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Sonia Tandon Date

Effects of Supplemental Calcium and Vitamin D on Toll-like Receptor 5 (TLR5) Expression in the Stroma of the Normal-Appearing Rectal Mucosa of Colorectal Adenoma Patients

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By

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B.S.

The Ohio State University

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Abstract

Effects of Supplemental Calcium and Vitamin D on Toll-like Receptor 5 (TLR5) Expression in the Stroma of the Normal-Appearing Rectal Mucosa of Colorectal Adenoma Patients

By Sonia Tandon

Despite major advances in screening and treatment, colorectal cancer (CRC) remains the third leading cause of death from cancer worldwide. Calcium and vitamin D have both gained appreciable interest as promising chemopreventive agents against colorectal neoplasms, likely due to their anti-inflammatory and cell cycle regulating properties. Recent studies suggest that dysregulation of Toll-like receptor 5 (TLR5), an innate immune sensor for flagellin, may trigger cytokine secretion and pro-inflammatory responses in the colon, contributing to colorectal carcinogenesis. Although some preliminary data suggest that TLR5 may be beneficially modified by calcium and vitamin D, no human data exist. Therefore, we conducted an adjunct biomarker study nested within a larger randomized, double-blind, placebo-controlled, partial 2 x 2 factorial chemoprevention trial to test the effect of supplemental calcium (1,200 mg daily) and vitamin D (1,000 IU daily) on TLR5 expression in the stroma of normal-appearing rectal mucosa of 105 colorectal adenoma patients. TLR5 expression was measured using standardized, automated immunohistochemistry and quantitative image analysis. Although not statistically significant, the preliminary results of this pilot study suggest that supplemental vitamin D_3, alone or in combination with calcium, may increase TLR5 expression. Our analysis of patient characteristics and biomarker expression at baseline showed that race (P=0.01), having serrated adenomas (P=0.06), and baseline total calcium intake (P=0.04) were associated with TLR5 expression. While not statistically significant, modest associations were also observed with baseline physical activity, BMI, and alcohol consumption, providing further support for TLR5 expression in the stroma of the normal-appearing rectal mucosa as a modifiable, pre-neoplastic marker of risk for colorectal neoplasms. These initial findings are promising and support the continued investigation of modifiable risk factors that may influence inflammatory pathways related to colorectal carcinogenesis.

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TABLE OF CONTENTS

BACKGROUND/LITERATURE REVIEW

Despite major advances in screening and treatment, colorectal cancer (CRC) remains the third leading cause of death from cancer worldwide, accounting for more than 1.2 million cases and over 600,000 deaths annually in men and women combined (1). The continuing large global burden suggests the need to identify treatable pre-neoplastic biomarkers of risk. While incidence of CRC varies by geographic region and is highest in developed settings such as North America, Europe, and Australia/New Zealand, regions with historically low rates of CRC, particularly in Asian countries, are now witnessing an increase in risk (2). This has primarily been attributed to shifts in dietary patterns and sedentary behaviors associated with the "westernized lifestyle", which is characterized by higher consumption of red meats and processed foods, high-fat and calorie-ridden foods, obesity, a sedentary lifestyle, and lower consumption of fiber, calcium, and vitamin D (2, 3). This change in trend, accompanied by migration studies showing increasing incidence rates among populations moving from low incidence to high incidence settings, has highlighted the importance of certain diet and environmental exposures in colorectal carcinogenesis along with gene-environment interaction in mitigating risk (1, 4).

Colorectal Carcinogenesis Pathway

The underlying causes of CRC are complex and multifactorial, with both genetic and environmental factors contributing to cancer risk. CRC arises in three predominant forms: inherited, sporadic, and familial (5). Roughly 70% of cases are sporadic, while approximately 25% are familial, and 5-10% are hereditary in nature (6). This is a prime example of a multistage carcinogenesis that develops over many years. Although not

proven directly, the ‘adenoma-carcinoma sequence’ has been supported by various epidemiologic, genetic, and clinical studies to explain the stepwise progression from normal to dysplastic epithelium to carcinoma (7). The first step in the carcinogenesis sequence is thought to be the formation of aberrant crypt foci (ACF) in the normal rectal mucosa. Subsequently, inactivation of the adenomatous polyposis coli (APC) tumor suppressor gene activates the Wnt pathway. Progression to early adenoma, advanced adenoma, and carcinoma then generally occurs as a result of activation of mutations in the KRAS gene and additional mutations in PIK3CA, TP53, and TGF-β pathway genes (5). Although almost all CRCs develop from adenomatous polyps, only a small percentage of polyps become malignant, resulting in histological changes and cancer development (8). Because progression of polyps to cancer can take several years to decades, pre-cancerous polyps are ideal targets for screening strategies to reduce CRC risk (9).

Risk Factors for Colorectal Cancer

There are a wide variety of risk factors for CRC, ranging from demographic, environmental, and lifestyle factors to presence of chronic medical conditions (1, 6). Prominent established risk factors include family history, inflammatory bowel disease, tobacco smoking, consumption of alcohol and red and processed meats, obesity, and diabetes (1). Demographic risk factors include age, race, and sex (10). Incidence rates of CRC rise drastically with age, as nearly 90% of cases occur in individuals age 50 or older (10). However, in recent years, incidence of CRC has notably been increasing among younger people, and in the United States, it has become one of the top ten most commonly diagnosed cancers among individuals ages 20-49 (11). Additionally, incidence

varies by race, ethnicity, and geographic region, with black men and women having the highest incidence and mortality rates in the United States (12). Consistently, higher incidence rates have been observed in higher income countries as compared to lower income countries, but incidence in lower income countries is rapidly beginning to increase. While incidence ranges from 30 or more per 100,000 people in the United States, Europe, and Australia, New Zealand, and Japan, incidence is limited to 5 or fewer per 100,000 in most of Africa and parts of Asia. These vast differences in incidence across geographic regions suggest the striking role of environmental influences in CRC risk (13). Because it is considered an environmental disease, it is thought that a large proportion of cases could theoretically be prevented, and it has been postulated that up to 70% of all cases may be attributable to a western diet characterized by poor diet and a sedentary lifestyle (10, 14). Although the introduction of screening and improvements in treatment have certainly reduced CRC incidence and mortality, their effectiveness has been limited, particularly in developing settings (15, 16). Thus, much research has focused on targeting other modifiable dietary and lifestyle risk factors for CRC (15).

Throughout the past few decades, findings from numerous observational studies and randomized clinical trials have demonstrated associations between multiple dietary factors and risk of colorectal neoplasms. According to a 2017 report from the World Cancer Research Fund, there is strong evidence that consumption of calcium, whole grains, dietary fiber, and dairy products reduces the risk of CRC, while consumption of red and processed meats and alcohol contribute to CRC risk (15, 17). In particular, preclinical experimental studies provide strong support for the role of calcium and vitamin D in inhibiting colorectal carcinogenesis, while the epidemiologic literature

suggest that calcium and vitamin D intake are associated with lower CRC risk. Furthermore, clinical trial data suggest that calcium and vitamin D reduce risk of colorectal neoplasms (3). Therefore, calcium and vitamin D have both gained appreciable interest as promising chemopreventive agents against colorectal neoplasms; however, the exact mechanisms through which this occurs are not well understood.

Calcium and Colorectal Carcinogenesis

Calcium is a critical micronutrient that plays an important role in muscular contraction, cellular growth, cell adhesion, and bone formation (13). It is thought to reduce CRC risk by binding with secondary bile acids and free fatty acids formed in the colon, protecting the colonic lumen against their toxic effects (4). Other protective mechanisms of calcium include direct effects on colonic epithelial cells through cell cycle regulating activities such as inhibition of cell proliferation, promotion of cell differentiation and apoptosis, and modulation of CRC cell signaling pathways via the calcium-sensing receptor (3, 15). These mechanisms have been abundantly supported by *in vitro* experimental studies (18, 19). In one animal model study, rodents fed a diet representing Western dietary intake of calcium, phosphate, vitamin D, and fat had higher incidence of hyperplasia and increased proliferation in the colon; however, these effects were reduced when intake of dietary calcium was increased (19).

Numerous observational cohort and case-control studies have supported these plausible mechanisms by examining the association between calcium intake and CRC risk, including a meta-analysis of 60 observational studies with data suggesting that increased intake of calcium may reduce risk of both colon and rectal cancers by up to 45% (4, 20, 21). Additionally, a pooled analysis of ten prospective cohort studies

assessing 534,536 individuals determined that higher consumption of dietary and supplemental calcium was inversely associated with risk of CRC (OR, 0.78; 95% CI, 0.69-0.88) (22).

Randomized controlled trial data have also largely supported this association, as supplemental calcium has been shown to be effective in reducing risk for adenoma recurrence (23-26). Specifically, multiple studies that randomized participants to either calcium carbonate or placebo reported that calcium supplementation was associated with significant, but moderate, reductions in risk of recurrent colorectal adenomas (23, 25). However, interestingly, in a 2 x 2 factorial trial of calcium and vitamin D, there was no statistically significant effect of calcium on risk of colorectal neoplasms (OR, 0.95; 95% CI, 0.85-1.06), although the authors had no strong explanation for these results (26). In summary, the inverse association between calcium and colorectal adenoma has been consistently observed in observational studies, and randomized controlled trials have largely found that calcium supplementation reduces recurrence of colorectal adenomas.

Vitamin D and Colorectal Carcinogenesis

Vitamin D is a precursor to the steroid hormone calcitriol, also known as 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), and governs many actions throughout the body including regulation of calcium homeostasis and phosphate metabolism, normal mineralization of bone, and gene expression in tissues (27, 28). Dietary vitamin D is available in two forms: vitamin D_2, or ergocalciferol (derived from plant sources), and vitamin D_3, or cholecalciferol (derived from animal sources) (28). Unless fortified, however, most foods contain very little vitamin D, leading most persons to primarily acquire vitamin D through cutaneous synthesis following exposure to ultraviolet B rays

(29). Additionally, it is now known that the vitamin D receptor (VDR) and CYP24 are highly expressed in the colon along with many other tissues, where, when activated, vitamin D can modulate more than 200 genes thought to be involved in colorectal carcinogenesis, including cell cycle regulation, growth factor signaling, cell adhesion, protection against oxidative stress, bile acid and xenobiotic metabolism, DNA repair, angiogenesis, and inflammation (15, 30).

The epidemiologic link between vitamin D and colon cancer was first established in 1980 by Garland, who discovered that colon cancer mortality rates varied geographically by exposure to sunlight and hypothesized that vitamin D status may be inversely related with risk of colon cancer (31). Although its potential anti-neoplastic effects were unknown at the time, since then, basic science and preclinical findings have provided strong support for the hypothesis that vitamin D reduces cancer risk. *In vitro* studies have demonstrated that vitamin D inhibits proliferation, induces differentiation, inhibits angiogenesis, and stimulates apoptosis in epithelial tissues (32).

In the epidemiologic literature, Vitamin D has been shown to be associated with lower risk for both colorectal adenoma and cancer. Several meta-analyses assessing serum 25(OH)D concentrations and CRC incidence in prospective cohort studies have been conducted, and all have found a statistically significant inverse association (33-35). A meta-analysis of eight prospective cohort studies (N =1,822 colon and 868 rectal cancers) found a statistically significant inverse association between circulating 25(OH)D levels and CRC risk (OR, 0.66; 95% CI, 0.54-0.81) (34). Additionally, a systematic review of nine prospective cohort studies assessing vitamin D intake and nine studies on blood 25(OH)D levels found pooled risk ratios for CRC of 0.88 (95% CI, 0.80-0.96) and

0.67 (95% CI, 0.54-0.80), respectively (35). Meta-analyses assessing the association between serum 25(OH)D levels and colorectal adenoma in both observational studies and randomized trials have consistently shown a statistically significant inverse association for adenoma incidence, but not recurrence (33, 36, 37). This data suggest that increased vitamin D intake may reduce risk of incident colorectal adenomas but, once removed, may not prevent formation of another adenoma. To date, the few large randomized controlled trials that have been conducted have found that vitamin D supplementation does not significantly reduce the risk for colorectal adenoma or cancer; however, many of these studies were limited by factors such as small sample size, low dose, timing of supplementation, and short follow-up time (38-41). The Women's Health Initiative randomized 36,282 women to 1000 mg of calcium and 400 IU vitamin D3 vs. placebo, but they found that daily supplementation over seven years had no significant effect on risk of incident CRC among post-menopausal women (41). Another study conducted in the United Kingdom randomized 2686 people to 100,000 IU/d oral vitamin D_3 or placebo every four months for five years, but no reduction in risk of CRC incidence or mortality was observed (40). Thus, while preclinical findings and observational studies have clearly supported an inverse association between vitamin D intake and risk of colorectal adenoma or cancer, the clinical trial data have been inconsistent and may require further research.

