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Effects of Supplemental Calcium and Vitamin D on Circulating Biomarkers of Gut Barrier Function in Colorectal Adenoma Patients: A Randomized Controlled Trial

By

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Master of Public Health

Epidemiology

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Veronika Fedirko, PhD, MPH

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Abstract

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Disruption of epithelial barrier integrity leading to translocation of bacterial components[lipopolysaccharide (LPS) and flagellin] and associated inflammation may promote colorectal carcinogenesis. LPS-binding protein (LBP) and intestinal fatty-acid binding protein (IFABP) are potential markers of exposure to bacterial products due to impaired gut barrier function. Experimental evidence supports vitamin D and calcium as chemopreventive agents that could improve gut barrier function in part due to their anti-inflammatory actions. We conducted a pilot adjunct biomarker study on 118 patients from the Vitamin D/Calcium Polyp Prevention Study randomized to 1,200 mg/day calcium, 1,000 IU/day vitamin D3 or both over 1 year to estimate the effects of vitamin D and calcium supplementation on plasma levels of these two biomarkers. We found no appreciable effects of calcium and/or vitamin D on LBP or IFABP levels over a 12-month treatment period, with a suggestion for a possible 25% reduction in LBP ($p=0.04$) in the vitamin D group compared to placebo. There was no evidence of synergy between vitamin D and calcium effects on investigated biomarkers. Secondary analyses suggested that reductions in LBP (20%; $p=0.09$) and IFABP (28%; $p=0.02$) following vitamin D supplementation could be limited to individuals with low baseline circulating levels of vitamin D (< 22.52 ng/ml). At baseline, women had 60% higher IFABP levels compared to men ($p=0.001$) and study participants with BMI $\geq$30 kg/m^2 had a 12% higher level of LBP compared to those with BMI <25 kg/m^2 ($p=0.16$). The study findings support further research into the role of vitamin D supplementation on gut barrier function and colorectal carcinogenesis, and continued investigation of potentially modifiable risk factors for colorectal cancer prevention.

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Chapter 1

Introduction

Background and Significance

Colorectal Cancer Incidence and Mortality

Colorectal cancer (CRC) continued to be within the top five leading cancers worldwide in 2018, ranking fourth in terms of incidence (6.1%) and second in terms of mortality (5.8%) among all cancers combined, according to GLOBOCAN data (1). There were an estimated 1.8 million new cases of CRC and 881,000 deaths globally. CRC is the third most common cancer in men (10.9% of 9.5 million new cancer cases) and the second most common cancer in women (9.5% of 8.6 million new cancer cases) of all cancer incidence. Worldwide, the age-standardized rate (ASR; per 100,000 person-years) for CRC incidence was 13.1 for men and 10.1 for women, while ASR for CRC mortality was 6.4 and 4.6 among males and females respectively (1).

In the United States, CRC is the second most common cancer in men and women combined, with an estimated 140,250 new cases (8.1% of all cancer incidence, ASR = 39.4) and 50,630 deaths (8.3% of all cancer deaths, ASR = 14.5) due to CRC in 2018 (2).

Molecular Mechanisms of Colorectal Cancer

Colorectal cancer mostly arises from benign neoplasms (i.e., tubular adenomas and serrated polyps) that undergo a malignant transformation over many years. While not all benign neoplasms become malignant, most cancer arises from benign neoplasms. Various factors play a direct role in this transformation, particularly gene mutations, epigenetic alterations and local inflammatory changes (3). The largest fraction of CRC cases is considered to be due to environmental factors, and hence sporadic, rather than hereditary.

CRCs develop from one or a combination of three different and often overlapping mechanisms: (a) chromosomal instability (CIN), microsatellite instability (MSI) and CpG island methylator phenotype (CIMP), with one predominant type of genomic instability in the development of a specific CRC, although MSI and CIMP often coexist (4, 5). Chromosomal instability pathway is characterized by early mutational deactivation of the adenomatous

polyposis coli (APC) gene and disruption of the canonical Wnt signaling pathway, which results in β-catenin activation and nuclear localization. For progression to carcinomas, this is accompanied or followed by deregulation of other pathways such as activation of the oncogenes KRAS and BRAF, inactivation of tumor suppressors such as p53, activin receptors, transforming growth factor (TGF)-β receptor (R)II and pro-apoptotic protein Bax and increased expression of cyclooxygenase 2 (COX2) enzyme in the prostaglandin biosynthesis pathway (6). The MSI pathway for carcinogenesis involves inherited germline mutations in or epigenetic gene inactivation of DNA mismatch repair (MMR) proteins, which prevents corrections of DNA replication errors and causes accumulation of somatic mutations in tumor-related genes (4, 7). The CIMP pathway is characterized by hypermethylation of promoter CpG island loci and resulting inactivation of tumor suppressor or tumor-related genes (4). About 70-85% of sporadic CRCs are CIN type, while MSI accounts for about 15% of sporadic CRCs (5).

The Gut Barrier and Colon Microenvironment

The environment of the colon is complex and unique, consisting of the intestinal epithelial layer and the gut microbiota comprising trillions of bacteria and viruses, and their interactions with host's immune system. These components function collectively to maintain tissue homeostasis. The intestinal epithelial cells (IEC) are different types of specialized cells such as enterocytes, goblet cells and Paneth cells, which along with mucosal and submucosal cells, immune system cells and overlaying mucus layer, constitute a protective shield against hazardous material in the lumen, including foreign microorganisms and their toxins (8, 9).

The primary function of the IECs is maintenance of epithelial barrier integrity, and each of the cell types performs different functions to achieve this, such as impeding microbial entry to lumen, mucus secretion, repair of epithelial layer following damage, regulation of incoming antigens, secretion of IgA, phagocytosis of bacteria, secretion of anti-microbial peptides, neutralization of bacterial toxins (9) and production of inflammatory mediators such as cytokines and chemokines (10). Intestinal microflora perform important metabolic functions

such as metabolizing unabsorbed carbohydrates, mucus, bile acids and dead epithelial cells, affect the proliferation and survival of epithelial cells and regulate inflammatory response (6). Innate immune cells in the gut contribute to homeostasis maintenance by promoting or suppressing T cell differentiation and activation, by various mechanisms including production of retinoic acid and TGF-β to induce gut immune tolerance and production of IL-10 in response to commensals to suppress production of pro-inflammatory cytokines (10). Cells of the innate immune system, including NK cells, macrophages, neutrophils and dendritic cells (DC), as well as cells of the adaptive immune response such as CD8+ T cells, CD4+ Th1, Th17 and T regulatory (Treg) cells participate in anti-tumor response by direct cytotoxicity of cancer cells, production of cytokines (IL-1, IL-6, IL-10, IFN-γ, TNF, TGF-β), presenting tumor antigens to T cells, production and activation of cytotoxic T lymphocytes (CTL), and other mechanisms (11). Breakdown of the coordination between epithelial cells, microbiota and immune cells in the intestine and sustained damage to the epithelial barrier leads to disruption of homeostasis, pathological inflammatory responses and tumorigenesis (8).

Chronic Inflammation and Immune Response in the Gut

Gut homeostasis is maintained by a balance between immunosurveillance and tumor-promoting inflammation. Immunosurveillance is the process of immune cells patrolling the body to recognize and eliminate cancerous cells before they cause harm (11). It helps in early detection and elimination of aberrant crypt foci (ACF), that would otherwise progress into adenomas and adenocarcinomas (6). Chronic inflammation creates an environment that outcompetes immunosurveillance mechanisms and favors inhibition of anti-tumor responses (12). Activated inflammatory cells produce reactive oxygen species (ROS) and active nitrogen intermediates that can induce DNA damage. Cytokines and other factors can also stimulate epithelial cells to produce ROS, causing epigenetic silencing of tumor suppressor genes (6).

Gut Microbiome and Gut Barrier Dysfunction

A change in the intestinal microbiome, called gut microbial dysbiosis, is associated with the development of CRC (8). Intestinal barrier dysfunction may be exacerbated by chronic inflammation and other risk factors such as diet and body weight leading to exposure of colonic epithelium to endotoxins and leakage of these endotoxins into systemic circulation. Overabundance of lipopolysaccharide (LPS), an integral part of the outer membrane of gram negative bacterial cell wall, and flagellin, the primary structural component of flagella, and their translocation across colon tissue promotes colon carcinogenesis through mechanisms such as increased production of inflammatory factors, activation of NF-κB or increased Wnt signaling (13). These mechanisms can be triggered by both pathogenic and commensal bacteria (8).

Risk Factors for Colorectal Cancer

Diet, particularly consumption of red meat has been associated with increased CRC risk (8). Consumption of vegetables and high fiber foods were found to substantially lower colon cancer risk in many case control studies, but not prospective cohort studies. The weight of epidemiological evidence suggests alcohol and tobacco use are strong risk factors for CRC. Low consumption of calcium, dietary anti-oxidants such as Vitamins C, D and E, and folate have been associated with increased CRC risk but studies have found mixed results. Excess intake of energy-supplying macronutrient components of diet could also increase colon cancer risk, potentially by contributing to obesity (14).

Higher body weight, usually assessed by BMI, is associated with higher CRC risk, while physical activity is associated with decrease in risk, possibly due to underlying risk factor of insulin resistance and the resulting hyperinsulinemia. Among women, use of post-menopausal hormones has been found to be associated with reduced risk. Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin have consistently been found to have anti-carcinogenic effects in colon and rectums (14). Inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis (UC), is a widely established risk factor for CRC, with

risk increasing with longer duration, extent of colitis, family history of CRC, and degree of inflammation (15).

Biomarkers of Gut Barrier Dysfunction

Gut barrier dysfunction can be assessed by using several measurable biomarkers in blood. These include bacteria-related markers such as LPS in peripheral blood, circulating serum antibodies to LPS (IgG, IgM, IgA), lipopolysaccharide-binding protein (LBP), an acute-phase protein involved in innate immune response to LPS, and plasma D-lactate, a bacterial fermentation product. Measurable markers for epithelial cell integrity include plasma levels of α-glutathione S-transferase (GST) and plasma intestinal fatty-acid binding protein (IFABP) (16). The following sections discuss some relevant findings from studies using selected biomarkers for gut barrier dysfunction and carcinogenesis.

