7	2.65	4.13	2.37	4.06	-0.06 (-1.17, 1.28)	0.92	0.94
Men								
Placebo	16	1.28	3.61	1.48	4.79	N/A	N/A	1.00
Calcium	16	0.90	2.98	0.79	3.51	-0.28 (-0.74, 0.19)	0.24	0.76
Vitamin D	16	1.34	2.70	0.99	2.04	-0.45 (-0.90, -0.01)	0.05	0.63
Calcium + Vit D	16	1.65	2.42	2.14	2.74	0.11 (-0.36, 0.57)	0.64	1.12
Non-NSAID users[#]								
Placebo	19	1.37	3.59	1.51	4.13	N/A	N/A	1.00
Calcium	20	1.13	3.80	1.07	4.47	0.22 (-0.70, 0.37)	0.43	1.25
Vitamin D	20	1.55	2.64	1.02	1.90	-0.53 (-1.05, -0.01)	0.05	0.59
Calcium + Vit D	16	2.19	2.83	3.05	2.82	-0.17 (-0.34, 0.78)	0.53	0.84
Inflammation Z-Score[§]								
Women								
Placebo	7	2.15	3.91	2.32	3.94	N/A	N/A	1.00
Calcium	7	-1.39	2.78	-1.45	3.39	-0.23 (-1.91, 1.44)	0.77	0.79
Vitamin D	6	-1.07	2.50	-1.68	3.70	-0.78 (-2.52, 0.96)	0.36	0.46
Calcium + Vit D	7	-0.06	3.74	0.37	3.12	-0.14 (-1.54, 1.81)	0.87	0.87
Men								
Placebo	16	-0.72	1.94	-0.12	3.05	N/A	N/A	1.00
Calcium	16	0.17	2.75	-0.03	2.69	-0.85 (-2.09, 0.39)	0.18	0.42
Vitamin D	16	-0.71	2.79	-1.82	2.44	-1.75 (-2.95, -0.55)	0.01	0.17
Calcium + Vit D	16	0.88	2.77	0.86	3.03	-0.67 (-1.91, 0.57)	0.29	0.51
Non-NSAID users[#]								
Placebo	19	-0.10	2.84	0.20	3.19	N/A	N/A	1.00
Calcium	20	-0.60	3.57	-0.84	2.88	-0.56 (-1.57, 0.46)	0.28	0.57
Vitamin D	20	-0.80	2.51	-1.89	2.67	-1.42 (-2.41, -0.45)	0.01	0.42
Calcium + Vit D	16	0.02	2.42	1.08	3.01	-0.08 (-1.14, 0.98)	0.89	0.92

Abbreviations: C-reactive protein, CRP; nonsteroidal anti-inflammatory drug, NSAID.

[†] Absolute treatment effect is the absolute change from baseline to follow-up in the treatment group minus the absolute change from baseline to follow-up in the placebo group from mixed model.

[‡] Relative treatment effect is defined as: (treatment group follow-up /treatment group baseline)/(placebo follow-up /placebo baseline).

* P values for difference between each treatment group and the placebo group from mixed model.

[§] Inflammation z-score: Z-score of pro- and anti-inflammatory markers (CRP, IL6, IL-1β, TNF-α, IL-8 and IL-10) calculated by 1) subtracting the mean and dividing by the standard deviation (thus creating a mean of zero and standard deviation of 1.0) for each participants individual biomarker value at each visit, and then 2) summing the biomarker z-score values for each participant at each visit (IL-10 was included with a negative sign).
[#] NSAID user status at baseline.

CHAPTER 5. CONCLUSIONS AND FUTURE DIRECTIONS

Observational epidemiological studies have indicated that colorectal cancer is due in large part to modifiable risk factors such as diet, physical activity, and smoking. Currently the only reliable biomarker of risk for colorectal cancer is a colorectal adenoma, detected and removed through the only reliable screening method, the colonoscopy. However, even high risk individuals may choose to avoid this expensive, invasive, and uncomfortable procedure. Less invasive biomarkers of risk for neoplasia could increase the likelihood of detecting early-stage adenomas, more clearly pinpointing those patients in need of a colonoscopy. Although biomarkers in the colon tissue could be very effective in creating risk profiles, obtaining this tissue is still invasive, and analysis of tissue biomarkers remains more time consuming and expensive than comparable biomarker measurements in blood samples. Blood biomarkers of risk are minimally invasive, inexpensive, and easy to obtain and analyze. Optimized blood biomarkers that are associated with colorectal cancer risk and could be used to monitor the effectiveness of preventative interventions, analogous to cardiovascular disease biomarkers, such as cholesterol or blood pressure, would be indispensible tools for primary preventative medicine.