Synergism/Antagonism of Calcium and vitamin D

Several previous studies have suggested that calcium and vitamin D may modify each other's activity in affecting risk for CRC (42-44). Mouse models have shown that vitamin D and calcium both, individually and synergistically, have an inhibitory effect on

the development of polyps (28). Furthermore, evidence from both observational and large randomized controlled trials propose a possible synergistic effect of calcium and vitamin D supplementation on risk of recurrent colorectal adenoma (45). The Calcium Polyp Prevention Study showed that calcium supplementation can reduce the recurrence of colon polyps, but the magnitude of effect relies on serum vitamin D levels (46). That is, the association between calcium intake and colorectal neoplasms may be limited to those with high vitamin D intake. However, other studies suggest a possible antagonistic effect of calcium and vitamin D; one animal study determined that calcium and vitamin D served as better inhibitors of colorectal carcinogenesis separately rather than combined (47). There is a need to better understand the magnitude and implications of the potential synergistic or antagonistic effects that calcium and vitamin D may have on risk of colorectal adenoma and cancer.

Inflammation, Gut Microbiome, TLR5, and Colorectal Carcinogenesis

While inflammation is well known to be linked to CRC progression, the molecular mechanisms by which this occurs are not fully understood (48). Studies show that immune cells, cytokines, and other immune mediators play important roles in nearly every step of the colorectal carcinogenesis sequence, including initiation, promotion, progression, and metastasis (49). Furthermore, increasing evidence supports the role of the gut microbiota in CRC (50). Biologic plausibility for the role of the gut microbiota has been established by the large presence of microbes in the large intestine as well as the known importance of gut microbiota in maintaining gut barrier integrity and promoting immune homeostasis in the colon (50). Subtle inflammation may contribute to the conversion from a healthy to dysplastic colon, leading to epithelium barrier disruption,

bacterial translocation, and activation of immune signaling pathways by bacterial stimuli, ultimately resulting in the production of a pro-neoplastic environment (51). In summary, disruption of the normal intestinal microbiota may lead to a chronic inflammatory state, contributing to carcinogenic metabolite production and possibly neoplasia (52). However, it is not yet clear whether aberrations in commensal microbial communities are the cause or consequence of inflammation (53).

Due to vastly different gut microbial structures between populations consuming different diets, it is thought that the gut microbiota may modulate dietary associations with CRC risk and vice versa (50). Controlled diet intervention studies have shown that changes in dietary composition result in rapid alterations in the gut microbiota (54, 55). This is also well exemplified by the case of rural Africans, who consume diets rich in fiber and low in fat, and have strikingly different gut microbial compositions from African Americans and Europeans who consume a western diet. Interestingly, these differences in microbial composition align with the lower rates of CRC seen in African versus western countries (56, 57). Furthermore, researchers have identified multiple microbial groups that are associated with CRC that may account for differences in healthy and disease gut microbiomes, including significantly higher populations of the groups *Bacteroides* and *Prevotella* and genera *Enterococcus, Escherichia/Shigella, Klebsiella, Streptococcus*, and *Peptostreptococcus* in CRC patients (58-60).

One of the aims of the research group is to determine potential pre-neoplastic biomarkers of risk for colorectal neoplasms. Toll-like receptor 5 (TLR5) is one such potential biomarker. TLR5 is an innate immune sensor for flagellin, the structural component of bacterial flagella that stimulate host defenses. Because alterations in gut

microbial composition may lead to additional recognition of bacterial stimuli (61), by recognizing pathogen-associated molecular patterns (PAMPs) such as flagellin, TLR5 plays an important role in regulating the innate immune response to microbial agents. TLR5 acts via the MYD88 pathway, leading to NF-kB activation and secretion of cytokines, chemokines, and anti-microbial peptides that help establish a tightly regulated inflammatory state (48, 53). One research group has shown that the colorectal carcinogenesis sequence was accompanied with a rise in Toll-like receptors (62). Furthermore, recent studies suggest that dysregulated TLR signaling may trigger pro-inflammatory responses contributing to the development of CRC; however, the molecular mechanism remains poorly understood (63). Studies have shown that SNPs in the TLR5 gene are associated with the survival of CRC patients and other clinic-pathological measures (64). Specifically, the TLR5 variants rs5744174 and rs2072493 are associated with changes in the sensing of flagellin and impact secretion of IL-1β and IL-6, both of which play essential roles in colorectal carcinogenesis (64, 65). Thus, perturbations in TLR5 signaling may modify the intestinal inflammatory state and either promote or inhibit colorectal carcinogenesis.

Effect of Calcium and Vitamin D on TLR5 Expression

While the interplay between calcium, vitamin D, and TLR5 expression in CRC has not been explicitly studied, multiple *in vitro* studies have shown a link between these nutrients and Toll-like receptors, particularly through inactivation of the NF-kB pathway. *In vitro* studies suggest that the anti-inflammatory effects of Vitamin D3 may be mediated by inhibition of the nuclear factor-kB (NF-kB) and mitogen-activated protein kinase (MAPK) signaling pathways (66, 67). Furthermore, studies have shown inverse

associations between 25(OH)D levels and expression of TLRs, particularly in diseases characterized by inflammation, such as Type I Diabetes and Behçet's disease (66). These observations have given rise to the hypothesis that optimal vitamin D levels may reduce inflammation. Calcium is hypothesized to exert its anti-neoplastic effects by binding free fatty acids and bile acids in the colon; when unbound, bile acids are metabolized by the gut microbiome, producing secondary bile acids that may promote colorectal carcinogenesis via interaction with nuclear factor-kB (NF-kB) and cyclooxygenase-2 (COX2)/prostaglandin 401 synthase-2 pathways (15). While the mechanisms are currently unclear, there is great potential that calcium and vitamin D may have an effect on TLR5 expression, and TLR5 may serve as a pre-neoplastic biomarker of risk for colorectal adenoma and cancer.

Significance of the Stroma

Although clinical practice predominantly focuses on the epithelial cells, there is growing evidence that the tumor microenvironment, including the hematopoietic and non-hematopoietic stromal cells of the colon ("immune stroma"), plays a major role in CRC progression and metastasis through immune responses and initiation of inflammation (68, 69). Furthermore, its proximity to the colonic lumen may also increase exposure to harmful bacterial products. Thus, it is becoming increasingly important to study biomarkers of CRC risk in the stroma. Prior studies have shown that TLR5 is expressed by stromal immune cells, providing rationale for investigating its expression in relation to inflammation and colorectal carcinogenesis (70). Currently, no studies have examined the effect of calcium and vitamin D supplementation on expression of TLR5 in the stroma; therefore, this study will aim to further explore the hypothesized relationship

by assessing the effect of vitamin D and calcium supplementation on TLR5 expression in the stroma of the normal rectal mucosa of colorectal adenoma patients.

METHODS

Research Aims

1) To assess the effect of Vitamin D and calcium supplementation (alone and in combination) on TLR5 expression in the stroma of normal rectal mucosa;
2) In a cross-sectional analysis, to assess the association of TLR5 expression in the stroma of normal rectal mucosa at baseline with demographic and lifestyle characteristics chosen *a priori* due to biologic plausibility and their association with CRC risk

Hypothesis

We hypothesize that vitamin D and calcium supplementation (alone and in combination) over one year will reduce TLR5 expression in the stroma of normal rectal mucosa of colorectal adenoma patients.

Clinical Trial Protocol

Data for this study ("adjunct biomarker study") was obtained from 105 study participants who were participating in a larger 11-center randomized, placebo-controlled, partial 2 x 2 factorial chemoprevention clinical trial ("parent study"; Vitamin D/Calcium Polyp Prevention Study) assessing the effect of supplemental calcium and Vitamin D, alone and in combination, over 3-5 years on adenoma recurrence in colorectal adenoma patients (26). The parent study protocol, in addition to inclusion and exclusion criteria, has been published elsewhere (26).

The complete study protocol for the adjunct biomarker study has also been previously published (71). Patients were eligible to participate in the biomarker study if they visited two of the eleven clinical centers (South Carolina and Georgia) between May

2004 and July 2008 and agreed to provide rectal biopsy tissue samples at baseline and after one year of supplementation with the assigned study agent. Out of 231 eligible participants, 109 met final eligibility criteria, signed consent, and had rectal biopsy tissue samples taken at baseline. Of these, rectal biopsy tissue samples sufficient for biomarker measurement at baseline and 1-year follow-up were available for 105 participants. All participants signed a consent form at enrollment, and the research was approved by the Institutional Review Board at both clinical centers.

At enrollment in the parent study, information was collected from each study participant on medical history, medication and nutritional supplement use, lifestyle, and diet, assessed using the Block Brief 2000 food frequency questionnaire (Nutritionquest, Berkeley, CA). Blood levels of 25-hydroxyvitamin D [25(OH)D] were obtained at both baseline and year 1 follow-up; meanwhile, calcium and 1,25(OH)2 vitamin D [1,25(OH)2D] levels were only obtained at baseline.

Participants were randomized to one of four treatment groups following the placebo run-in period: placebo, 1200 mg/d calcium supplementation (calcium carbonate in equal doses twice daily), 1000 IU/d vitamin D_3 supplementation (500 IU twice daily), and 1200 mg/d elemental calcium plus 100 IU/d vitamin D_3 supplementation ("full factorial randomization"). Women who did not forego calcium supplementation were randomized to calcium or calcium plus vitamin D_3 through 2-arm randomization. Randomization was conducted using computer-generated random numbers with permuted blocks, and were stratified by sex, clinic center, scheduled colonoscopic follow-up of 3 or 5 years, and 4- versus 2-arm randomization. All study staff and participants were blinded to treatment assignments. Participants agreed not to take vitamin D or calcium

supplements outside of their treatment groups, although personal supplements of up to 1,000 IU vitamin D and/or 400 mg elemental calcium were allowed beginning in April 2008. Bottles of study tablets were mailed to participants every four months. Interviews were conducted via telephone every six months to assess participant adherence to their assigned treatment, illnesses, use of medicines or supplements, and any colorectal endoscopic or surgical procedures.

Rectal Biopsy Tissue Collection and Quantification of TLR5

Biopsies of normal-appearing rectal mucosa were taken at baseline and one-year follow-up without preceding bowel-cleansing preparation or procedure. TLR5 expression in the stroma/ immune cells in 105 rectal mucosa biopsies was measured at baseline and 1 year follow-up using standardized, automated immunohistochemistry and quantitative image analysis. The rectal biopsy and immunohistochemistry protocol has also been previously published (71). Five slides with three levels of 3 μm-thick biopsy sections taken 40 μm apart were prepared and placed in a preheated Pretreatment Module (Lab Vision Corp., Fremont, CA) with 100x Citrate Buffer pH 6.0 (DAKO S1699, DAKO Corp., Carpinteria, CA) and steamed for 40 minutes. They were then placed in a DakoCytomation Autostainer Plus System automated immunostainer and processed using a labeled streptavidin-biotin method (LSAB2 Detection System [DAKO K0675]) for TLR5 by applying a rabbit polyclonal antibody against TLR5 (catalog no.: MAB6704, dilution 1:200; R&D Systems, Minneapolis, MN). Baseline and follow-up biopsy slides for each participant were stained in the same batch, and each batch included an equal distribution of participants from each treatment group along with positive and negative controls.

A quantitative image analysis method ("scoring") was used to measure levels of TLR5 in both the colon crypts and stroma surrounding colon crypts in each sample. First, the slides were scanned using the Aperio Scanscope CS digital scanner (3DHISTECH, Budapest, Hungary); subsequently, the baseline and follow-up digital images for all 105 participants were reviewed in a custom-developed software, the CellularEyes program (DivEyes LLC, Atlanta, GA), by a trained technician to identify colon crypts that were acceptable for analysis. "Scorable" crypts were defined as intact crypts extending from the muscularis mucosa to the colon lumen. To score a selected hemicrypt (half of a crypt), the technician used a digital drawing board to trace its outline. The CellularEyes program then divided the outline into equally spaced segments with the average width of normal colonocytes and measured the background-corrected optical density of the biomarker labeling across the entire hemicrypt and in each segment. The obtained data was automatically transferred from the software to the MySQL database (Sun Microsystems Inc., Redwood Shores, CA). This process was continued until a total of 8 to 40 hemicrypts had been scored for each baseline and follow-up visit. If possible, both hemicrypts within a scorable crypt were scored.