Antibodies to LPS and Flagellin

Ziegler et al. (2008) reported higher levels of flagellin-specific serum IgM, IgA and IgG in parenteral nutrition (PN)-dependent patients with short bowel syndrome (SBS), hypothesized to contribute to gut barrier dysfunction, but not in the two control groups of healthy adults without SBS and PN-dependent patients without SBS (17). Two case control studies nested in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort analyzed levels of serum antibodies to LPS and flagellin and their association with cancer risk (13, 18). Kong et al. (2016) found statistically significant association between total anti-LPS+flagellin and increased CRC risk among men, and not among women (13). Fedirko et al. (2017) showed statistically significant association between antibody response to LPS and flagellin and risk of hepatocellular carcinoma (HCC) (18).

Lipopolysaccharide-binding Protein (LBP)

Lipopolysaccharide-binding protein (LBP) is a glycoprotein synthesized in the liver that forms a complex with LPS and triggers a cascade of inflammatory cytokines in response to bacteremia

or endotoxemia, that is increased concentrations of circulating LPS and other bacterial antigens. LBP binds with plasma lipoproteins and acts as a cofactor for neutralization of LPS (19).

Studies have assessed LBP levels as indicators for systemic infectious complications, particularly to monitor interactions between innate immune system and LPS (20). Observational studies have found associations between increased LBP levels and conditions associated with chronic inflammation such as CVD risk (21) and obstructive sleep apnea (22). Others have studied associations between LBP levels and risk factors for CRC: Gonzalez-Quintela et al. (2013) and Nien et al. (2018) found positive associations between increased LBP and obesity (20, 23); Gonzalez-Quintela et al. (2013) and Avoyemi et al. (2018) found positive associations between LBP levels and metabolic syndrome (20, 21); whereas Umoh at al. (2016) found no association between diet and change in serum LBP levels, although this could be due to the short study period of 6 months (24). Ciubotaru et al. (2016) used levels of serum LBP and antibodies to LPS as markers to study leaky gut-associated inflammation and impact of 25-hydroxyvitamin D (25D) on it, among patients receiving Vitamin D3 supplementation, randomized to weekly ergocalciferol (vitamin D2) or placebo arms over 12 months. The study found significantly higher levels of LBP in the highest 25D quartile and high 25D levels (>50 ng/dl) were required to impact LBP and anti-LPS levels (25). Another nested case control study based on the Multiethnic Cohort study (MEC) looked at association between LBP levels, as marker of exposure to LPS, and CRC risk, but found no statistically significant association overall or stratified by BMI, fiber intake, saturated fat intake, cancer site or cancer stage (26). While the results of these studies are varied, the majority of these studies found positive associations between LBP levels and CRC or its risk factors and underlying conditions, demonstrating that LBP is well-documented and widely used as a biomarker for gut inflammation and CRC risk.

Intestinal fatty-acid binding protein (IFABP)

Fatty acid binding proteins (FABP) are intracellular proteins that protect cells from side effects of fatty acids and increase in conditions of inflammation and ischemia (27). Intestinal fatty-acid binding protein (IFABP), a water-soluble protein found predominantly in the jejunum, is often studied as an indicator for enterocyte function. Animal studies have measured serum IFABP levels as indicator of disrupted gut barrier integrity. Lau et al. (2016) investigated suitability of IFABP as a marker of intestinal injury and inflammation in obesity in standard and high-fat (HF) diet fed rats, where HF feeding was found to be associated with obesity, insulin resistance and increased plasma levels of pro-inflammatory cytokines IL1-β and monocyte chemoattractant protein 1 (MCP-1). However, while they found increased IFABP expression in the jejunum of HF-fed rats, the serum IFABP levels were decreased compared to standard-fed rats (28). Liu at al. (2016) observed increased levels of blood endotoxins and IFABP in rats fed HF diet compared to controls with basic feed, and also observed significant reductions in both endotoxins and IFABP levels among experimental group of rats that were given HF diet but additionally received intervention of *Saccharomyces boulardii,* which is believed to reduce inflammatory response and protect against mucosal barrier injury (29).

Observational human studies have also analyzed IFABP levels in different conditions associated with gut barrier dysfunction and inflammation. A cross-sectional study assessing IFABP as a marker in diagnosis of abdominal pathology found that patients presenting with mesenteric ischemia and intra-abdominal mass had significantly higher serum IFABP levels compared to healthy controls (27). Undseth et al. (2016) measured IFABP, LPS, a co-receptor of LPS soluble cluster of differentiation (sCD) and MCP-1 in intestinal bowel syndrome (IBS) patients and compared them with healthy controls before and after lactulose ingestion but found no association with any of the biomarkers and IBS symptom development. Contrary to expectation, they observed lower IFABP levels in IBS patients compared to healthy controls both before and after the lactulose challenge test (30). A double-blind placebo-controlled crossover study with 18 male participants reported an increase in plasma IFABP levels among

the placebo group post-exercise, believed to be a result of increased intestinal permeability induced by the exercise, but not in the intervention group that received bovine colostrum supplementation, which blunts exercise-induced intestinal permeability (31). Studies on antiretroviral therapy (ART)-induced gut barrier dysfunction in HIV-positive patients found increased serum IFABP levels among patients with chronic HIV on antiretroviral therapy (ART), which was consistent across all ART arms (32), and significantly different compared to HIV-positive ART-naïve patients and HIV-negative controls (33). Cheru at al. (2018) also reported positive association of IFABP increase with dietary intake of added sugar and saturated fatty acids among HIV-positive ART patients (33). These studies show the association between IFABP and gut barrier dysfunction, making it a suitable biomarker for studying changes in intestinal permeability and gut barrier dysfunction associated with colorectal carcinogenesis.

Vitamin D and Calcium in Colorectal Cancer

Calcium and vitamin D have been studied extensively in several interventional studies as possible chemopreventative agents against CRC, although results are not conclusive from the evidence presently available. Calcium is hypothesized to decrease CRC risk by neutralizing toxic effects of bile acids and free ionized fatty acids by binding them and forming insoluble mineral-fat complexes. Activation of vitamin D receptors on colorectal cells by 1,25-hydroxyvitamin D, the active metabolite form of vitamin D, has anti-cancerous effects such as increasing differentiation, apoptosis and inhibiting proliferation, invasiveness, angiogenesis and metastatic potential. Another mechanism suggested for protective action of Vitamin D is increasing calcium absorption (34). Therefore, randomized clinical trials (RCTs) are being conducted to evaluate protective effect of supplementation with calcium and vitamin D alone and/or in combination in lowering cancer incidence and mortality for all cancers as well as specifically for colorectal cancer.

Vitamin D

Goulao et al. (2018) conducted a systematic review and meta-analysis of RCTs with vitamin D supplementation as intervention. Based on the 30 studies and 18,808 patients found eligible and included in the analysis, researchers found no evidence of effect of vitamin D supplementation alone on cancer incidence and cancer-related deaths, despite the long-term follow-up (minimum of 12 months) of the included trials (35). The Vitamin D and Omega-3 Trial (VITAL), a large-scale country-wide trial in the USA evaluated high dose vitamin D and marine n−3 fatty acids in a two-by-two factorial RCT. Results from analysis of the vitamin D supplementation arm compared with placebo found no significant differences between the groups for any of the cancer outcomes, which included any cancer incidence, any cancer-related death and site-specific cancer incidence (breast, colorectal, prostate) (36). However, RCTs of vitamin D supplementation have several limitations such as use of lower doses of vitamin D, insufficient statistical power for assessing site-specific cancer outcomes, shorter follow-up periods, or a combination of these factors (36). A recent pooling project of 17 cohort studies analyzed participant-level data on colorectal cancer cases and controls and found statistically significant inverse association between circulating 25-hydroxyvitamin D (25(OH)D) and colorectal cancer risk among women and similar but non-statistically significant association in men (37).

Calcium

A recent systematic review and meta-analysis of five randomized trials with a total of 2,234 patients with history of colorectal adenoma assessed the effects of calcium supplementation compared to placebo on colorectal adenoma recurrence and reported null results (38). However, a previous pooled analysis of 10 observational studies on calcium and colorectal cancer risk with 534,536 participants found statistically significant inverse association between calcium intake and risk of colorectal cancer (39). An RCT that tested two different doses of calcium on colorectal adenoma patients over 4 months found no effect of calcium supplementation on circulating levels of antibodies to LPS and flagellin (40) or on markers of oxidative stress (F2-isoprostanes) and inflammation (C-reactive protein [CRP], tumor necrosis factor [TNF]-α,

interleukin [IL]-1β, IL-4, IL-6, IL-8, IL-10, IL-12p40, IL-17, vascular endothelial growth factor [VEGF], and interferon [IFN]-γ) (41).

Vitamin D and calcium in combination

Since vitamin D and calcium are interrelated in their metabolic processes and both are associated with reduced risk of CRC neoplasms, it is possible that they act synergistically in order to prevent CRC. The Vitamin D/Calcium Polyp Prevention Study randomized patients with recently diagnosed adenomas who had undergone colonoscopy and had no colorectal polyps to receive daily vitamin D, calcium, both or neither over a period of 3 to 5 years and looked at colorectal adenoma recurrence. Serum 25(OH)D levels were measured to observe changes in serum concentrations with vitamin D intake. The authors reported that there was no effect observed on colorectal adenoma recurrence over the study period in any of the study arms (42). Adjunct studies of this parent study looked at associations between treatment arms and biomarkers of proliferation, differentiation and apoptosis (mib-1, p21, bax and bcl-2) (43) and proinflammatory biomarkers (toll-like receptor [TLR]-4 & 5 and phosphor IKKα [pIKKα]) (44) but found no significant effect of vitamin D and calcium alone or in combination on these biomarkers.

Rationale for Present Study

The previous sections in this review have outlined numerous studies that support use of LBP and IFABP as potentially suitable markers for colonic inflammation and gut barrier dysfunction under different chronic conditions, including CRC. Evidence from both observational and interventional studies suggest that vitamin D and calcium might affect gut barrier function and inflammation, but not much is known about this association and it warrants further research. In order to answer this question, we conducted a pilot study to investigate the impact of calcium and vitamin D on circulating biomarkers of gut barrier function and intestinal permeability.

The present analysis is an adjunct biomarker study to the parent trial, the Vitamin D/Calcium Polyp Prevention Study, where we assess effects of vitamin D and calcium, alone or in combination, on circulating biomarkers related to impaired gut barrier function (LBP and IFABP). In addition, to understand factors associated with these biomarkers, we investigated whether LBP and IFABP levels at baseline differ by established and potential CRC risk factors such as age, sex, race, BMI, aspirin or NSAID use, alcohol intake, smoking status, and family history of CRC.