The first study was a pilot, randomized, controlled chemoprevention trial of an antioxidant micronutrient cocktail (*dl*-α-tocopherol acetate 800 mg, β-carotene 24 mg, vitamin C 1.0 g, *1*-selenomethionine 200 μg, riboflavin 7.2 mg, niacin 80 mg, zinc 60 mg, manganese 5 mg) given daily over 4 months to sporadic colorectal adenoma patients. This antioxidant cocktail had different effects in smokers and non-smokers. In non-

smokers, the antioxidant cocktail had positive effects on TNF-α (an inflammatory cytokine) and CySS (an oxidative stress biomarker), statistically significantly lowering the circulating levels of both. However, in smokers these positive effects were not found, in fact IL-6 (an inflammatory cytokine) and F2-isoprostanes (an oxidative stress biomarker) were present at higher circulating concentrations when participants where given the antioxidant cocktail. Although the increase in inflammatory and oxidative stress biomarkers in those who smoked was not statistically significant, biological evidence and many other studies point to the validity of this finding. The CARET (106) and ATBC (104) studies, which administered a similar dose of β-carotene to smokers, found an increased risk of lung cancer in the supplementation group. Also, in a mouse model, when exposed to smoke, IL-6 expression increased in lung tissue, and caused lung epithelial cell oxidative DNA damage. The results of this study indicate that antioxidant micronutrients may be beneficial only in certain populations. This underscores the need for reliable biomarkers of risk which can differentiate the risk and benefits of certain preventative interventions in distinct populations.

This study was unique in its ability to look retrospectively at the unexpected findings in the large studies of smokers given β-carotene. If oxidative stress biomarkers or IL-6 had been measured in a prior biomarker endpoint trial, it is possible that the potential for deleterious effects to smokers of this supplement in supraphysiological doses would have been recognized. The use of surrogate endpoints in intervention trials may be more important for detecting and preventing negative outcomes than fully elucidating positive outcomes. In the CARET and ATBC studies, β-carotene supplementation could have been halted early, and deaths from lung cancer might have

been avoided. This study highlights not only the need for surrogate endpoints, or biomarkers, but also the need to assess preventative strategies in different subpopulations. Each person, and each cancer, has a unique chemistry, and therefore more personalized preventative strategies, as well as rapid and effective biomarkers are key for the prevention of cancer.

For example, curcumin is a promising anti-inflammatory agent proposed to be particularly effective for colorectal cancer prevention due to the mechanistic pathways of its action, and the increased bioavailability of this compound in the gut. The use of blood and tissue biomarkers from participants prior to the development of adenoma could be highly effective in testing the effectiveness of this preventative treatment. Little is known about the effects of curcumin supplementation in a largely healthy population, and potential positive or negative reactions should be noted early, in a small, pilot biomarker trial, before a full adenoma recurrence trial deemed warranted.

The second study investigated the association of biomarkers of inflammation with risk factors for colorectal cancer. We assessed several risk factors for colorectal cancer associated with either increased or decreased risk, including BMI, central adiposity, NSAID use, physical activity, total energy intake, calcium intake, and serum vitamin D, for their association with biomarkers of inflammation in colorectal adenoma patients. This study also assessed the association of a combined inflammation z-score with these colorectal cancer risk factors. CRP and IL-6 were significantly associated with increased BMI, as well as a sex-specific high waist-hip ratio (WHR). The inflammation z-score was also statistically significantly associated with a higher BMI and WHR. This study was a small, cross sectional study, which could explain why statistically significant

associations of the inflammatory biomarkers with other risk factors were not found. Inflammation is known to play a key role in carcinogenesis, and certain inflammatory cytokines, *i.e.*, CRP, IL-6, and TNF-α, are associated with inflammatory bowel diseases, colorectal adenomas, and colorectal cancer progression. The combined inflammation z-score may give a more global picture of the systemic inflammatory environment of an individual. Although this study showed only a modestly stronger association with a major risk factor for colorectal cancer, adiposity, it provides needed background for future studies of similar combined inflammation scores.

The third study was a pilot, randomized, controlled chemoprevention trial of calcium and/or vitamin D (1,000 mg/d of calcium, 800 IU/d of vitamin D_3) given over 6 months to sporadic colorectal adenoma patients. In this study, plasma concentrations of pro-inflammatory markers (CRP, TNF-α, IL-6, IL-1β, and IL-8) and an anti-inflammatory marker (IL-10) were measured. Surprisingly, there were no indications of synergistic effects of vitamin D and calcium, with inflammatory markers being reduced only when these supplements were given separately. We found statistically significant reductions in CRP in men and non-NSAID users in the vitamin D supplementation group, and although other inflammatory biomarkers showed a pattern of reduced levels with either vitamin D or calcium supplementation, no other significant reductions were found except in a combined inflammation Z-score. This combined inflammation z-score represents a snapshot of several inflammatory biomarkers in a summary score, which is hypothesized to better represent the interdependent nature of inflammation. This combined inflammation z-score was statistically significantly reduced with vitamin D

supplementation. Calcium supplementation also reduced the inflammation z-score, although this finding was not statistically significant.