To score the stromal region between two scorable crypts, hereby referred to as "stromal region", a second technician would identify previously scored crypts and inspect it to see if the width of the lamina propria region surrounding the hemicrypt was wide enough for scoring. If deemed appropriate, the technician would outline the region, avoiding epithelial cells, muscle tissue, and staining artifacts **(Figure 1)**. The technician would continue this process until, if possible, a minimum of eight stromal regions were scored for each patient at each visit.

To determine intra-reader scoring reliability, reliability control samples previously analyzed by the technician were re-analyzed. The intra-class correlation coefficient for TLR5 in the stroma was 0.96.

Statistical Analysis

The treatment groups were assessed for similarity in baseline characteristics including demographic information, medical history, habits, anthropometrics, and dietary intakes at baseline and follow-up using χ^2 tests for categorical variables and ANOVA or t-tests for continuous variables. Treatment effects were determined by assessing the differences in TLR5 expression from baseline to 1-year follow-up between participants in the treatment group of interest and appropriate comparison group using a general MIXED linear model. The model included the intercept, visit (baseline and 1-year follow-up), treatment group, and a treatment-by-visit interaction term. First, we assessed changes in TLR5 expression in the stroma by treatment assignment, where the 4-arm group received (i) calcium vs. placebo, (ii) vitamin D vs. placebo, or (iii) calcium + vitamin D vs. placebo and the 2-arm group received vitamin D vs. placebo. Next, we assessed changes in TLR5 expression in the stroma after randomization to treatment groups that received (i) vitamin D relative to those that did not ("vitamin D vs. no vitamin D"), (ii) calcium relative to those that did not ("calcium vs. no calcium"), and (iii) calcium plus vitamin D relative to those that received only calcium ("calcium + vitamin D vs. calcium"). In addition to assessing changes in biomarker expression in the whole stromal region, we also assessed changes within the upper 40% and lower 60% of the stroma. The upper 40% is normally considered the differentiation zone in the crypt but was selected in the stroma due to its proximity to the colon lumen and hence, higher exposure to flagellin

and other bacterial products. In conjunction, the lower 60% of the stromal region, normally considered the proliferation zone of crypts, and the ratio of the upper 40% of the stromal region to the whole stromal region (ϕh) were also assessed. To better explain the magnitude of the estimated treatment effects, relative and absolute treatment effects were calculated using the following formulas: relative effect = [(treatment group at follow-up)/ (treatment group at baseline)]/ [(comparison group at follow-up)/ (comparison group at baseline)]; absolute effect = [(treatment group follow-up)-(treatment group baseline)]–[(control group follow-up)-(control group baseline)]. For example, a relative effect of 1.4 would signify a 40% increase in TLR5 biomarker expression in the treatment group relative to the control group. Through all analyses, participants were retained in their original assigned treatment group, regardless of adherence to study treatment and procedures.

Potential confounders, identified through differences in baseline characteristics by treatment group or from participant inclusion in the adjunct biomarker study, were assessed by running one additional model. The first model controlled for age, sex, and study center, while the second model additionally controlled for current smoking status (current and former vs. never), multivitamin use (yes vs. no), physical activity (MET-min/week), dietary fiber (g/day), and total caloric intake (kcal/day). However, because adjustment for these potential confounders did not substantially affect the estimated treatment effects, only the results from model 1 are reported.

Exploratory stratified analyses were conducted to assess potential treatment effect modification by baseline characteristics such as regular NSAID and/or aspirin use and baseline total calcium intake, and blood 25(OH)D. We also examined if patients'

characteristics, chosen *a priori* due to biologic plausibility, were associated with baseline expression of TLR5 in the stroma through a generalized linear model adjusted for age, sex (by study arm), study center, and batch. All statistical analyses were conducted using SAS 9.4 statistical software (Cary, NC), and a two-sided p-value of ≤ 0.05 was considered statistically significant.

RESULTS

Baseline Characteristics of Study Participants

Selected baseline characteristics of the 105 study participants are presented in **Table 1**. The mean age across all treatment groups was 59 years, 47% of study participants were male, and 79% were white. Most participants were at least high school graduates, overweight (79%), and not currently smokers (92%). 8.9% of study participants had a family history of CRC. At baseline, there were significant differences in physical activity ($P = 0.03$) and dietary fiber intake ($P = 0.04$) across treatment groups. During the first year after randomization, 76% of participants reported taking 80% or more of their assigned study tablets. There was a mean increase in serum 25(OH)D levels 9.0 (SD = 1.6; $P = 0.03$) ng/ml at year 1 in subjects randomized to vitamin D compared to those who were not.

TLR5 Expression by Treatment Assignment and Agent

The effect of one year of calcium and vitamin D supplementation, alone and in combination, on TLR5 expression by treatment assignment is shown in **Table 2**. In the 4-arm group, treatment with calcium *versus* placebo decreased TLR5 expression in the stromal region by an estimated 13% ($P = 0.77$). A similar decrease was noted in the upper 40% (23%, $P = 0.59$), whereas there was an increase in expression in the lower 60% (19%, $P = 0.77$) of the stroma. For vitamin D *versus* placebo, TLR5 expression increased by 52% ($P = 0.37$). Likewise, increases in expression were observed in the upper 40% and lower 60% of the stroma (27% ($P = 0.61$) and 170% ($P = 0.10$), respectively). There was no significant effect on TLR5 expression for calcium and vitamin D *versus* placebo ($P = 0.99$). Finally, there was a significant effect of treatment with vitamin D on the ratio

of the upper 40% of the stromal region to the whole stromal region (ϕh) ($P = 0.01$). In the 2-arm group, among women who declined to forego calcium supplementation, treatment with vitamin D *versus* placebo resulted in an increase in TLR5 expression in the stromal region by an estimated 36% ($P = 0.43$). Similar results were found for changes in TLR5 expression in the upper 40% and lower 60% of the stroma. To better describe how TLR5 expression varied across the stroma, we graphed the distribution of mean TLR5 biomarker density across the stroma in calcium vs. placebo, vitamin D vs. placebo, calcium and vitamin D vs. placebo, and the 2-arm vitamin D vs. placebo groups (**Figures S.1-S.4**).

The effect of one year of calcium and vitamin D supplementation, alone and in combination, on TLR5 expression by treatment agent is shown in **Table 3**. After one year of treatment with the study agent, neither calcium nor vitamin D, alone or in combination, significantly affected expression of TLR5 in the stroma of normal-appearing rectal mucosa.

Treatment with calcium *versus* no calcium decreased TLR5 expression by an estimated 27% in the stromal region ($P = 0.31$). Similar decreases were observed in the upper 40% and lower 60% of the stroma (27% ($P = 0.30$) and 31% ($P = 0.37$), respectively). For vitamin D *versus* no vitamin D, TLR5 expression increased by an estimated 32% in the stromal region ($P = 0.24$). Similar increases in expression were noted in the upper 40% (28%, $P = 0.30$) and lower 60% (58%, $P = 0.12$) of the stroma. Treatment with both calcium and vitamin D *versus* calcium increased TLR5 expression by an estimated 23% ($P = 0.47$). An increase in TLR5 expression of 26% ($P = 0.42$) was observed in the upper 40% of the stroma, while an increase of 29% ($P = 0.48$) was

observed in the lower 60% of stroma. There were no significant effects of combined treatment with calcium and vitamin D on the ratio of the upper 40% of the stromal region to the whole stromal region (ϕh) ($P = 0.78$). Non-significant treatment effects on ϕh were also observed for calcium *versus* no calcium ($P = 0.90$) and vitamin D *versus* no vitamin D ($P = 0.36$).

Stratified Analyses

We conducted secondary analyses to assess potential treatment effect modification by baseline characteristics such as regular NSAID and/or aspirin use, baseline vitamin D intake, and baseline calcium intake **(Tables 4-7)**. In the stratified analyses by frequency of NSAID use, there was no statistically significant evidence of interaction for treatment effects of calcium, vitamin D, or calcium plus vitamin D (all $P > 0.19$) **(Table 4)**. When the treatment groups were stratified by frequency of aspirin use, there were no statistically significant differences in treatment effects of calcium, vitamin D, or calcium plus vitamin D; however, the data suggest that calcium supplementation was more effective in non-aspirin users (-57%, $P = 0.08$). **(Table 5)**. We also performed a stratified analysis by baseline serum vitamin D intake **(Table 6)**. Although no statistically significant interaction was observed ($P_{interaction} = 0.65$), among study participants with below median baseline serum vitamin D levels, treatment with vitamin D *versus* no vitamin D increased TLR5 expression in the stromal region by 87% ($P = 0.08$); among study participants with above median serum vitamin D levels, however, TLR5 expression was largely unaffected ($P = 0.85$), suggesting that participants below median intake were not achieving an optimal amount of vitamin D. Finally, we stratified the study population by baseline total calcium intake **(Table 7)**. While there was no evidence of statistically

significant interaction ($P_{interaction} = 0.59$), we found that, among those who had below median total calcium intake, treatment with vitamin D *versus* no vitamin D increased TLR5 expression in the stromal region by 62% ($P = 0.16$); among those with above median total calcium intake, however, TLR5 stromal expression was not affected ($P = 0.88$). Stratified comparisons of TLR5 stromal expression by sex-specific total calcium intake are available in **Table S.1**.

TLR5 Expression by Baseline Characteristics

We also examined if participant characteristics, chosen *a priori* due to biologic plausibility and their association with CRC risk, were associated with baseline expression of TLR5 in the stroma through a cross-sectional analysis. The associations between these selected baseline characteristics and baseline TLR5 expression in the whole stromal region are shown in **Table 8**. Data for TLR5 expression in the upper 40%, lower 60%, and ratio of the upper 40% of the stromal region to the whole stromal region (ϕh) are available in the supplementary tables (**Tables S.2- S.4**). On average, although not statistically significant, women had 14% lower TLR5 expression in the stromal region relative to men ($P = 0.57$). There was no significant association of baseline TLR5 stroma expression with age ($P = 0.80$). Furthermore, on average, white study participants had 46% lower TLR5 expression in the whole stromal region ($P = 0.01$), regular NSAID use was associated with 14% lower expression of TLR5 stroma ($P = 0.45$), and regular aspirin use was associated with 4% lower expression ($P = 0.84$). High physical activity was associated with 27% lower expression of TLR5 in the stroma ($P = 0.14$). Study participants who were overweight (25-30 kg/m^2) had 35% higher expression of TLR5 in the stroma, and obese participants (> 30 kg/ m^2) had 4% higher expression compared to

those who were of normal weight (<25 kg/ m^2) ($P_{trend} = 0.52$). Baseline TLR5 expression in the stroma was 49% higher among those who had a history of sessile serrated adenoma ($P = 0.06$). Achieving the daily recommended dietary fiber intake (≥ 14 g/1000 kcal per day) was associated with 5.3% lower expression of TLR5 ($P = 0.83$), while achieving the daily recommended intake of fruits and vegetables (≥ 5 servings/d) was associated with 13% lower baseline expression of TLR5 in the stroma ($P = 0.47$). Alcohol intake of more than 1 drink per week was associated with 49% higher TLR5 expression in the stromal region ($P_{trend} = 0.17$). Lastly, study participants in the highest tertile of baseline calcium intake had 36% lower expression of TLR5 in the stroma relative to those in the lowest tertile ($P = 0.04$).

DISCUSSION

Discussion of Primary Findings

Although not statistically significant, the preliminary results of this pilot study suggest that (i) supplemental vitamin D_3, alone or in combination with calcium, may increase TLR5 expression; and (ii) provide further support for TLR5 expression in the stroma of the normal-appearing rectal mucosa as a modifiable, pre-neoplastic marker of risk for colorectal neoplasms. While our small sample size likely limited our ability to detect statistically significant differences in biomarker expression, we observed relatively large treatment effects in the analysis by treatment agent. Because our small sample size in the placebo group (N=12) may have led to unstable estimates of the treatment effect by treatment assignment, our analysis was primarily focused on the effect on TLR5 expression in the stroma by treatment agent. However, of note, we did observe similar results in our analysis by treatment assignment.