Chapter 2

Manuscript

Effects of Supplemental Calcium and Vitamin D on Circulating Biomarkers of Gut Barrier Function in Colorectal Adenoma Patients: A Randomized Controlled Trial

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Abstract

Disruption of epithelial barrier integrity leading to translocation of bacterial components[lipopolysaccharide (LPS) and flagellin] and associated inflammation may promote colorectal carcinogenesis. LPS-binding protein (LBP) and intestinal fatty-acid binding protein (IFABP) are potential markers of exposure to bacterial products due to impaired gut barrier function. Experimental evidence supports vitamin D and calcium as chemopreventive agents that could improve gut barrier function in part due to their anti-inflammatory actions. We conducted a pilot adjunct biomarker study on 118 patients from the Vitamin D/Calcium Polyp Prevention Study randomized to 1,200 mg/day calcium, 1,000 IU/day vitamin D3 or both over 1 year to estimate the effects of vitamin D and calcium supplementation on plasma levels of these two biomarkers. We found no appreciable effects of calcium and/or vitamin D on LBP or IFABP levels over a 12-month treatment period, with a suggestion for a possible 25% reduction in LBP ($p=0.04$) in the vitamin D group compared to placebo. There was no evidence of synergy between vitamin D and calcium effects on investigated biomarkers. Secondary analyses suggested that reductions in LBP (20%; $p=0.09$) and IFABP (28%; $p=0.02$) following vitamin D supplementation could be limited to individuals with low baseline circulating levels of vitamin D (< 22.52 ng/ml). At baseline, women had 60% higher IFABP levels compared to men ($p=0.001$) and study participants with BMI $\geq$30 kg/m^2 had a 12% higher level of LBP compared to those with BMI <25 kg/m^2 ($p=0.16$). The study findings support further research into the role of vitamin D supplementation on gut barrier function and colorectal carcinogenesis, and continued investigation of potentially modifiable risk factors for colorectal cancer prevention.

Introduction

Microbial dysbiosis, the imbalance created in the intestinal microbiome as a result of infection or lifestyle and metabolic factors such as diet and obesity, is known to trigger chronic inflammation, a known precursor to colorectal cancer (CRC) development (45). Disruption of epithelial barrier integrity, bacterial persistence in the inner mucus layer lining the gut epithelium and translocation of bacterial components such as lipopolysaccharide (LPS), an integral part of gram-negative bacterial cell wall outer membrane, and flagellin, the primary structural component of bacterial flagella, promote colon carcinogenesis through increased production of pro-inflammatory cytokines and reactive oxygen species and subsequent activation of NF-κB transcription factor (45). LPS-binding protein (LBP) is a glycoprotein synthesized in the liver that binds LPS to induce inflammatory response to infection. While low LBP concentrations enhance responses to LPS, high concentrations can inhibit LPS activity (46). High serum LBP levels, that occur during acute inflammation and infection, have also been reported in study subjects with chronic conditions such as obesity and metabolic syndrome, which are risk factors for CRC (20, 23). Another molecule, intestinal fatty-acid binding protein (IFABP), a water-soluble protein found predominantly in the jejunum, is often studied as an indicator for enterocyte function and inflammation. Studies have found increased serum IFABP levels in patients with abdominal pathology diagnosis compared to controls (27) and in HIV-positive patients with ART-induced gut barrier dysfunction (32, 33). These biomarkers are strongly associated with chronic inflammation and gut barrier dysfunction and are potential indicators for changes in gut microenvironment leading to colon carcinogenesis.

A number of observational studies have found evidence suggesting protective role for vitamin D and calcium in CRC (14, 37, 47). Mechanisms proposed for their protective action include binding of 1,25-hydroxyvitamin D (25(OH)D), the circulating active metabolite form of vitamin D, to vitamin D receptor (VDR) on colorectal cells and their subsequent activation and anti-cancerous effects on cell differentiation, proliferation and apoptosis, anti-inflammatory effects of vitamin D by promoting cytokine production and inhibition of NF-κB

signaling (48), maintenance of epithelial barrier integrity and increasing mucosal regeneration and healing capacity by VDR (49) and binding and neutralizing of bile acids and free ionized fatty acids by calcium to reduce their toxic effects on gut epithelium (34). Vitamin D also regulates production of anti-microbial peptides in response to microbial infections, and in particular acts synergistically with LPS to induce cathelicidin antimicrobial peptide (camp) expression in neutrophils (50).

To our knowledge, no human studies have tested the effects of vitamin D and calcium supplementation, alone and in combination, on circulating biomarkers of intestinal permeability. Our goal was to address this gap in literature and obtain preliminary data on the effects of supplemental vitamin D and calcium, alone and in combination, on plasma concentrations of LBP and IFABP, biomarkers of microbial exposure and gut barrier disruption, in colorectal adenoma patients.

Methods

Study Participants

We conducted an "adjunct biomarker study" to a larger 11-center, randomized, placebo-controlled, partial 2x2 factorial chemoprevention clinical trial, the Vitamin D/Calcium Polyp Prevention Study (NCT00153816; referred to as "parent study"). The parent study enrolled participants of ages 45-75 years with at least one colorectal adenoma removed within 120 days prior to enrollment and with scheduled 3-year or 5-year colonoscopic follow-up examinations (42). Participants at 2 of the 11 study centers (South Carolina and Georgia) who were found eligible for the adjunct biomarker study were recruited and signed biomarker study consents, with additional exclusion criteria being unable to be off aspirin for 7 days, history of bleeding disorder, or current use of anticoagulant medication (51). Detailed study protocols and participant eligibility and exclusion criteria have been previously published for both studies (42, 51).

Clinical Trial Protocol

Of 2,259 patients randomized in the parent study, 118 patients met the additional eligibility criteria at the two study centers and agreed to provide blood samples and rectal biopsy tissues at baseline and after 1 year of supplementation with study agent and were consented and recruited into adjunct biomarker study. Institutional Review Boards at both centers approved the study protocol.

The parent study collected baseline information on demographic data, medical history, medications, nutritional supplements, behavioral factors, and diet (using the Block Brief 2000 food frequency questionnaire [Nutritionquest]) from all participants. Following a 56-84 day placebo run-in period, participants were randomized into four treatment arms: placebo, 1,200 mg/day calcium supplementation (as calcium carbonate in equal doses twice daily), 1,000 IU/day vitamin D3 supplementation (500 IU twice daily), and 1,200 mg/day elemental calcium plus 1,000 IU/day vitamin D3 supplementation ("4-arm randomization"). Women who refused to forego calcium supplementation were randomized to calcium or calcium plus vitamin D ("2-arm randomization"). All participants agreed to not take vitamin D or calcium supplementation outside the study, though personal supplements up to 1,000 IU vitamin D and/or 400 mg elemental calcium were permitted from April 2008.

Randomization was through computer-generated random numbers with permuted blocks and stratified by sex, clinical center, scheduled colonoscopic follow-up of 3 or 5 years and 4 or 2-arm randomization. All study staff and participants were blinded to treatment assigned. During follow-up, bottles of study tablets were delivered to participants every 4 months, and interviews via telephone were conducted every 6 months to gather information on adherence to study treatment, illnesses, use of medications and supplements, and colorectal endoscopic or surgical procedures. Blood levels of serum calcium and 25-hydroxyvitamin D were measured at baseline and 1-year follow-up (which is the study period for the adjunct study).

Blood Collection and Biomarker Measurements

Blood was collected, handled and stored according to a standardized protocol at baseline and 1-year follow-up. Briefly, peripheral venous blood was collected into pre-chilled Vacutainer tubes for whole blood, plasma and serum and immediately placed on ice. Tubes were immediately processed in laboratory according to a strictly timed protocol. Centrifugation was in refrigerated centrifuge. Blood fractions were aliquoted and immediately stored in -80°C freezer.

Plasma LBP concentrations were measured using electrochemiluminescent Human LBP Assay kit from Meso Scale Discovery (Catalog No. L451 IYB, MSD®) at the Emory Multiplexed Immunoassay Core (EMIC). Briefly, frozen plasma samples were thawed, diluted 1:200 with 1% blocker A solution (Blocker A Kit, MSD®) and added to ELISA plates pre-coated with LBP antibodies (Multi-Array 96-well Human LBP Plate, MSD®). Following incubation and washing, the Antibody Detection Solution (SULFO-TAG™ Anti-hLBP Detection Antibody, MSD®), diluted with diluent provided in kit (Diluent 15, MSD®), was added to the wells and incubated. After washing, read buffer (Read Buffer T (4X), MSD®) was added to the wells and the plates were loaded and read immediately in the MSD Sector® imager. Quantification of LBP is by measuring the light intensity emitted by the bound detection antibody, in response to voltage applied to the plates inside the Sector instrument. Controls were included in each plate in the form of blank wells, samples from patients with Crohn's disease obtained from a biobank and the "pooled" samples. The average within-batch and between-batch coefficients of variation (CVs) for LBP were 8% and 10%, respectively.

For plasma IFABP measurement, we created a custom singleplex sandwich ELISA using a U-PLEX Technology from MSD Diagnostics (Catalog No. K15227N) and antibodies from the Human FABP2/I-FABP (Catalog No. DY3078, R&D Systems™) kit. Briefly, biotinylated mouse anti-human FABP2 capture antibodies from the R&D Systems kit were coupled to unique U-PLEX linkers and coated onto the U-PLEX plates. The linkers self-

assemble on unique spots in the U-PLEX plate. Plasma samples were added to each of the wells along with Reagent Diluent (1% BSA in PBS, pH 7.2-7.4, 0.2 μm filtered [R&D Systems, Catalog No. DY995]). Biotinylated goat anti-human FABP2 detection antibodies were added to the wells, followed by working dilution of streptavidin conjugated to horseradish peroxidase (Streptavidin-HRP) and Substrate Solution (1:1 mixture of Color Reagent A (H2O2) and Color Reagent B [Tetramethylbenzidine] [R&D Systems, Catalog No. DY999]), with incubation and washing per protocol after each step. After addition of Stop Solution (2 N H2SO4 [R&D Systems, Catalog No. DY994]), IFABP concentrations were measured by optical density using microplate reader. Controls were included in each plate in the form of blank wells, samples from patients with Crohn's disease obtained from a biobank and the same two "pooled" samples. The average within-batch and between-batch coefficient of variation (CV) for IFABP were 5% and 12%, respectively.