This study demonstrated that either calcium or vitamin D supplementation may reduce systemic biomarkers of inflammation, suggesting that these agents could reduce adenoma recurrence by reducing inflammation. Also, identifying early response, either positive or negative, to preventative treatments can lead to earlier identification of treatment outcomes. Development of cancer can take years, and with an abundance of new chemopreventive strategies, and the potential risks of these strategies unknown, fast and reliable detection of treatment effects is necessary. In the future, I hope to conduct a large case-control study of the inflammation z-score to test its association with colorectal adenomas. In addition, adding the measurement of this inflammation z-score to ongoing cohort adenoma recurrence trials could further determine the predictive capacity of this score.

One limitation of the combined inflammation z-score is that it assumes an additive relationship of all cytokines included in the score. Inflammation is a very complex process, and this simplified measure may not be an accurate representation of actual physiology. Because of this, the inflammation z-score needs more extensive examination to determine its relationship with colorectal neoplasia, and its predictive and treatable capacity.

To develop a robust combined inflammation z-score, a large case-control study of colorectal adenoma patients should measure a wide variety of inflammatory biomarkers. Each biomarker could be assessed though a sensitivity analysis for the level of contribution it makes to the overall combined z-score which would then be assessed for

its association with adenoma risk. Through this method, a validated, practical, and data-driven combined inflammation z-score could be created. I hypothesize that a score created via this method would be highly reliable, have flexible application, and lead to better assessment of colorectal cancer prevention strategies.

In conclusion, this dissertation demonstrates that calcium and vitamin D may modulate biomarkers of inflammation in colorectal adenoma patients, and that an antioxidant micronutrient cocktail can reduce biomarkers of oxidative stress and inflammation in colorectal adenoma patients who do not smoke. These biomarkers of inflammation are also associated with a prominent risk factor for colorectal cancer, increased adiposity, further supporting their use as biomarkers for risk of colorectal cancer and chemopreventive intervention response.

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APPENDIX. Laboratory Procedures

Measurement of CySS

As discussed in chapter 2, CySS was measured as a plasma biomarker of oxidative stress in response to an antioxidant micronutrient combination. A shift from high free thiols (cysteine) towards more oxidized disulfides (CySS) is seen in aging and in cardiovascular disease, diabetes, rheumatoid arthritis, and other disorders involving inflammation (82). Plasma CySS levels are on average between 60-66 μM/ml in a population with a mean age of 60 (216), however, mean values observed in my study were 32-58 μM/ml. The levels of plasma Cys, GSH, and GSSG observed in this study were much lower than the normal viable range at a mean age of 60. This indicates that degradation of the samples during ten years of storage has occurred. Redox measurements are usually taken in a preservation solution that immediately derivatives thiol groups, preparing the samples to be analyzed by HPLC. Normally the samples are run on an HPLC within a few months after blood draw and preservation, and the values of Cys, CySS, GSH, and GSSG are shown to be consistent. However, as the length of storage increases, the samples begin to degrade and the levels of CySS, Cys, GSH and GSSG decrease. It is hypothesized that the acid in the preservation solution is the cause of this degradation. Individual blood samples degrade at different rates, making comparison of different samples stored in presentation solution for long periods of time impossible. The samples analyzed in the antioxidant micronutrient supplementation trial were treated with BHT, an antioxidant preservative. Without acid, and in the presence of BHT, our hypothesis was that the concentration of CySS would be preserved even over a period of ten years at -80º C.

Prior to analysis, the samples were thawed only twice to remove aliquots, then frozen again at -80º C. All samples during handling were blinded and were treated identically. In 2009, plasma samples were analyzed by HPLC for CySS and quantified relative to an internal standard, γ-glutamyl glutamate, 10 μM. The plasma samples were processed according to the protocol available in Jones et al (171). Unfortunately, the blood ideally would have been collected in a preservation solution of 100 mM serine Borate Buffer to properly measure glutathione, glutathione disulfide, cysteine and cysteine (CySS). However, because this study was performed on stored samples, this was not possible. The blood was preserved with BHT, which we hypothesized would give some protection form oxidative damage over the time of storage. The blood samples were then processed as in Jones et al (217). Each plasma sample was diluted one-to-one with 10% perchloric acid solution containing 0.2 M boric acid with 165 μMg γ-glutamyl glutamate as an internal standard. This solution adds hydroxyl-methyl groups to all free thiols in the sample. The pH of each sample was then adjusted to 9.0 with 1 M KOH/potassium tetra-borate, and then left at room temp to incubate for 20 min. 300 μl of a 20 mg/ml dansyl chloride solution in acetone was added to the samples, which were vortexed, and then left overnight to incubate at room temperature in the dark. The next day, 500 μl chloroform was added to each sample to extract the acetone and unbound dansyl chloride from the aqueous phase. The samples were vortexed, and then spun at 13,000 rpm for 2 mins. to separate the organic from the aqueous phase. The upper, or aqueous, phase contained the dansyl chloride bound thiols, which were then separated by HPLC for analysis. HPLC separation was done on a 3-aminopropyl column (5 μm; 4.6 mm×25 cm; Custom LC, Houston).

I found statistically significant changes in CySS levels in response to antioxidant micronutrient supplementation. More research is needed to determine the usefulness of CySS as a biomarker of oxidative stress in plasma samples that have been in long-term storage.