Multiple calcium and vitamin D supplementation trials have been conducted previously, but to our knowledge, this is the first study to measure TLR5 biomarker expression in the colon tissue following supplementation. While the inverse association of both calcium intake and blood levels of vitamin D with risk of colorectal adenoma and cancer has been extensively shown in the epidemiologic literature (4, 20-22, 33-35), the human clinical trial evidence has been largely inconsistent, especially for vitamin D. This lack of consensus may stem from the fact that the current human data comes from clinical trials limited by small sample size, short follow-up time, and inconsistent dosing and routes of administration for supplemental calcium and vitamin D. Furthermore, studies have only assessed the effect of calcium and vitamin D on serum inflammatory markers,

which may not accurately represent the localized inflammatory activity in the tissue of the colon. Because inflammation has been shown to be a key risk factor in CRC progression, and the cell cycle regulation activities of calcium and anti-inflammatory properties of vitamin D have been well documented, both separately and combined, in multiple experimental studies, it was hypothesized that supplementation with both potential chemopreventive agents would decrease expression of the pro-inflammatory biomarker TLR5 in the stroma of normal-appearing rectal mucosa (28). While our results do not statistically support this primary hypothesis, in the analysis by treatment agent, we found that supplementation with calcium relative to no calcium decreased TLR5 expression by 23% and supplementation with vitamin D relative to no vitamin D increased TLR5 expression by 32%. Furthermore, we found that supplementation with calcium and vitamin D relative to calcium only increased TLR5 stroma expression by 23%, suggesting a potential antagonistic effect of calcium and vitamin D in combination. This is further supported by the results of the stratified analyses, where consistently, supplementation with vitamin D and calcium in combination resulted in TLR5 expression that was higher than supplementation with calcium but lower than supplementation with vitamin D.

Factors Associated with TLR5 Expression

We also examined the cross-sectional association of *a priori* selected patient characteristics with TLR5 expression at baseline. Our results suggest that women had lower, albeit not statistically significant, TLR5 expression at baseline relative to men. Female sex hormones have been hypothesized to have a protective immunological effect on inflammatory responses, and progression of CRC has been linked to estrogen

receptors in the rectal mucosa (72). These findings may also be explained by the accumulating evidence indicating that sex-specific alterations in the gut microbiota may drive differences in intestinal inflammatory diseases (73).

In our population, we found that compared to those who were non-white, white participants had statistically significantly lower baseline TLR5 expression. Racial disparities have been well observed in observational studies of CRC, with incidence, aggressiveness, and mortality higher among African-Americans (12). While the biological causes of this discrepancy remain to be elucidated, multiple studies have examined racial disparities in differential gene expression and microbial composition and how they correspond to CRC risk. One such study compared gene expression profiles of tumors from African Americans and European Americans and found that three of the six differentially expressed genes and their pathways were related to immune and inflammatory response, suggesting that differential gene expression and inflammation may underlie racial disparities in CRC (74).

High physical activity, defined as more than 3,306 met-min/week for men and more than 2,106 met-min/week for women, was associated with lower TLR5 expression at baseline. The link between physical activity and CRC has been extensively shown in the literature, with some studies suggesting that individuals with low physical activity have a 27% increased risk of CRC (75). These associations are supported by plausible biological mechanisms including influencing inflammation-stimulated cell growth. Emerging evidence further suggests that the association with CRC progression may be mediated through modulation of energy metabolism and inflammatory pathways (76).

Overall, BMI was associated with increased expression of TLR5 at baseline, a finding that aligns with a large and continuously growing body of literature. Obesity is considered a chronic state of inflammation, characterized by the release of free fatty acids, pro-inflammatory cytokines including IL-8, IL-6, or IL-2, and polypeptide growth factors from obese adipose tissue (77-79). Dysregulated release of these factors subsequently contributes to malignant transformation, tumor initiation, and CRC progression (77, 79). In obese individuals, overproduction of leptin, a hormone that helps regulate appetite and body weight, has been found to promote cell motility and invasiveness in colon cancer through activation of multiple signal-transduction pathways (80). *In vitro* models simulating leptin overproduction have shown increased proliferation of CRC cell lines, angiogenesis, TLR5 signaling, and pro-inflammatory cytokines including IL-6 (79, 81). These findings may also be explained by diet-driven differences in the gut microbiome of obese and normal weight individuals. Recent mouse model studies have found that both genetic and diet-induced obesity disrupt gut homeostasis and alter abundance of flagellated bacterial species such as *E.coli*, promoting intestinal inflammation and colorectal tumorigenesis (82). While the exact mechanisms through which this occurs require further study, our results support the link between obesity, inflammation, and colorectal carcinogenesis.

We also found that, relative to no alcohol consumption, alcohol intake of more than 1 drink per week was associated with 49% higher TLR5 expression in the stroma. Although the mechanisms through which alcohol consumption promotes colorectal carcinogenesis remains unclear, these findings may be explained by mucosal inflammation in the intestine. Oxidative stress derived from ethanol metabolism may

increase intestinal permeability and exposure to microbial byproducts, leading to the expression of pro-inflammatory cytokines including IL-6 (83).

High baseline calcium intake was statistically significantly associated with lower TLR5 expression in the stroma, aligning with our main findings that supplementation with calcium *versus* no calcium decreased TLR5 expression. Calcium is hypothesized to exert its anti-neoplastic effects by binding free fatty acids and bile acids in the colon, inhibiting the production of secondary bile acids that promote colorectal carcinogenesis via inflammatory signaling pathways such as NF-kB (15). Thus, these results add support for our hypothesis that, although the mechanism remains to be fully elucidated, there may be an association between calcium and the pro-inflammatory biomarker TLR5.

Finally, compared to patients with no history of serrated adenomas, patients with previously removed serrated adenomas had 49% higher baseline expression of TLR5 in the stroma. Sessile serrated adenomas are considered precursor lesions to CRC, characterized by mutations of the BRAF gene and higher susceptibility to hypermethylation (84). These mutations have been implicated as key events in colorectal carcinogenesis through activation of the mitogen-activated protein kinase (MAPK) pathway, which has been shown to modulate TLR5 signaling (85) and contribute to increased proliferation and decreased apoptosis (86, 87).

Overall, our findings from the analysis of participant characteristics and baseline biomarker expression suggest that some modifiable risk factors may influence inflammatory pathways contributing to colorectal carcinogenesis.

Strengths and Limitations

This study had several strengths and limitations. The major strengths include high protocol adherence by study participants, the automated immunostaining and novel image analysis software to quantify crypt biomarker distribution, and the resulting high biomarker measurement reliability. Additionally, to our knowledge, this adjunct biomarker study is the first randomized, double-blind, placebo-controlled trial to report on the effect of calcium and vitamin D supplementation, alone and in combination, on TLR5 expression in the stroma of normal colorectal tissue. The main limitation of this study is the small sample size, as we may have lacked sufficient power to detect small differences in TLR5 expression across treatment groups and to conduct subgroup analyses. Because we only measured biomarker expression in the rectal mucosa, the treatment effects of calcium and vitamin D on other parts of the colon are unknown. Furthermore, because we were only able to obtain colorectal tissue samples from study participants at baseline and after one year of supplementation, we were not able to assess the long-term effects of calcium and vitamin D supplementation on TLR5 stroma expression. Additionally, it is poorly understood whether calcium and vitamin D may affect pre-cancerous and cancerous tissue in the human colon differently. If calcium and vitamin D only have a treatment effect on neoplastic tissue, this may explain our null findings in a population of individuals with normal colorectal tissue. To better understand the effect of supplementation on biomarker expression throughout the colorectal carcinogenesis sequence, further studies with larger sample sizes, longer supplementation periods, and more extensive follow-up are needed.

Public Health Impact

As previously mentioned, the goal of this study and of the research group is to identify chemopreventive agents that can be used to prevent CRC. Studies have estimated that regular screening can reduce CRC incidence by an estimated 17-54% (88); however, its effectiveness is hindered by limited uptake, as 30-50% of eligible individuals in the United States never begin the screening process (89). Additionally, screening is associated with inconveniences including their invasive nature, cost, and morbidity (90). Thus, much interest has arisen in the research and development of effective chemopreventive treatment agents that would allow for primary prevention. While chemoprevention would never replace traditional screening strategies such as colonoscopy, it could greatly enhance screening strategies by targeting missed lesions, decreasing the number of adenomas that need to be removed, and inhibiting or slowing the growth of early stage cancers, ultimately reducing the high morbidity and mortality burden currently observed.

A secondary objective of this study was to identify potential treatable pre-neoplastic biomarkers of risk for colorectal neoplasms. Although tissue and blood-based biomarker tests would never replace colonoscopies, they could help physicians screen many more patients at risk. That is, biomarker tests could be used to distinguish between those who are at higher risk and require more invasive screening or those who could wait longer intervals between screenings, reducing the burden for practicing physicians while enhancing the health of the population. Last, because adenomatous polyps and colorectal adenomas are precursors to CRC and can be detected up to decades earlier, there is great

potential to identify cases early in the cancer progression sequence, reducing utilization of resources and economic burden on the healthcare system.

Conclusion and Future Directions

In conclusion, although not statistically significant, the preliminary results of this pilot study suggest that (i) supplemental vitamin D_3, alone or in combination with calcium, may increase TLR5 expression; and (ii) provide further support for TLR5 expression in the stroma of the normal-appearing rectal mucosa as a modifiable, pre-neoplastic marker of risk for colorectal neoplasms. Our analysis of patient characteristics and biomarker expression at baseline suggest that multiple modifiable risk factors are associated with expression of the pro-inflammatory biomarker TLR5 at baseline. Given that our study was mainly limited by small sample size, further studies with larger sample sizes, longer supplementation periods, and more extensive follow-up may be needed to detect differences in expression. These initial findings are promising and support the continued investigation of modifiable risk factors that may influence inflammatory pathways related to colorectal carcinogenesis.

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Table 1. Select Baseline Characteristics of Clinical Trial Participants by Treatment Group Assignment (n=105)[a]

	Randomization to Calcium and Vitamin D (4-arm)					Randomization to Vitamin D Only (2-arm)		
Baseline Characteristics	Placebo (n=12)	Calcium (n=16)	Vitamin D (n=17)	Calcium + Vitamin D (n=18)	*p value*[b]	Placebo (n=23)	Vitamin D (n=19)	*p value*[c]
Age, years	59.9 (7.2)	59.9 (6.5)	59.2 (7.8)	58.0 (7.1)	*0.86*	58.2 (5.3)	59.2 (7.3)	*0.60*
Men, %	75.0	81.3	70.6	83.3	*0.83*	0.0	0.0	--
White, %	83.3	75.0	70.6	94.4	*0.29*	69.6	84.2	*0.30*
College graduate[d], %	66.7	37.5	64.7	55.6	*0.37*	47.8	36.8	*0.54*
1° family history of CRC, %***	0.0	12.5	20.0	5.6	*0.37*	4.4	11.1	*0.57*
Regular[e] non-aspirin NSAID users, %	8.3	18.8	17.7	5.6	*0.59*	8.7	10.5	*0.84*
Regular[e] aspirin users, %	41.7	68.8	29.4	33.3	*0.10*	21.7	31.6	*0.47*
If woman (n=56), HRT users, %	100.0	0.0	20.0	33.3	*0.11*	21.7	31.6	*0.50*
Current smoker, %	25.0	6.3	0.0	5.6	*0.10*	0	15.8	*0.08*
Multivitamin users, %	41.7	81.3	47.1	66.7	*0.10*	69.57	89.5	*0.15*
Physical activity, MET-min/wk[f]*	1620 (1195)	2128 (2378)	2782 (2764)	4042 (2456)	*0.03*	1458 (1235)	3021 (3469)	*0.05*
BMI, kg/m^2	29.4 (4.9)	32.3 (7.6)	28.7 (5.5)	30.2 (4.4)	*0.31*	29.7 (5.6)	27.5 (4.7)	*0.18*
Adenomas removed at colonoscopy	1.6 (0.7)	1.6 (1.0)	1.4 (0.8)	1.4 (0.7)	*0.70*	1.2 (0.7)	1.6 (1.0)	*0.11*
Had advanced adenoma, %***	36.4	6.7	23.5	27.8	*0.30*	9.1	15.8	*0.65*
Dietary Intakes								
Total energy intake, kcal/d***	1314 (381)	1737 (556)	1437 (527)	1569 (565)	*0.21*	1254 (549)	1429 (595)	*0.33*
Total fat, g/d***	57.1 (22.3)	68.9 (25.6)	60.5 (27.3)	61.6 (26.8)	*0.69*	50.3 (25.9)	61.5 (36.1)	*0.25*
Calories from fat, %***	38.9 (8.1)	35.8 (7.0)	37.2 (6.6)	35.2 (8.0)	*0.58*	35.6 (7.7)	36.0 (8.3)	*0.61*
Dietary fiber, g/d***	9.5 (4.1)	15.8 (5.6)	13.7 (6.2)	15.1 (5.7)	*0.04*	13.8 (5.4)	17.2 (5.0)	*0.04*
Red and/or processed meat, servings/d	1.2 (0.9)	1.0 (0.7)	0.9 (0.8)	1.0 (0.7)	*0.74*	0.6 (0.5)	0.7 (0.6)	*0.59*
Fruits and vegetables, servings/d***	3.0 (1.7)	4.4 (2.0)	4.5 (2.5)	4.3 (1.7)	*0.28*	4.7 (1.7)	6.0 (2.4)	*0.04*
Alcohol intake, drinks/day	0.7 (0.7)	0.8 (1.0)	0.9 (0.9)	0.9 (0.9)	*0.95*	0.5 (1.0)	0.3 (0.5)	*0.42*
Total vitamin D[g], IU/d	354 (307)	457 (189)	313 (278)	421 (296)	*0.48*	521 (354)	634 (276)	*0.34*
Total calcium, mg/d	641 (284)	863 (284)	663 (272)	656 (251)	*0.16*	938 (466)	1213 (553)	*0.09*
Serum levels								
25-OH-vitamin D_3, ng/mL	22.4 (8.2)	24.5 (13.4)	23.1 (8.7)	22.7 (6.4)	*0.93*	24.8 (8.9)	26.5 (9.6)	*0.54*
Ca^{2+}, mg/dL	9.2 (0.2)	9.3 (0.3)	9.3 (0.3)	9.4 (0.3)	*0.25*	9.5 (0.3)	9.4 (0.3)	*0.52*