Inflammatory biomarkers in plasma were measured previously in duplicate using electrochemiluminescence detection-based immunoassays from MSD at the Emory Multiplexed Immunoassay Core (EMIC) facility. The average within-batch and between-batch coefficients of variation (CVs) were 3%-9% and 8%–15%, respectively.

All samples were treated identically by technician, who was blinded to treatment assignment. Baseline and 1-year follow-up samples from each participant were processed in the same batch.

Statistical Analyses

We compared baseline characteristics of participants across treatment arms using the χ^2 test for categorical data and ANOVA or Student's T-test for continuous variables within the 4-arm and 2-arm randomization groups, respectively.

We used a generalized linear model adjusted for age, center, gender and BMI to assess baseline association of LBP and IFABP levels with categories of *a priori* selected factors based on biological plausibility (age, sex, race, BMI, regular aspirin/NSAID use, smoking status, daily alcohol intake, presence of serrated adenoma, family history of CRC, and daily red/processed meat consumption, vegetable and fruit intake and dietary fiber intake) and with baseline levels of selected inflammatory cytokines that showed high degree of correlation with LBP and IFABP (IFN-γ, IL-6, TNF-α and summary z-score for collective panel of inflammatory cytokines). An individual z-score was first calculated for each cytokine by the formula $z=(x-\mu)/\delta$, where x is the natural log-transformed value of each biomarker, and μ and δ are the sex-specific mean and standard deviation of the natural log-transformed biomarker value at baseline, respectively. The summary z-score was then calculated by summing the individual score for the following biomarkers, determined by the proportion of observations for which cytokine samples were above the lower level of detection: IL-6, IL-8, IL-10, TNF-α, IL-12p40, IFN-γ, IL-17A, IL-2. IL-10 was included with a negative sign due to its anti-inflammatory properties. Each individual z-score was normally distributed with a mean of 0 and SD of 1 at baseline.

The proportional difference was calculated to assess the differences between concentrations of each biomarker and demographic and lifestyle factors at baseline. Proportional difference was calculated as follows: Proportional difference = [(comparison mean - reference mean) / reference mean] * 100%.

Treatment effects were evaluated by assessing differences in plasma concentrations of LBP and IFABP from baseline to year 1 by treatment arm versus placebo, separately for 4-arm and 2-arm randomization categories, using general MIXED linear models. IFABP values were log transformed. The predictors in the model included the treatment arm (4-arm: placebo, calcium, vitamin D, calcium + vitamin D; 2-arm: Vitamin D, placebo), visit (baseline or 1-year follow-up) and an interaction term for treatment arm and visit. We also conducted analysis by

grouping treatment arms into categories based on treatment agent received versus comparison group to increase sample size. The treatment groups were coded as those who received vitamin D versus no vitamin D, received calcium versus no calcium, and received both vitamin D and calcium versus calcium.

Relative treatment effect was calculated as [(treatment group follow-up)/(treatment group baseline)]/[(control group follow-up)/(control group baseline)] and absolute treatment effect was calculated as [(treatment group follow-up)-(treatment group baseline)]–[(control group follow-up)-(control group baseline)]. Both absolute and relative treatment effects are reported with confidence intervals on difference scale (absolute) for LBP and ratio scale (relative) for IFABP. Potential confounding of treatment effects was assessed by running multiple additional models adjusted for different combinations of age, sex, center, number of adenomas at baseline and BMI. However, the adjusted estimates were all very similar to the unadjusted, hence only the unadjusted results are presented.

In order to assess potential effect modification by selected covariates, we performed secondary stratified analyses of treatment groups divided as follows: serum 25-hydroxyvitamin D and total calcium were stratified at the median; BMI was categorized as less than versus greater than or equal to 30 kg/m^2; and aspirin and/or NSAID use was stratified according to less than once versus once or more per week.

All analyses were intention-to-treat (ITT) and statistical analysis was conducted in SAS 9.4 (Cary, NC). A two-sided P - value ≤ 0.05 was considered statistically significant.

Results

Baseline Patient Characteristics

Baseline demographic, lifestyle and adenoma characteristics of all study participants by treatment group assignment are shown in Table 1. The mean age of all participants was 59 years

and mean BMI was 29 kg/m^2. Demographic distribution was 50% male, 79% white, and 81% with some college education (no degree) or higher. Among the two study centers from which participants were recruited, 64% were at the Georgia center and the rest from South Carolina center. Many of the participants used aspirin (60%), NSAID (66%) and multivitamin (67%) at baseline. Majority of them had no family history of CRC (91%), 20% had advanced adenomas and 25% had sessile serrated adenomas. Alcohol consumption was <1 drink/day on average and 58% of the participants never smoked. There were some statistically significant differences across treatment groups in daily vegetable and fruit and dietary fiber consumption.

Estimated treatment effects on plasma LBP and IFABP levels are presented in Table 2.

Treatment Effects of Calcium and/or Vitamin D on LBP

Mean LBP levels reduced over 1 year in vitamin D arm compared to placebo in the 4-arm randomization and the difference was statistically significant (Absolute effect = -907.02; 95% CI: -1769.79, -44.2495; p = 0.04). Effect of combined treatment with calcium and vitamin D was in similar direction, but of far lesser magnitude (Absolute effect = -378.14; 95% CI: -1252.8, 496.53) and did not attain statistical significance. Randomization to only calcium showed mild increase in plasma LBP levels which was also not statistically significant. The 2-arm randomization comparison showed effect in opposite direction from anticipated, with LBP levels increasing over 1 year for Vitamin D.

Results of analysis by treatment group is presented in Supplementary Table S1. These yielded similar results as reported above for analysis by randomization arms and none of the results were statistically significant. Mean plasma LBP levels decreased among vitamin D group compared to no vitamin D group (-315.75; 95% CI: -836.85, 205.35) over 1 year and increased in calcium versus no calcium groups (322.01; 95% CI: -395.86, 1039.89). Intervention group receiving both vitamin D and calcium compared to group receiving only calcium showed no effect (23.99; 95% CI: -573.74, 621.82). Results of secondary analyses

stratified by baseline median 25-hydroxyvitamin D, median total calcium, BMI (≥ 30) and use of aspirin and/or NSAID at least once per week are presented in Supplementary Tables S2 to S5. Stratification by vitamin D showed stronger effects for both LBP and IFABP in strata with lower serum vitamin D, whereas the effects were attenuated in the strata with higher vitamin D at baseline. but increased magnitude as above. Stratified effects for calcium, BMI and aspirin/NSAID were similar to non-stratified effects.

Treatment Effects of Calcium and/or Vitamin D on IFABP

In the 4-arm randomization groups, administration of calcium alone showed a not statistically significant increase of 10% in mean IFABP levels over 1 year (Relative effect = 1.10; 95% CI: 0.75, 1.62). The treatment arms administered vitamin D alone and in combination with calcium showed reduction in IFABP levels by 16% (Relative effect = 0.84; 95% CI: 0.58, 1.21) and 13% (Relative effect = 0.87; 95% CI: 0.60, 1.27), respectively. The 2-arm randomization group administered vitamin D showed the highest reduction in mean IFABP levels by 18% (Relative effect = 0.82; 95% CI: 0.57, 1.18). None of the effects were statistically significant.

Mean IFABP levels and treatment effects by treatment group are presented in Supplementary Tables S1. IFABP levels showed 18% and 20% reduction respectively in comparisons for vitamin D versus no vitamin D (0.82; 95% CI: 0.66, 1.01) and calcium and vitamin D combined versus calcium alone (0.80; 95% CI: 0.62, 1.05), similar to effects observed in randomization arm comparisons reported above. The calcium versus no calcium comparison showed no treatment effect over 1 year (1.06; 95% CI: 0.81, 1.40). None of the treatment effects observed were statistically significant.

Results of secondary analysis stratified by baseline median 25-hydroxyvitamin D, median total calcium, BMI (≥ 30) and use of aspirin and/or NSAID at least once per week are presented in Supplementary Tables S2 to S5. Similar to LBP, stronger effects on IFABP levels were observed in participant group with lower serum vitamin D, with attenuation of effect in

higher serum vitamin D group. Stratification by total calcium, BMI and aspirin and NSAID use showed similar effects as unstratified comparisons.

Associations Between Baseline LBP and IFABP Plasma Levels and Selected Participant Characteristics

Baseline mean LBP and IFABP plasma concentrations by categories of selected participant characteristics, adjusted for age, BMI, gender and center are presented in Table 3. Mean LBP levels at baseline were similar for men and women, whereas mean IFABP levels were statistically significantly higher among women (60%) compared to men ($P_{trend} = 0.001$).

LBP and IFABP were lower by 14% and 60%, respectively, among other races compared to white. For BMI, obese patients compared to normal and underweight had 12% higher LBP but 74% lower IFABP. Regular aspirin use and NSAID use (defined as ≥ 1/wk) was associated with 3% and 6%, respectively, higher LBP. However, while aspirin use was associated with 8% higher IFABP, NSAID use was associated with 11% lower IFABP. LBP levels were 8% lower among patients with more than 1 adenoma removed at baseline compared to those with 1 adenoma removed. This association was reversed in IFABP, with 33% higher IFABP levels in those with more than 1 adenoma removed. Similarly, trend in sessile serrated adenoma was reversed with participants who had sessile serrated adenomas having 13% higher LBP but 13% lower IFABP. None of these associations was statistically significant.

Increase in consumption of red/processed meat was associated with statistically significantly lower IFABP (9-30%; $P_{trend} = 0.02$). There was strong positive correlation between LBP and other inflammatory markers at baseline with LBP increasing by 21% and 28% with increasing tertile of inflammatory z-score ($P_{trend} = 0.03$). Particularly, LBP increased by 3% and 24% with increasing tertile of IFN-γ ($P_{trend} = 0.05$) and by 20% and 46% with increasing tertile of IL-6 ($P_{trend} < 0.001$).

Discussion

Overall, our study findings demonstrate decrease in plasma LBP and IFABP levels with administration of vitamin D over 1 year, with the magnitude of effect reduced or in opposite direction when vitamin D was given in combination with calcium. The effect was more evident when stratified by baseline serum hydroxyvitamin D levels, with participants in lower-than-median levels showing strong protective effect on LBP and IFABP levels with 1-year vitamin D treatment, and participants with median or higher levels showing no effect. While not all the results achieved statistical significance, the consistency of this finding across different comparisons suggests protective effect of vitamin D in reducing gut inflammation associated LBP and IFABP levels, and that it is particularly beneficial for patients who have low serum hydroxyvitamin D levels.