Abbreviations: CRC= colorectal cancer; HRT= hormone replacement therapy; NSAID= non-steroidal anti-inflammatory drug; BMI= body mass index; g= grams; IU= international units; kcal= kilocalories; d= day; wk= week; MET=metabolic

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

[b] Chi squared for categorical variables; ANOVA for continuous variables

[c] Chi squared for categorical variables; Student t-tests for continuous variables

[d] Received a Bachelor's degree or higher

[e] At least four times a week

[f] Metabolic equivalent of task

[g] Dietary vitamin D plus supplemental vitamin D. Missing information for 3 placebo patients, 2 calcium, 2 vitamin D, one combined, 6 placebo (2-arm) and 5 vitamin D (2-arm)

[*] One patient missing per asterisk

Table 2. Comparison of TLR5 Expression in the Stroma of Normal-Appearing Rectal Mucosa of Study Participants (n=105) by Treatment Assignment[a]

	Baseline				1-Yr Follow-Up				Relative Treatment Effect[d]			Absolute[e]
	Geometric[b]				*Geometric*							
Treatment Group	n	Mean	(95% CI)	*p value*	n	Mean	(95% CI)	*p value*	Rx Effect	(95% CI)	*p value*	Rx Effect
Whole Stromal Region												
4-Arm												
Placebo	12	212.7	(102.1-443.0)		12	122.8	(59.0-255.8)					
Calcium	16	161.0	(83.3-311.3)	*0.56*	16	81.1	(41.9-156.7)	*0.38*	0.87	(0.34-2.23)	*0.77*	9.93
Vitamin D	17	107.8	(58.0-200.4)	*0.15*	17	94.8	(51.0-176.2)	*0.58*	1.52	(0.60-3.85)	*0.37*	76.87
Calcium + Vitamin D	18	136.0	(73.1-253.3)	*0.34*	18	77.9	(41.8-145.0)	*0.33*	0.99	(0.40-2.48)	*0.98*	31.71
2-Arm												
Placebo	23	38.3	(23.8-61.4)		23	53.9	(37.0-78.4)					
Vitamin D	19	36.4	(25.1-53.0)	*0.25*	19	67.8	(40.7-112.9)	*0.74*	1.36	(0.62-2.97)	*0.43*	15.69
Upper 40% of Stromal Region												
4-Arm												
Placebo	12	132.1	(63.1-276.6)		12	87.0	(41.6-182.2)					
Calcium	16	100.8	(51.9-195.7)	*0.57*	16	51.3	(26.4-99.6)	*0.27*	0.77	(0.30-1.99)	*0.59*	-4.40
Vitamin D	17	71.2	(38.2-132.9)	*0.19*	17	59.4	(31.8-110.9)	*0.42*	1.27	(0.50-3.22)	*0.61*	33.27
Calcium + Vitamin D	18	87.9	(47.0-164.3)	*0.38*	18	51.7	(27.6-96.6)	*0.27*	0.89	(0.36-2.24)	*0.81*	8.88
2-Arm												
Placebo	23	67.8	(40.7-112.9)		23	38.9	(23.3-64.8)					
Vitamin D	19	42.9	(24.7-74.4)	*0.23*	19	34.2	(19.7-59.3)	*0.73*	1.39	(0.64-2.99)	*0.39*	20.17
Lower 60% of Stromal Region												
4-Arm												
Placebo	12	63.7	(27.1-149.8)		12	23.2	(9.9-54.6)					
Calcium	16	42.2	(19.6-90.9)	*0.46*	16	18.3	(8.5-39.4)	*0.67*	1.19	(0.36-3.99)	*0.77*	16.60
Vitamin D	17	24.0	(11.7-49.4)	*0.08*	17	23.6	(11.5-48.7)	*0.97*	2.70	(0.82-8.92)	*0.10*	40.13
Calcium + Vitamin D	18	35.7	(17.3-73.5)	*0.29*	18	16.8	(8.2-34.6)	*0.55*	1.29	(0.40-4.21)	*0.66*	21.63
2-Arm												
Placebo	23	30.8	(17.9-53.0)		23	17.5	(10.1-30.1)					
Vitamin D	19	18.1	(10.1-32.6)	*0.19*	19	16.1	(9.0-29.1)	*0.84*	1.57	(0.64-3.84)	*0.31*	11.35
ϕh[c]												
4-Arm												
Placebo	12	63.1	(57.5-68.7)		12	71.1	(65.6-76.7)					
Calcium	16	63.0	(58.1-68.0)	*0.99*	16	63.8	(58.8-68.8)	*0.05*	-7.28	(-16.18-1.63)	*0.11*	0.90
Vitamin D	17	66.9	(62.2-71.6)	*0.28*	17	63.9	(59.2-68.6)	*0.05*	-11.04	(-19.84--2.25)	*0.01*	0.85
Calcium + Vitamin D	18	65.2	(60.5-69.9)	*0.56*	18	66.7	(62.1-71.4)	*0.22*	-6.45	(-15.14-2.24)	*0.14*	0.91
2-Arm												
Placebo	23	63.4	(58.6-68.2)		23	62.9	(58.2-67.7)					
Vitamin D	19	61.7	(56.5-66.9)	*0.63*	19	61.8	(56.7-67.0)	*0.75*	0.57	(-8.5-9.64)	*0.90*	1.01

[a] The effect of treatment assignment on TLR5 expression was modeled using PROC MIXED in SAS 9.4 (Cary, NC), controlling for age, gender (if 4-arm) and study center

[b] Data were log-transformed. Reported values are geometric means (95% CI) of optical density

[c] Defined as the percent of expression in the distribution zone (expression in upper 40% of crypts over expression in the whole crypts). Values are means of the optical density (not geometric means)

[d] Relative Treatment effect= [(Tx Y1)/(Tx BL)]/[(Pl Y1)/(Pl BL)]

[e] Absolute Treatment effect= [(Tx Y1)-(Tx BL)]-[(Pl Y1)-(Pl BL)]

Table 3. Comparison of TLR5 Expression in the Stroma of Normal-Appearing Rectal Mucosa of Study Participants (n=105) by Treatment Agent[a]

	Baseline				1-Yr Follow-Up				Relative Treatment Effect[g]			Absolute[h]
	Geometric[f]				*Geometric*							
Treatment Effect Comparisons	n	Mean	(95% CI)	*p value*	n	Mean	(95% CI)	*p value*	Rx effect	(95% CI)	*p value*	Rx Effect
Whole Stromal Region												
No calcium	29	201.2	(130.6-310.0)		29	148.7	(83.1-266.1)					
Calcium[b]	34	211.0	(141.6-314.5)	*0.87*	34	113.7	(66.4-194.6)	*0.50*	0.73	(0.39-1.35)	*0.31*	-44.76
No vitamin D	51	179.1	(127.8-251.0)		51	99.1	(66.0-148.9)					
Vitamin D[c]	54	122.4	(88.2-169.9)	*0.11*	54	89.6	(60.4-133.1)	*0.73*	1.32	(0.82-2.12)	*0.24*	47.21
Calcium only	39	153.7	(105.3-224.3)		39	84.0	(53.7-131.4)					
Vitamin D and calcium[d]	37	110.2	(74.7-162.4)	*0.22*	37	74.2	(46.9-117.4)	*0.70*	1.23	(0.70-2.17)	*0.47*	33.73
Upper 40% of Stromal Region												
No calcium	29	126.8	(83.0-193.7)		29	95.9	(53.6-171.7)					
Calcium	34	130.9	(88.5-193.6)	*0.91*	34	71.9	(42.0-123.1)	*0.47*	0.73	(0.39-1.34)	*0.30*	-28.08
No vitamin D	51	110.5	(79.0-154.7)		51	63.1	(41.8-95.3)					
Vitamin D	54	76.6	(55.2-106.2)	*0.12*	54	55.9	(37.5-83.5)	*0.68*	1.28	(0.80-2.05)	*0.30*	26.79
Calcium only	39	95.3	(65.2-139.4)		39	52.1	(33.1-81.9)					
Vitamin D and calcium	37	67.9	(46.0-100.3)	*0.22*	37	46.7	(29.3-74.3)	*0.74*	1.26	(0.72-2.21)	*0.42*	22.02
Lower 60% of Stromal Region												
No calcium	29	54.6	(33.1-90.3)		29	35.6	(17.7-71.7)					
Calcium	34	59.9	(37.7-95.2)	*0.79*	34	27.1	(14.2-51.7)	*0.57*	0.69	(0.31-1.55)	*0.37*	-13.75
No vitamin D	51	52.0	(35.4-76.5)		51	24.4	(15.4-38.8)					
Vitamin D	54	31.6	(21.8-46.0)	*0.07*	54	23.5	(15.0-36.8)	*0.91*	1.58	(0.88-2.85)	*0.12*	19.46
Calcium only	39	43.3	(28.2-66.5)		39	22.0	(13.4-36.2)					
Vitamin D and calcium	37	29.4	(18.9-45.8)	*0.22*	37	19.2	(11.5-32.1)	*0.71*	1.29	(0.63-2.61)	*0.48*	11.09
ϕh[e]												
No calcium	29	64.0	(60.4-67.6)		29	65.5	(61.9-69.1)					
Calcium	34	62.6	(59.3-65.9)	*0.56*	34	63.7	(60.4-67.1)	*0.47*	-0.37	(-6.47-5.73)	*0.90*	0.99
No vitamin D	51	62.6	(59.6-65.5)		51	64.5	(61.7-67.2)					
Vitamin D	54	63.6	(60.7-66.4)	*0.63*	54	63.2	(60.5-65.8)	*0.50*	-2.30	(-7.30-2.70)	*0.36*	0.96
Calcium only	39	62.8	(59.5-66.1)		39	62.8	(59.9-65.8)					
Vitamin D and calcium	37	62.6	(59.2-66.0)	*0.94*	37	63.4	(60.4-66.5)	*0.78*	0.79	(-4.85-6.42)	*0.78*	1.01

[a] The effect of treatment agent on TLR5 expression was modeled using PROC MIXED in SAS 9.4 (Cary, NC), controlling for age, gender (by study arm), and study center
[b] Calcium group were patients assigned to either calcium or to calcium + vitamin D (combined) in the 4-arm group. Patients in the 2-arm group were excluded
[c] Vitamin D group consisted of patients assigned to vitamin D or to calcium + vitamin D (combined) in the 4-arm group, or to vitamin D in the 2-arm group
[d] Vitamin D and Calcium group consisted of patients assigned to calcium + vitamin D (combined) of the 4-arm group, or to vitamin D of the 2-arm group
[e] Defined as the percent of expression in the differentiation zone. Values are means of the optical density (not geometric means)
[f] Data were log-transformed. Reported values are geometric means (95% CI) of optical density
[g] Relative Treatment effect= [(Tx Y1)/(Tx BL)]/[(Pl Y1)/(Pl BL)]
[h] Absolute Treatment effect= [(Tx Y1)-(Tx BL)]-[(Pl Y1)-(Pl BL)]

Table 4. Stratified Comparisons of TLR5 Expression in the Whole Stromal Region of Normal-Appearing Rectal Mucosa of Study Participants (n=105)[a] by Treatment Agent and Frequency of NSAID Use

	Baseline				1-Yr Follow-up				Relative Treatment Effect[f]			Absolute[g]
		Geometric[e]				*Geometric*						
Frequency of NSAID use	n	Mean	95% CI	p value	n	Mean	95% CI	p value	Rx effect	95% CI	p value	Rx Effect
Less than every other day												
No calcium	21	211.3	(126.9-352.0)		21	145.6	(70.6-300.3)					
Calcium[b]	22	260.9	(158.5-429.5)	*0.55*	22	128.4	(63.3-260.6)	*0.80*	0.71	(0.35-1.47)	*0.35*	-66.72
No vitamin D	34	180.4	(114.6-283.9)		34	100.7	(58.7-172.6)					
Vitamin D[c]	39	125.1	(81.9-191.1)	*0.24*	39	83.6	(50.6-138.4)	*0.62*	1.20	(0.66-2.17)	*0.55*	38.25
Calcium only	26	154.2	(92.8-256.3)		26	81.3	(44.4-148.9)					
Vitamin D and calcium[d]	26	107.3	(64.6-178.2)	*0.31*	26	70.0	(38.3-128.2)	*0.73*	1.24	(0.58-2.63)	*0.57*	35.69
Every other day or greater												
No calcium	8	176.8	(74.0-422.2)		8	157.0	(53.2-463.4)					
Calcium	12	143.0	(70.3-291.1)	*0.70*	12	91.0	(37.6-220.1)	*0.42*	0.72	(0.19-2.65)	*0.60*	-32.27
No vitamin D	17	176.5	(110.1-282.9)		17	96.1	(52.7-175.3)					
Vitamin D	15	115.5	(69.9-190.8)	*0.22*	15	107.3	(56.6-203.4)	*0.80*	1.71	(0.76-3.84)	*0.19*	72.16
Calcium only	13	152.6	(87.4-266.5)		13	89.6	(47.2-169.8)					
Vitamin D and calcium	11	117.3	(64.0-215.01)	*0.51*	11	85.0	(42.4-170.4)	*0.91*	1.23	(0.52-2.92)	*0.62*	30.71

[a] The effect of treatment agent on TLR5 expression was modeled using PROC MIXED in SAS 9.4 (Cary, NC), controlling for age, gender (by study arm), and study center

[b] Calcium group were patients assigned to either calcium or to calcium + vitamin D (combined) in the 4-arm group. Patients in the 2-arm group were excluded

[c] Vitamin D group consisted of patients assigned to vitamin D or to calcium + vitamin D (combined) in the 4-arm group, or to vitamin D in the 2-arm group

[d] Vitamin D and Calcium group consisted of patients assigned to calcium + vitamin D (combined) of the 4-arm group, or to vitamin D of the 2-arm group

[e] Data were log-transformed. Reported values are geometric means (95% CI) of optical density

[f] Relative Treatment effect= [(Tx Y1)/(Tx BL)]/[(Pl Y1)/(Pl BL)]

[g] Absolute Treatment effect= [(Tx Y1)-(Tx BL)]-[(Pl Y1)-(Pl BL)]

Table 5. Stratified Comparisons of TLR5 Expression in the Whole Stromal Region of Normal-Appearing Rectal Mucosa of Study Participants (n=105)[a] by Treatment Agent and Frequency of Aspirin Use

	Baseline				1-Yr Follow-up				Relative Treatment Effect[f]			Absolute[g]
		Geometric[e]				*Geometric*						
Frequency of Aspirin use	n	Mean	95% CI	p value	n	Mean	95% CI	p value	Rx effect	95% CI	p value	Rx Effect
Less than every other day												
No calcium	16	197.3	(110.3-352.9)		16	145.8	(62.8-338.3)					
Calcium[b]	15	184.5	(101.2-336.3)	*0.87*	15	58.9	(24.7-140.6)	*0.14*	0.43	(0.17-1.12)	*0.08*	-74.06
No vitamin D	27	177.1	(113.1-277.3)		27	90.2	(50.8-160.0)					
Vitamin D[c]	33	103.4	(68.9-155.2)	*0.08*	33	61.2	(36.4-102.8)	*0.32*	1.16	(0.60-2.24)	*0.65*	44.69
Calcium only	21	134.5	(82.0-220.5)		21	69.0	(38.6-123.1)					
Vitamin D and calcium[d]	23	97.6	(60.8-156.6)	*0.35*	23	47.3	(27.2-82.2)	*0.35*	0.94	(0.44-2.02)	*0.88*	15.17
Every other day or greater												
No calcium	13	206.1	(103.3-411.3)		13	152.3	(68.5-338.4)					
Calcium	19	234.6	(132.5-415.5)	*0.77*	19	191.0	(98.7-369.8)	*0.66*	1.10	(0.50-2.41)	*0.80*	10.23
No vitamin D	24	181.4	(106.8-307.9)		24	110.3	(63.0-193.2)					
Vitamin D	21	159.4	(90.5-280.7)	*0.74*	21	163.3	(89.7-297.4)	*0.34*	1.68	(0.84-3.37)	*0.14*	74.98
Calcium only	18	179.6	(97.0-332.6)		18	105.7	(53.9-207.1)					
Vitamin D and calcium	14	134.4	(66.8-270.2)	*0.53*	14	155.6	(72.6-333.6)	*0.44*	1.97	(0.83-4.65)	*0.12*	95.13

[a] The effect of treatment agent on TLR5 expression was modeled using PROC MIXED in SAS 9.4 (Cary, NC), controlling for age, gender (by study arm), and study center

[b] Calcium group were patients assigned to either calcium or to calcium + vitamin D (combined) in the 4-arm group. Patients in the 2-arm group were excluded

[c] Vitamin D group consisted of patients assigned to vitamin D or to calcium + vitamin D (combined) in the 4-arm group, or to vitamin D in the 2-arm group

[d] Vitamin D and Calcium group consisted of patients assigned to calcium + vitamin D (combined) of the 4-arm group, or to vitamin D of the 2-arm group

[e] Data were log-transformed. Reported values are geometric means (95% CI) of optical density

[f] Relative Treatment effect= [(Tx Y1)/(Tx BL)]/[(Pl Y1)/(Pl BL)]

[g] Absolute Treatment effect= [(Tx Y1)-(Tx BL)]-[(Pl Y1)-(Pl BL)]

Table 6. Stratified Comparisons of TLR5 Expression in the Whole Stromal Region of Normal-Appearing Rectal Mucosa of Study Participants (n=105)[a] by Treatment Agent and Baseline Serum Level

	Baseline				1-Yr Follow-up				Relative Treatment Effect[f]			Absolute[g]
		Geometric[e]				*Geometric*						
Baseline Serum Vitamin D Level	n	Mean	95% CI	p value	n	Mean	95% CI	p value	Rx effect	95% CI	p value	Rx Effect
Below Median												
No calcium	17	245.8	(150.2-402.4)		17	212.0	(97.0-463.4)					
Calcium[b]	16	269.8	(162.3-448.4)	*0.79*	16	145.8	(65.1-326.4)	*0.50*	0.63	(0.25-1.54)	*0.30*	-90.14
No vitamin D	27	178.4	(119.7-265.9)		27	83.4	(45.6-152.7)					
Vitamin D[c]	25	174.8	(115.5-264.5)	*0.94*	25	152.8	(81.5-286.4)	*0.17*	1.87	(0.94-3.74)	*0.08*	73.05
Calcium only	19	142.1	(89.4-226.0)		19	62.2	(29.7-130.3)					
Vitamin D and calcium[d]	16	161.0	(97.1-266.9)	*0.71*	16	113.0	(50.5-253.0)	*0.27*	1.60	(0.67-3.86)	*0.28*	31.90
Above Median												
No calcium	12	151.4	(69.6-329.5)		12	90.0	(36.5-221.9)					
Calcium	18	169.6	(89.9-320.0)	*0.82*	18	91.2	(43.6-190.6)	*0.98*	0.91	(0.36-2.25)	*0.82*	-16.91
No vitamin D	24	179.8	(103.4-312.7)		24	120.4	(71.2-203.8)					
Vitamin D	29	90.0	(54.4-148.9)	*0.07*	29	56.6	(35.1-91.3)	*0.04*	0.94	(0.48-1.83)	*0.85*	26.02
Calcium only	20	165.6	(91.6-299.4)		20	111.7	(65.5-190.5)					
Vitamin D and calcium	21	82.5	(46.23-147.1)	*0.10*	21	53.8	(32.0-90.62)	*0.05*	0.97	(0.45-2.1)	*0.93*	25.19

[a] The effect of treatment agent on TLR5 expression was modeled using PROC MIXED in SAS 9.4 (Cary, NC), controlling for age, gender (by study arm), and study center
[b] Calcium group were patients assigned to either calcium or to calcium + vitamin D (combined) in the 4-arm group. Patients in the 2-arm group were excluded
[c] Vitamin D group consisted of patients assigned to vitamin D or to calcium + vitamin D (combined) in the 4-arm group, or to vitamin D in the 2-arm group
[d] Vitamin D and Calcium group consisted of patients assigned to calcium + vitamin D (combined) of the 4-arm group, or to vitamin D of the 2-arm group
[e] Data were log-transformed. Reported values are geometric means (95% CI) of optical density
[f] Relative Treatment effect= [(Tx Y1)/(Tx BL)]/[(Pl Y1)/(Pl BL)]
[g] Absolute Treatment effect= [(Tx Y1)-(Tx BL)]-[(Pl Y1)-(Pl BL)]
[h] Medium Baseline Serum Vitamin D level: 22.7 ng/mL

Table 7. Stratified Comparisons of TLR5 Expression in the Whole Stromal Region of Normal-Appearing Rectal Mucosa of Study Participants (n=105)[a] by Treatment Agent and Baseline Total Calcium Intake

	Baseline				1-Yr Follow-up				Relative Treatment Effect[g]			Absolute[h]
		Geometric[f]				*Geometric*						
Baseline Total Calcium Intake	n	Mean	95% CI	p value	n	Mean	95% CI	p value	Rx effect	95% CI	p value	Rx Effect
Below Median [b]												
No calcium	21	194.9	(118.0-321.8)		21	133.5	(67.8-263.0)					
Calcium [c]	19	243.7	(143.8-412.8)	0.54	19	111.5	(54.7-227.3)	0.71	0.67	(0.29-1.53)	0.33	-70.86
No vitamin D	27	235.9	(149.6-372.2)		27	106.2	(59.1-191.0)					
Vitamin D[d]	31	127.3	(83.2-194.7)	0.05	31	92.9	(53.7-160.7)	0.74	1.62	(0.82-3.21)	0.16	95.36
Calcium only	18	225.7	(127.8-398.7)		18	99.0	(46.5-210.8)					
Vitamin D and calcium[e]	19	111.0	(63.8-193.2)	0.08	19	70.9	(34.0-148.0)	0.53	1.46	(0.62-3.43)	0.38	86.64
Above Median												
No calcium	8	218.7	(87.4-547.7)		16	197.1	(57.2-679.3)					
Calcium	15	175.8	(89.9-343.7)	0.69	20	116.6	(47.2-287.9)	0.48	0.74	(0.27-1.99)	0.53	-37.57
No vitamin D	24	131.3	(78.8-218.8)		31	91.7	(51.1-164.6)					
Vitamin D	23	116.0	(68.9-195.5)	0.73	36	85.4	(47.0-155.2)	0.86	1.05	(0.54-2.04)	0.88	8.93
Calcium only	21	110.6	(66.3-184.3)		25	73.0	(42.1-126.4)					
Vitamin D and calcium	18	109.3	(62.9-189.7)	0.97	26	77.8	(43.0-140.8)	0.87	1.08	(0.49-2.38)	0.85	6.15