Our findings of increased LBP levels with vitamin D treatment in patients with disrupted gut barrier integrity are consistent with at least one previous study that reported high concentrations of vitamin D administration over 1 year lowering LBP and anti-LPS antibody levels in serum of African-American hypovitaminosis D, pre-diabetes, and obesity (52).

Contrary to expectations, our study results suggest that administration of calcium alone increases both LBP and IFABP levels. Calcium also appears to reduce impact of vitamin D when given in combination, suggesting a possible antagonistic effect. A similar antagonistic effect of calcium and vitamin D was observed in treatment effects on gut permeability-related biomarkers in colon tissue, published by our group previously (53). In stratified analysis, patients with lower serum vitamin D levels responded to calcium treatment with smaller increases in mean LBP and IFABP, than patients with high serum vitamin D levels. Interestingly, the combination treatment with calcium and vitamin D was protective for LBP and IFABP levels in participants with low serum vitamin D but the association moved to the opposite direction in participants with higher serum vitamin D. One possibility for these

findings is that the effect of calcium on combination treatment with vitamin D might be related to serum vitamin D concentrations and hence vitamin D dose dependent.

We also conducted a cross-sectional analysis at baseline of association of LBP and IFABP levels with selected *a priori* participant characteristics hypothesized to be associated with colorectal cancer. Women in our study had statistically significantly higher IFABP levels than men at baseline, while no gender difference was observed in LBP levels. This observed difference could potentially be a result of sex difference in intensity of immune response or due to differences in gut microbial composition that consequently influence the inflammatory response in colon (44). Being obese was associated with increased LBP levels in our study, a finding largely aligned with established literature. Obesity is considered a state of low-grade inflammation, and there are many reported associations of increased inflammatory markers and being obese. For example, a study previously published by our group found increased anti-LPS and anti-flagellin levels in very obese participants compared to underweight/normal BMI participants (40).

We found that plasma IFABP levels were lowest in the highest age group of individuals (≥ 65 years). One previous animal model study has reported reduction in IFABP levels with aging (54). We also found significantly lower IFABP levels in participants with higher alcohol and red/processed meat consumption, a finding largely conflicting with established literature. One study reported significant increase in IFABP levels following acute alcohol intoxication followed by significant decrease in following hours (55), and a possible explanation for the findings in our study might be a result of shorter time periods of alcohol consumption in the high intake category. As expected, participants with higher concentrations of inflammatory cytokines, specifically IFN-γ, IL-6 and the overall summary z-score of all inflammatory cytokines, showed significantly increased LBP levels at baseline.

This study has some strengths and limitations. Protocol adherence by study participants was high in this study. This is the first randomized, double-blind, placebo-controlled clinical trial to look at effects of vitamin D and calcium treatment, alone and in combination, on plasma LBP and IFABP levels. The primary limitation of this study is the small sample size, which limited the power to detect differences in circulating biomarker expression and the ability to conduct stratified analysis, especially by randomization arms. We could not assess race differences in biomarker expression, as the majority of our study population was white. As circulating biomarker levels were measured only for baseline and Year 1, this study could not analyze longer-term effects of vitamin D and calcium supplementation.

In conclusion, the results of our pilot study suggest that 1-year vitamin D_3 supplementation of 1,000 IU/day may reduce plasma levels of LBP in patients at high risk for developing CRC, and particularly among patients with low serum concentrations of vitamin D. Our study adds to existent literature supporting the use of LBP and IFABP, as sensitive non-invasive circulating biomarkers for gut inflammation and colon carcinogenesis.

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Table 1: Selected baseline characteristics of the study population (N=118), by treatment group assignment[a]

Characteristic	Randomization to Vitamin D and to Calcium (4-arm)					Randomization to Vitamin D Only (2-arm)		
	Placebo (n=21)	Calcium (n=17)	Vitamin D (n=20)	Calcium + Vit D (n=19)	p[b]	Placebo (n=22)	Vitamin D (n=19)	p[c]
Age, years	60.1 (5.9)	58.6 (6.9)	59.3 (7.7)	58.2 (6.9)	0.82	58 (5.2)	58.8 (6.6)	0.65
Men, %	85.7	70.6	65.0	84.2	0.36	0.0	0.0	-
White[d], %	81.0	70.6	75.0	94.4	0.28	68.2	84.2	0.29
College graduate[e], %	85.7	58.8	85.0	84.2	0.19	95.5	73.7	0.08
Study center, %								
Georgia	47.6	76.5	70.0	63.2	0.27	72.7	52.6	0.18
South Carolina	52.4	23.5	30.0	36.8		27.3	47.4	
Smoking status, %								
Never	52.4	76.5	60.0	47.4	0.30	63.6	52.6	0.21
Former or occasionally	33.3	17.7	40.0	47.4		36.4	31.6	
Current	14.3	5.9	0.0	5.3		0.0	15.8	
Family history of CRC[f], %	10.0	5.9	16.7	5.3	0.67	4.6	11.1	0.58
Physical activity, MET-min./wk[d]	2702.3 (2120.2)	2069.9 (2419.2)	2659.2 (2716.6)	4027.0 (2387.5)	0.10	1587.5 (1221.0)	2961.5 (3470.3)	0.09
Regular NSAID users[g], %	19.1	47.1	25.0	26.3	0.27	27.3	31.6	0.76
Regular aspirin users[g], %	52.4	70.6	45.0	42.1	0.32	27.3	26.3	0.95
Multivitamin users, %	52.4	76.5	45.0	68.4	0.18	72.7	89.5	0.25
BMI, kg/m^2	28.5 (4.3)	32.8 (7.3)	29.2 (5.2)	30.3 (4.4)	0.09	29.0 (4.7)	27.4 (4.8)	0.29
No. of adenomas	1.5 (0.6)	1.6 (0.9)	1.4 (0.7)	1.4 (0.7)	0.67	1.2 (0.7)	1.6 (1.0)	0.13
Had advanced adenoma[f,h], %	40.0	12.5	20.0	26.3	0.30	9.5	16.7	0.65
Had sessile serrated adenoma, %	23.8	29.4	15.0	31.6	0.64	27.3	21.1	0.73
Dietary Intakes								
Alcohol intake, drinks/d	0.8(0.8)	0.8(1.0)	0.7(0.9)	0.8(0.9)	0.98	0.6(1.0)	0.4(0.5)	0.40
Vegetable and fruit intake[i], svgs/d	3.6 (2.2)	4.5 (2.1)	4.4 (2.4)	4.4 (1.7)	0.54	4.9 (1.6)	6.1 (2.3)	0.05
Red/processed meat, svgs/d	1.0 (0.8)	1.0 (0.7)	0.8 (0.7)	1.1 (0.7)	0.78	0.7 (0.7)	0.7 (0.6)	0.94
Total calcium[j], mg/d	603.9 (366.8)	940.2 (346.7)	646.5 (260.9)	679.8 (265.4)	0.01	993.5 (460.5)	1227.2 (547.5)	0.15
Total vitamin D[k], IU/d	340.6 (262.1)	477.8 (196.2)	307.7 (255.2)	426.9 (280.0)	0.16	476.9 (340.1)	577.6 (267.2)	0.30
Total energy intake, kcal/d	1294.6 (416.9)	1693.3 (577.9)	1414.7 (496.5)	1625.7 (602.3)	0.08	1332.4 (548.6)	1433.9 (589.8)	0.57
Total fat, g/d	57.1 (25.7)	66.5 (26.1)	59.9 (25.3)	64.4 (28.9)	0.69	53.0 (26.7)	61.8 (35.7)	0.37
Dietary fiber, g/d	11.0 (4.6)	15.5 (5.4)	13.4 (5.9)	15.3 (5.6)	0.04	14.6 (5.5)	17.3 (4.9)	0.12
Serum Concentrations								
LBP[d], ng/ml	2793.1 (1468.4)	2988.7 (1059.1)	3652.7 (2090.9)	2779.7 (1155.4)	0.23	2716.8 (1486.9)	2754.8 (1053.9)	0.93
IFABP (geometric mean)[l], pg/ml	270.4 (2.0)	365.0 (2.7)	270.4 (2.2)	244.7 (2.0)	0.44	445.9 (2.0)	544.6 (2.0)	0.32
25-OH-Vitamin D, ng/ml	22.7 (7.3)	23.6 (13.2)	24.4 (10.3)	22.5 (6.3)	0.91	25.1 (8.9)	26.2 (9.5)	0.70
Calcium, mg/dl	9.2 (0.2)	9.3 (0.3)	9.4 (0.3)	9.4 (0.3)	0.35	9.5 (0.3)	9.4 (0.3)	0.35

Characteristic	Randomization to Vitamin D and to Calcium (4-arm)					Randomization to Vitamin D Only (2-arm)		
	Placebo (n=21)	Calcium (n=17)	Vitamin D (n=20)	Calcium + Vit D (n=19)	p[b]	Placebo (n=22)	Vitamin D (n=19)	p[c]

Abbreviations: BMI = body mass index; CRC = colorectal cancer; d = day; g = gram; IFABP = Intestinal fatty acid binding protein; IU = international unit; kcal = kilocalorie; LBP = LPS-binding protein; MET = metabolic equivalent of task; min = minute; NSAID = non-steroidal anti-inflammatory drug; No. = number; svgs = servings; wk = week.

[a]Data are given as mean (SD) unless otherwise specified.

[b]$\chi 2$ for categorical variables; general linear model for continuous variables.

[c]$\chi 2$ test for categorical variables; Student's t test for continuous variables.

[d]Missing data on 1 patient.

[e]Received some college education (no degree) or higher.

[f]Missing data on 4 patients.

[g]At least once a week. NSAIDs do not include aspirin.

[h]Defined as those with high-grade dysplasia, more than 25% villous features, or an estimated diameter of at least 1 cm.

[i]Missing data on 5 patients.

[j]Dietary calcium plus supplemental calcium (extra calcium + multivitamin).

[k]Dietary vitamin D plus supplemental vitamin D.

[l]Missing data on 2 patients.

Table 2. LBP and IFABP levels in study participants, by treatment groups (N=118)[a]

	Baseline			1-Yr follow-up			Absolute treatment effect[b]		Relative effect[c]	
Treatment	**n**	**Mean (95%CI)**	**p**	**n**	**Mean (95% CI)**	**p**	**Mean (95% CI)**	**p**	**Mean (95% CI)**	**p**
LBP										
4-arm										
Placebo	21	2793.11 (2167.50,3418.71)	Ref.	19	3692.56 (2900.34,4484.78)	Ref.	Ref.		Ref.	
Calcium	17	2988.74 (2293.42,3684.06)	0.68	15	4027.12 (3140.23,4914.01)	0.58	138.92 (-779,1056.85)	0.76	1.02	-
Vitamin D	20	3652.68 (3011.63,4293.73)	0.06	19	3645.11 (2844.50,4445.72)	0.93	-907.02 (-1769.79,-44.2495)	0.04	0.75	-
Calcium + Vitamin D	19	2779.74 (2122.03,3437.44)	0.98	18	3301.05 (2479.00,4123.10)	0.50	-378.14 (-1252.8,496.53)	0.39	0.90	-
2-arm										
Placebo	21	2716.76 (2091.15,3342.36)	Ref.	21	2607.39 (1837.13,3377.65)	Ref.	Ref.		Ref.	
Vitamin D	19	2754.77 (2097.07,3412.48)	0.93	18	2959.37 (2137.32,3781.42)	0.54	313.97 (-540.86, 1168.80)	0.47	1.12	-
IFABP										
4-arm										
Placebo	20	263.14 (189.27,365.84)	Ref.	19	305.36 (212.98,437.86)	Ref.	Ref.	-	Ref.	
Calcium	16	369.74 (255.80,534.43)	0.18	15	472.43 (315.36,707.76)	0.11	60.48	-	1.10 (0.75,1.62)	0.62
Vitamin D	20	270.16 (194.32,375.59)	0.91	19	262.59 (183.13,376.53)	0.56	-49.78	-	0.84 (0.58,1.21)	0.34
Calcium + Vitamin D	19	247.72 (176.67,347.37)	0.80	18	251.11 (172.55,365.40)	0.46	-38.83	-	0.87 (0.60,1.27)	0.48
2-arm										
Placebo	22	433.89 (316.94,594.07)	Ref.	21	525.58 (372.90,740.78)	Ref.	Ref.	-	Ref.	
Vitamin D	19	540.12 (385.18,757.33)	0.35	18	538.13 (371.70,778.99)	0.93	-93.68	-	0.82 (0.57,1.18)	0.29

Abbreviations: CI = Confidence interval; SE = standard error; Yr = year.

[a]The effect of treatment agent on biomarker level was modeled using PROC MIXED in SAS 9.4 (Cary, NC).

[b]For LBP, absolute effect was obtained directly from model. For IFABP, it was calculated as: Absolute effect = [(treatment group follow-up) - (treatment group baseline mean)] - [(placebo group follow-up) - (placebo group baseline mean)].

[c]As IFABP was log transformed, relative effect was obtained directly from model. For LBP, it was calculated as: Relative effect = (treatment group follow-up mean/treatment group baseline mean)/(placebo group follow-up mean/placebo group baseline mean).

Table 3. Categorical baseline LBP and IFABP levels by selected baseline participant characteristics (N=118).[a]

Baseline characteristics	LBP[b]			IFABP[c]		
	n	Diff(%)[d]	p value	n	Diff(%)[d]	p value
Age, years						
<55	36	Ref.		35	Ref.	
55-59	28	-7.52		27	-22.73	
60-64	27	2.44		28	-11.67	
≥65	26	1.19	0.95	26	-23.06	0.07
Sex						
Men	59	Ref.		59	Ref.	
Women	58	-0.20	0.85	57	60.00	0.001
Race						
White	91	Ref.		91	Ref.	
Other	25	-14.18	0.56	24	-59.74	0.26
BMI, kg/m^2						
<25	24	Ref.		24	Ref.	
25-29.9	47	0.00		47	-32.00	
>30	46	11.75	0.16	45	-73.52	0.26
Regular aspirin use (≥ 1/wk)						
No	66	Ref.		65	Ref.	
Yes	51	3.02	0.62	51	8.29	0.80
Regular NSAID use (≥ 1/wk)						
No	84	Ref.		83	Ref.	
Yes	33	6.34	0.50	33	-11.47	0.94
Smoking status						
Never	69	Ref.		67	Ref.	
Former or occasionally	40	16.97		41	-12.02	
Current	8	-3.80	0.49	8	0.11	0.30
Alcohol intake, drinks/d						
0	41	Ref.		39	Ref.	
≤0.5	29	-0.35		30	15.37	
0.6-1	20	2.72		20	-26.88	
>1	27	-14.57	0.20	27	-17.44	0.02
No. of adenomas						
1	82	Ref.		81	Ref.	
>1	35	-7.93	0.53	35	33.29	0.80
Had serrated adenoma						
No	89	Ref.		87	Ref.	
Yes	28	13.32	0.29	29	-12.92	0.29
Family history of CRC[e]						
No	103	Ref.		102	Ref.	
Yes	10	24.39	0.21	10	49.90	0.16

Red/processed meat, svgs/d						
0	10	Ref.		10	Ref.	
≤0.5	39	6.17		37	-9.15	
0.6-1	29	-9.64		29	-30.42	
>1	39	-3.54	0.53	40	-22.91	0.02
Vegetable and fruit intake, svgs/d[f]						
Tertile 1	40	Ref.		40	Ref.	
Tertile 2	35	8.53		34	-13.27	
Tertile 3	37	3.66	0.88	38	2.66	0.11
Dietary fiber, g/d						
Tertile 1	39	Ref.		38	Ref.	
Tertile 2	43	4.16		42	-0.87	
Tertile 3	35	-0.34	0.91	36	16.30	0.14
Inflammatory score (zscore)						
Tertile 1	40	Ref.		40	Ref.	
Tertile 2	38	21.33		37	-26.16	
Tertile 3	39	27.17	0.01	39	16.46	0.62
IFN-γ						
Tertile 1	40	Ref.		40	Ref.	
Tertile 2	38	3.19		39	-3.19	
Tertile 3	39	24.03	0.05	37	3.74	0.84
IL-6						
Tertile 1	40	Ref.		39	Ref.	
Tertile 2	39	19.90		39	9.27	
Tertile 3	38	45.79	0.00	38	2.28	0.90
TNF-α						
Tertile 1	39	Ref.		38	Ref.	
Tertile 2	40	8.30		39	13.04	
Tertile 3	38	16.89	0.22	39	10.11	0.64

Abbreviations: BMI = body mass index; CI = confidence interval; d = day; Diff = proportional difference; g = grams; IFABP = Intestinal fatty acid binding protein; LBP = LPS-binding protein; min = minute; svgs = servings; wk = week.

[a]Proportional differences of multivariable-adjusted means and p-values calculated using general linear models (implemented using PROC GLM in SAS 9.4 [Cary, NC]), controlling for age (continuous), BMI (continuous), sex, and study center. All means, SEs and p values were calculated using ANCOVA.

[b]1 patient with missing value for LBP in placebo arm of 4-arm randomization

[c]2 patients missing values for IFABP, in calcium arm of 4-arm randomization and placebo arm of 2-arm randomization.

[d]Proportional difference = [(comparison mean- reference mean) / reference mean]* 100%.

[e]Missing data on 4 patients.

[f]Missing data on 5 patients in LBP group and on 4 patients in IFABP group.

Supplementary Table S1. LBP and IFABP levels in study participants, by treatment agent (N= 118)[a]

	Baseline			1-Yr follow-up			Absolute treatment effect[b]		Relative effect[c]	
Treatment Assignment	**n**	**Mean (95% CI)**	**p**	**n**	**Mean (95% CI)**	**p**	**Mean (95% CI)**	**p**	**Mean (95% CI)**	**p**
LBP										
No vitamin D	59	2822.30 (2447.15,3197.46)		55	3373.46 (2897.79,3849.12)					
Vitamin D[d]	58	3072.57 (2694.20,3450.95)	0.35	55	3307.98 (2830.60,3785.36)	0.85	-315.75 (-836.85,205.35)	0.23	0.90	-
No calcium	41	3212.41 (2735.66,3689.16)		38	3651.95 (3005.21,4298.69)					
Calcium[e]	36	2878.43 (2369.65,3387.22)	0.34	33	3639.99 (2947.63,4332.34)	0.98	322.01 (-395.86,1039.89)	0.37	1.11	-
Calcium	38	2838.44 (2449.97,3226.90)		36	3183.70 (2745.74,3621.65)					
Calcium plus vitamin D[f]	38	2767.25 (2378.79,3155.72)	0.80	36	3136.50 (2698.55,3574.46)	0.88	23.99 (-573.74,621.72)	0.94	1.01	-
IFABP										
No vitamin D	58	349.40 (284.77,428.70)		55	423.84 (339.30,529.44)					
Vitamin D[d]	58	329.49 (268.54,404.26)	0.69	55	327.24 (261.74,409.13)	0.11	-76.68	-	0.82 (0.66,1.01)	0.06
No calcium	40	266.62 (208.92,340.26)		38	282.62 (220.11,362.88)					
Calcium[e]	35	297.50 (229.22,386.11)	0.54	33	335.75 (256.37,439.72)	0.35	22.26	-	1.06 (0.81,1.40)	0.65
Calcium	38	405.63 (313.69,524.52)		36	505.31 (374.69,681.46)					
Calcium plus vitamin D[f]	38	365.79 (282.88,473.00)	0.57	36	365.75 (270.77,494.05)	0.13	-99.72	-	0.80 (0.62,1.05)	0.10

Abbreviations: CI = Confidence interval; SE = standard error; Yr = year; vs = versus.

[a]The effect of treatment agent on biomarker level was modeled using PROC MIXED in SAS 9.4 (Cary, NC).

[b]Absolute effect = [(treatment group follow-up mean) - (treatment group baseline mean)] - [(placebo group follow-up mean) -(placebo group baseline mean)].

[c]Relative effect = (treatment group follow-up mean/treatment group baseline mean)/(placebo group follow-up mean/placebo group baseline mean).

[d]Vitamin D group comprised patients assigned to vitamin D or to calcium + vitamin D in the 4-arm randomization, or to vitamin D in the 2-arm randomization.

[e]Calcium group comprised patients assigned to either calcium or to calcium + vitamin D in the 4-arm randomization; patients in the 2-arm randomization were excluded.

[f]Vitamin D + calcium group comprised patients assigned to calcium + vitamin D in the 4-arm randomization, or to vitamin D in the 2-arm randomization.