[a] The effect of treatment agent on TLR5 expression was modeled using PROC MIXED in SAS 9.4 (Cary, NC), controlling for age, gender (by study arm), and study center
[b] Median Total Calcium Intake: 763.5 mg/d
[c] Calcium group were patients assigned to either calcium or to calcium + vitamin D (combined) in the 4-arm group. Patients in the 2-arm group were excluded
[d] Vitamin D group consisted of patients assigned to vitamin D or to calcium + vitamin D (combined) in the 4-arm group, or to vitamin D in the 2-arm group
[e] Vitamin D and Calcium group consisted of patients assigned to calcium + vitamin D (combined) of the 4-arm group, or to vitamin D of the 2-arm group
[f] Data were log-transformed. Reported values are geometric means (95% CI) of optical density
[g] Relative Treatment effect= [(Tx Y1)/(Tx BL)]/[(Pl Y1)/(Pl BL)]
[h] Absolute Treatment effect= [(Tx Y1)-(Tx BL)]-[(Pl Y1)-(Pl BL)]

Table 8. Categorical Baseline Predictors of TLR5 Expression in Stromal Region at Baseline[a]

		Geometric[b]			
Baseline Characteristics	n	Mean	(95% CI)	Diff [c]	*p value[d]*
Age					
<55	33	118.7	(82.5-170.6)		
55-63	35	150.4	(106.9-211.8)	26.8	
>63	37	124.0	(88.9-173.0)	4.5	*0.80*
Sex					
Men	49	140.6	(91.9-215.1)		
Women	56	120.8	(88.0-165.9)	-14.1	*0.57*
Race					
Non-White	22	212.2	(136.1-330.8)		
White	83	114.3	(88.9-146.9)	-46.1	*0.01*
1° family history of CRC					
No	93	144.2	(112.4-185.1)		
Yes	9	105.4	(59.0-188.1)	-26.9	*0.30*
Regular NSAID use					
Less than once a week	73	139.0	(104.2-185.6)		
Once a week or greater	32	119.2	(85.1-166.9)	-14.3	*0.44*
Regular aspirin user					
Less than once a week	60	132.5	(100.4-174.9)		
Once a week or greater	45	127.5	(91.2-178.3)	-3.8	*0.84*
Current HRT user (women only, N=56)					
No	40	119.8	(83.1-172.6)		
Yes	16	90.9	(48.7-169.8)	-24.1	*0.48*
Smoking status					
Never	61	134.3	(100.1-180.0)		
Former	36	116.4	(82.2-165.0)	-13.3	
Current	8	169.6	(90.6-317.5)	26.3	*0.89*
Multivitamin user					
No	34	149.7	(109.8-204.1)		
Yes	71	114.4	(84.2-155.4)	-23.6	*0.18*
Physical activity (MET-min/wk)[t]*					
Low	33	125.6	(90.4-174.6)		
Moderate	36	169.8	(125.2-230.3)	35.1	
High	36	91.5	(64.9-129.0)	-27.2	*0.14*
BMI					
Normal (<25)	22	115.5	(74.7-178.8)		
Overweight (25-30)	43	153.9	(111.7-212.0)	33.2	
Obese (≥ 30)	40	120.2	(87.4-165.3)	4.0	*0.52*
Number of adenomas					
1 polyp	76	131.6	(99.9-173.4)		
> 1 polyp	29	128.5	(88.3-187.2)	-2.3	*0.91*
Had advanced adenomas					
No	83	128.1	(97.6-168.1)		
Yes	19	146.4	(93.6-229.0)	14.3	*0.59*
Had serrated adenomas					
No	79	116.3	(89.4-151.4)		
Yes	26	173.4	(119.1-252.4)	49.0	*0.06*
Total energy intake (kcal/d)[t]*					
Low	35	140.3	(100.6-195.6)		
Medium	36	152.2	(105.4-219.9)	8.5	
High	34	108.0	(77.2-151.2)	-23.0	*0.23*

Table 8. Categorical Baseline Predictors of TLR5 Expression in Stromal Region at Baseline[a] *(Cont.)*					
		Geometric[b]			
Baseline Characteristics	n	Mean	(95% CI)	Diff [c]	*p value[d]*
Percent daily calories from fat[t]*					
Low	35	128.4	(91.3-180.7)		
Moderate	35	187.4	(128.7-272.9)	45.9	
High	35	107.0	(77.9-147.0)	-16.7	*0.33*
Dietary fiber (g/1000kcal/d)[t]*					
Low	35	114.4	(82.3-158.9)		
Moderate	35	153.1	(107.3-218.4)	33.8	
High	35	132.3	(90.8-192.7)	15.7	*0.56*
Red and processed meat, servings/d					
< 0.5	37	137.1	(98.2-191.4)		
0.5-1	30	137.2	(92.4-203.7)	0.1	
≥ 1	38	119.8	(83.8-171.2)	-12.6	*0.57*
Achieved daily recommended intake of fruit and vegetables (≥ 5 servings/d)[t]*					
No	66	137.7	(104.2-182.0)		
Yes	39	119.2	(84.5-168.3)	-13.4	*0.47*
Alcohol intake					
None	36	113.0	(78.9-161.8)		
≤ 1 drink/wk	46	129.3	(90.7-184.2)	14.4	
> 1 drink/wk	23	168.3	(108.9-260.1)	48.9	*0.17*
Vitamin D Baseline Serum Levels (Tertiles)					
Low (12.90-17.90)	35	128.9	(91.-181.1)		
Medium (17.91-26.91)	36	133.0	(92.7-190.8)	3.1	
High (26.92-68.75)	34	130.5	(89.3-190.7)	1.2	*0.97*
Baseline Calcium Intake (Tertiles)					
Tertile 1	34	165.8	(121.2-226.8)		
Tertile 2	36	110.5	(77.4-157.6)	-33.4	
Tertile 3	35	106.8	(73.1-156.1)	-35.6	*0.04*

Abbreviations: Diff= proportional difference; CRC= colorectal cancer; HRT= hormone replacement therapy; NSAID= non-steroidal anti-inflammatory drug; BMI= body mass index; MET= metabolic equivalent of task; g= grams; IU= international units; kcal= kilocalories; d= day; wk= week

[a] The effect of the baseline characteristics on TLR5 expression was modeled using PROC GLM in SAS 9.4 (Cary, NC), controlling for age (continuous), gender (by study arm), study center and batch number, where appropriate.

[b] Reported means are geometric means (95% CI) and arithmetic means (95% CI) of optical density.

[c] Proportional Difference= (Comparison mean- reference mean)/reference mean*100%

[d] Reported p values are the Type III SS of the baseline characteristic. For baseline characteristics with more than two categories, the variables were treated as continuous and the reported p-value are of the overall trend

[t] Categorized by tertiles

*Missing values were replaced with treatment group- and sex-specific means

FIGURES

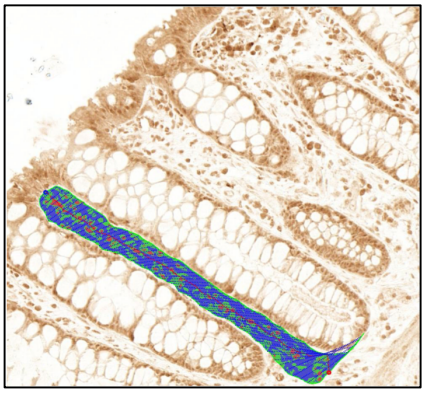

Figure 1. Measurement of TLR5 expression in the stroma surrounding the crypts of normal-appearing rectal mucosa using custom-designed quantitative image analysis software. The stroma scoring process involved identifying a previously scored crypt and determining if it was wide enough for scoring, tracing the outline of the region, and allowing the program to automatically divide the outline into equally-spaced segments and quantify TLR5 labeling optical density.

APPENDIX

Table S.1. Stratified Comparisons of TLR5 Expression in the Whole Stromal Region of Normal-Appearing Rectal Mucosa of Study Participants (n=105)[a] by Treatment Agent and Sex-specific Baseline Calcium Intake

	Baseline				1-Yr Follow-up				Relative Treatment Effect[g]			Absolute[h]
		Geometric[f]				*Geometric*						
Baseline Total Calcium Intake	n	Mean	95% CI	p value	n	Mean	95% CI	p value	Rx effect	95% CI	p value	Rx Effect
Below Median [b]												
No calcium	20	199.5	(119.6-332.7)		20	125.5	(64.1-245.7)					
Calcium [c]	18	216.4	(126.3-371.0)	0.83	18	90.4	(44.5-183.6)	0.50	0.66	(0.29-1.54)	0.33	-51.99
No vitamin D	29	210.8	(136.8-324.9)		29	99.9	(58.2-171.3)					
Vitamin D[d]	29	121.1	(78.6-186.6)	0.07	29	81.7	(47.6-140.1)	0.60	1.42	(0.73-2.80)	0.30	71.56
Calcium only	20	192.6	(115.4-321.5)		20	91.1	(47.0-176.6)					
Vitamin D and calcium[e]	18	101.5	(59.1-174.1)	0.09	18	62.1	(30.9-124.6)	0.42	1.29	(0.56-3.00)	0.5402	62.06
Above Median												
No calcium	9	205.0	(85.6-490.7)		9	216.7	(66.8-702.5)					
Calcium	16	205.1	(106.5-394.7)	0.99	16	147.2	(60.9-355.6)	0.59	0.68	(0.27-1.74)	0.40	-69.60
No vitamin D	22	144.4	(83.0-251.1)		22	98.2	(51.3-188.0)					
Vitamin D	25	123.8	(73.7-208.1)	0.69	25	99.8	(54.3-183.6)	0.9698	1.19	(0.60-2.33)	0.61	22.21
Calcium only	19	121.2	(68.0-216.0)		19	77.0	(40.7-145.7)					
Vitamin D and calcium	19	119.1	(66.8-212.2)	0.97	19	87.9	(46.5-166.1)	0.77	1.16	(0.52-2.60)	0.7102	12.94

[a] The effect of treatment agent on TLR5 expression was modeled using PROC MIXED in SAS 9.4 (Cary, NC), controlling for age, gender (by study arm), and study center

[b] Sex-specific total calcium intake cut-offs: Men- 743.4 mg/d; Women- 847.3 mg/d

[c] Calcium group were patients assigned to either calcium or to calcium + vitamin D (combined) in the 4-arm group. Patients in the 2-arm group were excluded

[d] Vitamin D group consisted of patients assigned to vitamin D or to calcium + vitamin D (combined) in the 4-arm group, or to vitamin D in the 2-arm group

[e] Vitamin D and Calcium group consisted of patients assigned to calcium + vitamin D (combined) of the 4-arm group, or to vitamin D of the 2-arm group

[f] Data were log-transformed. Reported values are geometric means (95% CI) of optical density

[g] Relative Treatment effect= [(Tx Y1)/(Tx BL)]/[(Pl Y1)/(Pl BL)]

[h] Absolute Treatment effect= [(Tx Y1)-(Tx BL)]-[(Pl Y1)-(Pl BL)]

Table S.2. Categorical Baseline Predictors of TLR5 Expression in Upper 40% of Stromal Region at Baseline[a]