Supplementary Table S2. LBP and IFABP levels in study participants by treatment agent, stratified by median baseline serum 25-(OH)-vitamin D level concentrations[a] (N= 118)

Treatment Assignment	Baseline			1-yr follow-up			Absolute Treatment Effect[b]		Relative Effect[c]	
	n	Mean (95% CI)	p	n	Mean (95% CI)	p	Mean (95% CI)	p	Mean (95% CI)	p
LBP										
25(OH)D < median[d]										
No vitamin D	31	2574.52(2066.58,3082.46)		27	3454.02(2758.07,4149.98)					
Vitamin D[e]	27	3139.81(2595.55,3684.08)	0.13	26	3386.29(2660.71,4111.87)	0.89	-633.03(-1378.20,112.14)	0.09	0.80	-
No calcium	22	3074.01(2392.67,3755.34)		20	3731.12(2809.87,4652.38)					
Calcium[f]	19	2785.40(2052.24,3518.55)	0.56	16	3646.42(2630.55,4662.29)	0.90	203.91(-907.63,1315.45)	0.71	1.08	-
Calcium	19	2586.27(2032.99,3139.56)		17	3201.64(2513.56,3889.71)					
Calcium plus vitamin D[g]	17	2812.80(2227.88,3397.73)	0.57	16	3185.56(2470.82,3900.31)	0.97	-242.61(-1108.63,623.41)	0.57	0.91	-
25(OH)D >= median[d]										
No vitamin D	28	3096.63(2530.58,3662.68)		28	3332.66(2655.23,4010.09)					
Vitamin D[e]	31	3014.01(2476.05,3551.97)	0.83	29	3243.81(2587.40,3900.21)	0.85	-6.23(-744.63,732.17)	0.99	1.00	-
No calcium	19	3372.67(2668.69,4076.65)		18	3559.50(2582.46,4536.54)					
Calcium[f]	17	2982.42(2238.18,3726.66)	0.44	17	3635.28(2615.16,4655.40)	0.91	466.03(-493.07,1425.13)	0.33	1.15	-
Calcium	19	3090.60(2524.19,3657.01)		19	3206.04(2606.01,3806.07)					
Calcium plus vitamin D[g]	21	2730.38(2191.62,3269.15)	0.36	20	3102.37(2520.29,3684.44)	0.80	256.54(-600.12,1113.21)	0.55	1.10	-
IFABP										
25(OH)D < median[d]										
No vitamin D	30	377.86(283.48,503.66)		27	491.20(376.75,640.42)					
Vitamin D[e]	27	336.11(248.28,455.03)	0.58	26	316.13(239.94,416.53)	0.02	-133.32	-	0.72(0.55,0.95)	0.02
No calcium	21	285.48(194.11,419.87)		20	340.80(237.65,488.70)					
Calcium[f]	18	401.46(264.66,608.98)	0.23	16	386.66(260.65,573.59)	0.63	-70.11	-	0.81(0.57,1.15)	0.23
Calcium	19	440.80(311.23,624.30)		17	576.22(396.56,837.27)					
Calcium plus vitamin D[g]	17	373.43(258.47,539.52)	0.51	16	322.10(217.84,476.24)	0.04	-186.76	-	0.66(0.46,0.94)	0.02
25(OH)D >= median[d]										
No vitamin D	28	321.29(237.68,434.30)		28	361.55(251.78,519.16)					
Vitamin D[e]	31	323.82(243.17,431.22)	0.97	29	336.47(236.80,478.09)	0.78	-27.61	-	0.92(0.67,1.28)	0.63
No calcium	19	247.23(187.37,326.21)		18	229.71(162.46,324.79)					
Calcium[f]	17	216.61(161.58,290.37)	0.51	17	288.25(199.71,416.04)	0.37	89.16	-	1.43(0.94,2.19)	0.10
Calcium	19	373.27(251.31,554.42)		19	442.79(275.90,710.64)					
Calcium plus vitamin D[g]	21	359.71(246.91,524.06)	0.89	20	405.59(256.50,641.36)	0.79	-23.64	-	0.95(0.64,1.41)	0.80

Abbreviations: CI = confidence interval; 25(OH)D = 25-hydroxyvitamin D.

[a]The effect of treatment agent on biomarker expression was modeled using PROC MIXED in SAS 9.4 (Cary, NC).

[b]Absolute effect = [(treatment group follow-up) - (treatment group baseline] - [(placebo group follow-up) - (placebo group baseline)].

[c]Relative effect = [(treatment group follow-up) / (treatment group baseline)] / [(placebo group follow-up) / (placebo group baseline)].

[d]Serum 25-(OH)-D concentration median = 22.52 ng/ml.

[e]Vitamin D group comprised patients assigned to vitamin D or to calcium + vitamin D in the 4-arm randomization, or to vitamin D in the 2-arm randomization.

[f]Calcium group comprised patients assigned to either calcium or to calcium + vitamin D in the 4-arm randomization; patients in the 2-arm randomization were excluded.

[g]Vitamin D + Calcium group comprised patients assigned to calcium + vitamin D in the 4-arm randomization, or to vitamin D in the 2-arm randomization.

Supplementary Table S3. LBP and IFABP levels in study participants by treatment agent, stratified by median baseline serum total calcium levels (N= 118)[a]

Treatment Assignment	Baseline			1-yr follow-up			Absolute Treatment Effect[b]		Relative Effect[c]	
	n	Mean (95% CI)	p	n	Mean (95% CI)	p	Mean (95% CI)	p	Mean (95% CI)	p
LBP										
Total Calcium < median[d]										
No vitamin D	28	2713.55(2057.09,3370.00)		26	3600.01(2796.38,4403.65)					
Vitamin D[e]	31	2923.39(2299.50,3547.27)	0.64	30	3032.82(2278.16,3787.47)	0.31	-777.04(-1638.72,84.65)	0.08	0.78	-
No calcium	31	3044.26(2436.28,3652.24)		29	3529.97(2706.78,4353.15)					
Calcium[f]	16	2748.99(1902.73,3595.26)	0.57	15	3617.56(2472.46,4762.66)	0.90	382.86(-707.42,1473.14)	0.48	1.13	-
Calcium	12	2760.15(1833.36,3686.94)		11	3137.01(2263.83,4010.18)					
Calcium plus vitamin D[g]	16	2444.40(1641.78,3247.03)	0.60	16	2925.45(2196.77,3654.12)	0.71	104.19(-1210.05,1418.42)	0.87	1.05	-
Total Calcium >= median[d]										
No vitamin D	31	2920.53(2519.00,3322.07)		29	3173.07(2632.41,3713.72)					
Vitamin D[e]	27	3243.86(2813.62,3674.11)	0.28	25	3628.23(3047.30,4209.16)	0.26	131.83(-453.06,716.71)	0.65	1.03	-
No calcium	10	3733.68(2913.02,4554.35)		9	4009.29(2849.08,5169.49)					
Calcium[f]	20	2981.99(2401.69,3562.28)	0.14	18	3666.78(2846.40,4487.17)	0.63	409.19(-594.51,1412.89)	0.41	1.15	-
Calcium	26	2874.57(2504.28,3244.86)		25	3230.24(2704.97,3755.52)					
Calcium plus vitamin D[g]	22	3002.06(2599.51,3404.61)	0.64	20	3270.92(2689.91,3851.93)	0.92	-86.81(-705.82,532.19)	0.78	0.97	-
IFABP										
Total Calcium < median[d]										
No vitamin D	27	330.27(243.93,447.18)		26	409.63(307.76,545.20)					
Vitamin D[e]	31	326.87(246.35,433.71)	0.96	30	293.95(225.19,383.71)	0.09	-112.27	-	0.73(0.52,1.01)	0.05
No calcium	30	266.58(200.70,354.08)		29	275.04(213.59,354.17)					
Calcium[f]	16	348.98(236.59,514.76)	0.27	15	357.70(252.17,507.40)	0.23	0.26	-	0.99(0.66,1.49)	0.97
Calcium	12	524.32(337.27,815.11)		11	666.96(419.45,1060.52)					
Calcium plus vitamin D[g]	16	342.05(233.42,501.22)	0.14	16	319.46(216.16,472.14)	0.02	-165.22	-	0.73(0.43,1.25)	0.24
Total Calcium >= median[d]										
No vitamin D	31	366.96(275.32,489.12)		29	432.48(307.18,608.88)					
Vitamin D[e]	27	332.51(244.39,452.40)	0.64	25	368.77(254.88,533.56)	0.53	-29.26	-	0.94(0.71,1.24)	0.66
No calcium	10	266.75(159.92,444.94)		9	314.46(169.19,584.44)					
Calcium[f]	19	260.08(179.44,376.97)	0.94	18	309.62(197.42,485.61)	0.97	1.84	-	1.01(0.67,1.53)	0.96
Calcium	26	360.32(260.39,498.59)		25	437.61(296.67,645.51)					
Calcium plus vitamin D[g]	22	384.08(269.82,546.73)	0.79	20	402.18(261.90,617.62)	0.77	-59.19	-	0.86(0.63,1.17)	0.34

Abbreviations: CI = confidence interval; Vit. D = vitamin D; 25(OH)D = 25-OH-vitamin D.

[a]The effect of treatment agent on biomarker expression was modeled using PROC MIXED in SAS 9.4 (Cary, NC).

[b]Absolute effect = [(treatment group follow-up) - (treatment group baseline] - [(placebo group follow-up) - (placebo group baseline)].

[c]Relative effect = [(treatment group follow-up) / (treatment group baseline)] / [(placebo group follow-up) / (placebo group baseline)].

[d]Serum total calcium median = 743.55.

[e]Vitamin D group comprised patients assigned to vitamin D or to calcium + vitamin D in the 4-arm randomization, or to vitamin D in the 2-arm randomization.

[f]Calcium group comprised patients assigned to either calcium or to calcium + vitamin D in the 4-arm randomization; patients in the 2-arm randomization were excluded.

[g]Vitamin D + Calcium group comprised patients assigned to calcium + vitamin D in the 4-arm randomization, or to vitamin D in the 2-arm randomization.

Supplementary Table S4. LBP and IFABP levels in study participants by treatment agent, stratified by baseline BMI (N= 118)[a]

Treatment Assignment	Baseline			1-yr follow-up			Absolute Treatment Effect[b]		Relative Effect[c]	
	n	Mean (95% CI)	p	n	Mean (95% CI)	p	Mean (95% CI)	p	Mean (95% CI)	p
LBP										
BMI < 30 kg/m^2										
No vitamin D	37	2701.69(2213.15,3190.24)		36	3156.60(2564.24,3748.97)					
Vitamin D[d]	34	2849.26(2339.62,3358.90)	0.68	34	3166.29(2553.18,3779.40)	0.98	-137.88(-800.24,524.47)	0.68	0.95	-
No calcium	27	2881.91(2282.61,3481.22)		26	3384.41(2551.92,4216.91)					
Calcium[e]	16	2448.36(1669.84,3226.88)	0.38	16	3437.62(2366.12,4509.12)	0.94	486.76(-461.77,1435.28)	0.31	1.20	-
Calcium	21	2804.99(2240.46,3369.53)		21	2978.85(2458.37,3499.33)					
Calcium plus vitamin D[f]	23	2613.96(2074.53,3153.39)	0.62	23	3058.78(2561.45,3556.12)	0.82	270.96(-546.01,1087.94)	0.51	1.10	-
BMI >= 30 kg/m^2										
No vitamin D	22	3025.14(2428.95,3621.33)		19	3773.25(2941.34,4605.17)					
Vitamin D[d]	24	3388.93(2818.12,3959.74)	0.38	21	3482.93(2689.46,4276.39)	0.61	-654.12(-1536.70,228.47)	0.14	0.82	-
No calcium	14	3849.80(3075.91,4623.69)		12	4197.96(3101.27,5294.65)					
Calcium[e]	20	3222.49(2575.01,3869.98)	0.21	17	3762.29(2842.01,4682.57)	0.54	191.64(-1035.86,1419.14)	0.75	1.07	-
Calcium	17	2879.75(2333.79,3425.71)		15	3542.55(2731.65,4353.44)					
Calcium plus vitamin D[f]	15	3002.30(2421.08,3583.52)	0.76	13	3214.12(2346.29,4081.95)	0.58	-450.98(-1362.88,460.92)	0.32	0.87	-
IFABP										
BMI < 30 kg/m^2										
No vitamin D	37	340.99(259.78,447.59)		36	405.37(296.03,555.10)					
Vitamin D[d]	34	349.90(263.45,464.71)	0.90	34	328.01(236.27,455.37)	0.36	-86.27	-	0.79(0.59,1.06)	0.11
No calcium	27	250.49(184.63,339.85)		26	259.75(187.82,359.23)					
Calcium[e]	16	283.26(190.57,421.02)	0.62	16	299.00(195.62,457.02)	0.60	6.49	-	1.02(0.67,1.55)	0.93
Calcium	21	428.31(293.37,625.30)		21	526.07(330.51,837.34)					
Calcium plus vitamin D[f]	23	413.17(287.81,593.15)	0.89	23	386.60(247.06,604.96)	0.34	-124.34	-	0.76(0.52,1.13)	0.17
BMI >= 30 kg/m^2										
No vitamin D	21	364.74(265.00,502.02)		19	454.80(340.84,606.86)					
Vitamin D[d]	24	302.59(224.43,407.97)	0.39	21	327.61(249.66,429.91)	0.10	-65.04	-	0.87(0.65,1.16)	0.34
No calcium	13	303.52(195.57,471.05)		12	337.71(221.52,514.83)					
Calcium[e]	19	310.04(215.54,445.99)	0.94	17	372.18(262.02,528.66)	0.72	27.95	-	1.08(0.74,1.57)	0.68
Calcium	17	379.27(267.88,536.98)		15	474.31(335.42,670.71)					
Calcium plus vitamin D[f]	15	303.46(209.58,439.41)	0.38	13	338.09(233.50,489.52)	0.18	-60.41	-	0.89(0.64,1.24)	0.48

Abbreviations: CI = confidence interval.

[a]The effect of treatment agent on biomarker expression was modeled using PROC MIXED in SAS 9.4 (Cary, NC).

[b]Absolute effect = [(treatment group follow-up) - (treatment group baseline] - [(placebo group follow-up) - (placebo group baseline)].

[c]Relative effect = [(treatment group follow-up) / (treatment group baseline)] / [(placebo group follow-up) / (placebo group baseline)].

[d]Vitamin D group comprised patients assigned to vitamin D or to calcium + vitamin D in the 4-arm randomization, or to vitamin D in the 2-arm randomization.

[e]Calcium group comprised patients assigned to either calcium or to calcium + vitamin D in the 4-arm randomization; patients in the 2-arm randomization were excluded.

[f]Vitamin D + Calcium group comprised patients assigned to calcium + vitamin D in the 4-arm randomization, or to vitamin D in the 2-arm randomization.

Supplementary Table S5. LBP and IFABP levels in study participants by treatment agent, stratified by aspirin or NSAID use once per week (N= 118)[a]

Treatment Assignment	Baseline			1-yr follow-up			Absolute Treatment Effect[b]		Relative Effect[c]	
	n	Mean (95% CI)	p	n	Mean (95% CI)	p	Mean (95% CI)	p	Mean 95% CI)	p
LBP										
Asp/NSAID < 1 per wk										
No vitamin D	19	2663.93(2076.22,3251.64)		18	2763.93(2148.98,3378.88)					
Vitamin D[d]	26	2936.47(2434.07,3438.88)	0.48	23	3187.18(2647.11,3727.25)	0.30	150.71(-675.83,977.26)	0.71	1.05	-
No calcium	15	2709.55(2132.87,3286.23)		13	3304.36(2446.36,4162.35)					
Calcium[e]	9	2739.47(1994.98,3483.96)	0.95	8	2973.10(1876.79,4069.41)	0.63	-361.18(-1662.43,940.07)	0.57	0.89	-
Calcium	12	2812.42(2018.16,3606.69)		12	2666.26(2007.47,3325.04)					
Calcium plus vitamin D[f]	18	2920.60(2272.08,3569.12)	0.83	16	2991.75(2433.37,3550.13)	0.45	217.32(-633.17,1067.80)	0.60	1.08	-
Asp/NSAID >= 1 per wk										
No vitamin D	40	2897.53(2402.62,3392.43)		37	3673.42(3020.12,4326.72)					
Vitamin D[d]	32	3183.15(2629.84,3736.47)	0.45	32	3440.49(2723.83,4157.15)	0.63	-518.56(-1196.81,159.68)	0.13	0.85	-
No calcium	26	3502.53(2840.03,4165.02)		25	3876.94(2995.83,4758.05)					
Calcium[e]	27	2924.76(2274.64,3574.87)	0.22	25	3856.14(2983.10,4729.18)	0.97	556.97(-333.32,1447.27)	0.21	1.19	-
Calcium	26	2850.44(2406.32,3294.56)		24	3449.82(2863.34,4036.29)					
Calcium plus vitamin D[f]	20	2629.25(2122.87,3135.62)	0.51	20	3245.79(2597.62,3893.97)	0.64	17.17(-796.81,831.15)	0.97	1.02	-
IFABP										
Asp/NSAID < 1 per wk										
No vitamin D	18	341.46(224.79,518.68)		18	391.44(243.18,630.07)					
Vitamin D[d]	26	331.15(233.86,468.91)	0.91	23	362.68(240.29,547.39)	0.81	-18.45	-	0.96(0.62,1.47)	0.83
No calcium	14	208.54(139.77,311.14)		13	242.21(162.34,361.39)					
Calcium[e]	9	279.54(169.71,460.42)	0.35	8	301.27(176.10,515.41)	0.51	-11.94	-	0.93(0.46,1.89)	0.83
Calcium	12	428.21(256.40,715.15)		12	498.36(258.69,960.05)					
Calcium plus vitamin D[f]	18	412.21(271.18,626.60)	0.91	16	413.63(237.54,720.26)	0.66	-68.73	-	0.86(0.51,1.47)	0.57
Asp/NSAID >= 1 per wk										
No vitamin D	40	353.04(279.82,445.41)		37	439.45(345.99,558.17)					
Vitamin D[d]	32	328.14(253.05,425.52)	0.68	32	304.40(233.92,396.10)	0.04	-110.16	-	0.75(0.59,0.94)	0.01
No calcium	26	304.34(222.63,416.03)		25	305.82(219.81,425.49)					
Calcium[e]	26	303.98(222.37,415.54)	1.00	25	353.36(253.98,491.64)	0.54	47.90	-	1.16(0.87,1.54)	0.31
Calcium	26	395.62(293.35,533.53)		24	506.78(369.32,695.41)					
Calcium plus vitamin D[f]	20	328.49(233.58,461.97)	0.41	20	324.23(227.25,462.60)	0.07	-115.42	-	0.77(0.57,1.04)	0.09

Abbreviations: Asp = Aspririn; CI = confidence interval; NSAID = Non-steroidal anti-inflammatory drug; wk = week.

[a]The effect of treatment agent on biomarker expression was modeled using PROC MIXED in SAS 9.4 (Cary, NC).

[b]Absolute effect = [(treatment group follow-up) - (treatment group baseline] - [(placebo group follow-up) - (placebo group baseline)].

[c]Relative effect = [(treatment group follow-up) / (treatment group baseline)] / [(placebo group follow-up) / (placebo group baseline)].

[d]Vitamin D group comprised patients assigned to vitamin D or to calcium + vitamin D in the 4-arm randomization, or to vitamin D in the 2-arm randomization.

[e]Calcium group comprised patients assigned to either calcium or to calcium + vitamin D in the 4-arm randomization; patients in the 2-arm randomization were excluded.

[f]Vitamin D + Calcium group comprised patients assigned to calcium + vitamin D in the 4-arm randomization, or to vitamin D in the 2-arm randomization.

Chapter 3

Conclusions and Future Directions

Conclusions and Future Directions

In conclusion, this pilot randomized clinical trial of vitamin D and calcium supplementation, alone and in combination, over 1 year on plasma levels of circulating biomarkers of microbial translocation and gut barrier permeability — LBP and IFABP — demonstrated that vitamin D supplementation alone may result in decrease in LBP levels, but combined treatment attenuates the effect. This study did not find beneficial effect of administering calcium on its own. These findings provide additional support for exploring use of LBP and IFABP as reliable, non-invasive biomarkers for colon permeability and CRC risk. Taken together with previous literature, our findings support further research into chemopreventative uses of vitamin D and calcium in larger observational and clinical trials.

Proposed studies for future investigation include a similar but larger scaled RCT to assess treatment effects of vitamin D and calcium on LBP and IFABP based on varying levels of baseline serum vitamin D and total calcium levels, by randomization arm and a trial to assess potential dose-dependent effect of calcium on vitamin D impact on these particular biomarkers.