		Upper 40% of stromal region			
		Geometric			
Baseline Characteristics	n	Mean	95% CI	Diff [c]	*p value*[d]
Age					
<55	33	76.7	(53.9-109.1)		
55-63	35	92.8	(66.5-129.3)	20.9	
>63	37	74.3	(53.8-102.6)	-3.2	*0.93*
Sex					
Men	49	81.2	(53.8-122.6)		
Women	56	75.5	(55.5-102.7)	-7.0	*0.78*
Race					
Non-White	22	118.4	(76.5-183.3)		
White	83	72.5	(56.6-92.9)	-38.7	*0.05*
1° family history of CRC					
No	93	89.1	(69.8-113.7)		
Yes	9	63.6	(36.1-112.1)	-28.6	*0.26*
Regular NSAID use					
Less than once a week	73	83.9	(63.4-111.2)		
Once a week or greater	32	76.8	(55.3-106.5)	-8.5	*0.65*
Regular aspirin user					
Less than once a week	60	83.1	(63.5-108.7)		
Once a week or greater	45	77.5	(56.0-107.3)	-6.7	*0.71*
Current HRT user (women only, N=56)					
No	40	74.5	(51.6-107.5)		
Yes	16	61.7	(32.9-115.5)	-17.2	*0.63*
Smoking status					
Never	61	80.5	(60.5-107.1)		
Former	36	75.1	(53.5-105.4)	-6.7	
Current	8	104.7	(56.9-192.7)	30.1	*0.69*
Multivitamin user					
No	34	90.5	(66.9-122.4)		
Yes	71	72.6	(53.9-97.9)	-19.8	*0.26*
Physical activity (MET-min/wk)[t]*					
Low	33	76.6	(55.9-104.9)		
Moderate	36	107.4	(80.2-143.9)	40.3	
High	36	56.1	(40.3-77.9)	-26.8	*0.14*
BMI					
Normal (<25)	22	69.7	(45.9-106.0)		
Overweight (25-30)	43	99.6	(73.2-135.4)	42.8	
Obese (≥ 30)	40	72.7	(53.6-98.7)	4.3	*0.39*
Number of adenomas					
1 polyp	76	81.2	(62.2-106.2)		
> 1 polyp	29	80.3	(55.8-115.7)	-1.1	*0.96*
Had advanced adenomas					
No	83	80.7	(61.9-105.3)		
Yes	19	84.6	(54.6-131.1)	4.8	*0.85*
Had serrated adenomas					
No	79	72.8	(56.4-94.0)		
Yes	26	105.0	(72.8-151.3)	44.2	*0.07*

Table S.2. Categorical Baseline Predictors of TLR5 Expression in Upper 40% of Stromal Region at Baseline[a] *(Cont.)*

		Upper 40% of stromal region			
		Geometric			
Baseline Characteristics	n	Mean	95% CI	Diff [c]	*p value*[d]
Total energy intake (kcal/d)[t]*					
Low	35	88.2	(63.9-121.7)		
Medium	36	92.8	(64.9-132.5)	5.2	
High	34	66.9	(48.3-92.7)	-24.2	*0.19*
Percent daily calories from fat[t]*					
Low	35	81.5	(58.6-113.3)		
Moderate	35	116.0	(80.7-166.6)	42.3	
High	35	65.1	(47.9-88.4)	-20.2	*0.23*
Dietary fiber (g/1000kcal/d)[t]*					
Low	35	70.2	(51.1-96.5)		
Moderate	35	95.8	(67.9-135.1)	36.5	
High	35	82.3	(57.2-118.5)	17.3	*0.51*
Red and processed meat, servings/d					
< 0.5	37	87.9	(63.6-121.3)		
0.5-1	30	86.4	(59.0-126.5)	-1.7	
≥ 1	38	70.7	(50.1-99.8)	-19.5	*0.34*
Achieved daily recommended intake of fruit and vegetables (≥ 5 servings/d)[t]*					
No	66	83.6	(63.7-109.6)		
Yes	39	76.6	(54.7-107.1)	-8.4	*0.65*
Alcohol intake					
None	36	69.8	(49.3-98.7)		
≤ 1 drink/wk	46	78.5	(55.8-110.5)	12.5	
> 1 drink/wk	23	107.8	(70.8-164.1)	54.6	*0.13*
Vitamin D Baseline Serum Levels (Tertiles)					
Low (12.90-17.90)	35	79.5	(57.2-110.5)		
Medium (17.91-26.91)	36	81.9	(57.7-116.3)	3.1	
High (26.92-68.75)	34	82.2	(56.9-118.9)	3.5	*0.88*
Baseline Calcium Intake (Tertiles)					
Tertile 1	34	101.5	(74.9-137.6)		
Tertile 2	36	68.9	(48.8-97.2)	-32.2	
Tertile 3	35	67.1	(46.4-97.0)	-33.9	*0.05*

Abbreviations: Diff= proportional difference; CRC= colorectal cancer; HRT= hormone replacement therapy; NSAID= non-steroidal anti-inflammatory drug; BMI= body mass index; MET= metabolic equivalent of task; g= grams; IU= international units; kcal= kilocalories; d= day; wk= week

[a] The effect of the baseline characteristics on TLR5 expression was modeled using PROC GLM in SAS 9.4 (Cary, NC), controlling for age (continuous), gender (by study arm), study center and batch number, where appropriate.

[b] Reported means are geometric means (95% CI) and arithmetic means (95% CI) of optical density.

[c] Proportional Difference= (Comparison mean- reference mean)/reference mean*100%

[d] Reported p values are the Type III SS of the baseline characteristic. For baseline characteristics with more than two categories, the variables were treated as continuous and the reported p-value are of the overall trend

[t] Categorized by tertiles

*Missing values were replaced with treatment group- and sex-specific means

Table S.3. Categorical Baseline Predictors of TLR5 Expression in Lower 60% of Stromal Region at Baseline[a]

		Lower 60% of stromal region			
		Geometric			
Baseline Characteristics	n	Mean	95% CI	Diff [c]	*p value*[d]
Age					
<55	33	30.8	(19.3-49.0)		
55-63	35	42.0	(27.1-65.1)	36.3	
>63	37	42.9	(28.0-65.7)	39.3	*0.23*
Sex					
Men	49	36.7	(21.4-63.2)		
Women	56	36.2	(24.2-54.3)	-1.3	*0.97*
Race					
Non-White	22	80.4	(45.9-141.0)		
White	83	31.9	(23.2-43.8)	-60.4	*0.00*
1° family history of CRC					
No	93	42.5	(31.2-58.0)		
Yes	9	31.7	(15.4-65.1)	-25.5	*0.44*
Regular NSAID use					
Less than once a week	73	44.8	(31.1-64.4)		
Once a week or greater	32	30.5	(20.0-46.7)	-31.8	*0.13*
Regular aspirin user					
Less than once a week	60	37.3	(26.2-53.2)		
Once a week or greater	45	40.1	(26.2-61.5)	7.5	*0.76*
Current HRT user (women only, N=56)					
No	40	34.9	(22.6-53.8)		
Yes	16	22.3	(10.6-46.7)	-36.2	*0.33*
Smoking status					
Never	61	40.8	(27.9-59.5)		
Former	36	33.9	(21.7-53.2)	-16.7	
Current	8	49.9	(22.2-111.9)	22.4	*0.98*
Multivitamin user					
No	34	49.2	(33.2-72.6)		
Yes	71	30.1	(20.5-44.3)	-38.7	*0.05*
Physical activity (MET-min/wk)[t]*					
Low	33	38.2	(24.8-58.9)		
Moderate	36	47.7	(31.9-71.3)	25.0	
High	36	27.5	(17.5-43.3)	-27.9	*0.239*
BMI					
Normal (<25)	22	30.5	(17.4-53.5)		
Overweight (25-30)	43	40.8	(27.0-61.7)	33.9	
Obese (≥ 30)	40	40.4	(26.8-60.9)	32.5	*0.64*
Number of adenomas					
1 polyp	76	37.8	(26.6-53.7)		
> 1 polyp	29	39.6	(24.5-63.9)	4.8	*0.86*
Had advanced adenomas					
No	83	37.5	(26.5-53.1)		
Yes	19	44.0	(24.8-77.9)	17.3	*0.62*
Had serrated adenomas					
No	79	32.1	(23.0-44.7)		
Yes	26	59.3	(37.0-95.2)	85.0	*0.02*

Table S.3. Categorical Baseline Predictors of TLR5 Expression in Lower 60% of Stromal Region at Baseline[a] *(Cont.)*

		Lower 60% of stromal region			
		Geometric			
Baseline Characteristics	n	Mean	95% CI	Diff [c]	*p value*[d]
Total energy intake (kcal/d)[t]*					
Low	35	42.0	(27.4-64.2)		
Medium	36	45.3	(28.3-72.6)	8.0	
High	34	30.8	(20.0-47.3)	-26.6	*0.26*
Percent daily calories from fat[t]*					
Low	35	39.3	(25.5-60.6)		
Moderate	35	61.2	(38.0-98.5)	55.6	
High	35	28.5	(19.0-42.6)	-27.6	*0.20*
Dietary fiber (g/1000kcal/d)[t]*					
Low	35	34.5	(22.6-52.7)		
Moderate	35	42.4	(26.9-66.9)	22.7	
High	35	40.3	(24.9-65.3)	16.7	*0.61*
Red and processed meat, servings/d					
< 0.5	37	39.6	(25.8-60.7)		
0.5-1	30	38.8	(23.4-64.3)	-2.0	
≥ 1	38	36.6	(23.2-57.8)	-7.5	*0.79*
Achieved daily recommended intake of fruit and vegetables (≥ 5 servings/d)[t]*					
No	66	42.6	(29.9-60.7)		
Yes	39	31.9	(20.6-49.5)	-25.0	*0.25*
Alcohol intake					
None	36	35.7	(22.5-56.8)		
≤ 1 drink/wk	46	38.5	(24.4-60.8)	7.8	
> 1 drink/wk	23	42.9	(24.5-75.2)	20.1	*0.62*
Vitamin D Baseline Serum Levels (Tertiles)					
Low (12.90-17.90)	35	40.4	(26.2-62.1)		
Medium (17.91-26.91)	36	40.9	(25.9-64.6)	1.3	
High (26.92-68.75)	34	32.2	(19.9-52.0)	-20.3	*0.41*
Baseline Calcium Intake (Tertiles)					
Tertile 1	34	55.0	(37.1-81.5)		
Tertile 2	36	29.6	(19.0-46.2)	-46.2	
Tertile 3	35	28.4	(17.6-45.7)	-48.4	*0.02*

Abbreviations: Diff= proportional difference; CRC= colorectal cancer; HRT= hormone replacement therapy; NSAID= non-steroidal anti-inflammatory drug; BMI= body mass index; MET= metabolic equivalent of task; g= grams; IU= international units; kcal= kilocalories; d= day; wk= week

[a] The effect of the baseline characteristics on TLR5 expression was modeled using PROC GLM in SAS 9.4 (Cary, NC), controlling for age (continuous), gender (by study arm), study center and batch number, where appropriate.

[b] Reported means are geometric means (95% CI) and arithmetic means (95% CI) of optical density.

[c] Proportional Difference= (Comparison mean- reference mean)/reference mean*100%

[d] Reported p values are the Type III SS of the baseline characteristic. For baseline characteristics with more than two categories, the variables were treated as continuous and the reported p-value are of the overall trend

[t] Categorized by tertiles

*Missing values were replaced with treatment group- and sex-specific means

Table S.4. Categorical Baseline Predictors of TLR5 Expression in ϕh at Baseline[a]					
		ϕh[b]			
Baseline Characteristics	n	Mean	95% CI	Diff [c]	*p value*[d]
Age					
<55	33	65.7	(61.2-70.3)		
55-63	35	62.7	(58.4-67.0)	-4.6	
>63	37	60.8	(56.7-65.0)	-7.5	*0.08*
Sex					
Men	49	58.8	(53.6-64.1)		
Women	56	63.4	(59.5-67.4)	7.9	*0.17*
Race					
Non-White	22	56.8	(51.2-62.4)		
White	83	64.4	(61.2-67.6)	13.3	*0.02*
1° family history of CRC					
No	93	62.8	(59.7-66.0)		
Yes	9	61.2	(53.9-68.6)	-2.6	*0.68*
Regular NSAID use					
Less than once a week	73	61.5	(58.0-65.1)		
Once a week or greater	32	65.2	(61.1-69.3)	5.9	*0.14*
Regular aspirin user					
Less than once a week	60	63.8	(60.4-67.2)		
Once a week or greater	45	61.7	(57.6-65.9)	-3.2	*0.38*
Current HRT user (women only, N=56)					
No	40	63.2	(58.3-68.0)		
Yes	16	68.8	(60.5-77.1)	8.9	*0.28*
Smoking status					
Never	61	60.8	(57.2-64.5)		
Former	36	65.7	(61.3-70.0)	7.9	
Current	8	63.0	(55.2-70.8)	3.5	*0.17*
Multivitamin user					
No	34	61.4	(57.5-65.2)		
Yes	71	64.6	(60.8-68.4)	5.3	*0.19*
Physical activity (MET-min/wk)[t]*					
Low	33	62.3	(58.0-66.6)		
Moderate	36	64.2	(60.2-68.2)	3.0	
High	36	62.1	(57.6-66.6)	-0.3	*0.95*
BMI					
Normal (<25)	22	62.1	(56.8-67.5)		
Overweight (25-30)	43	65.6	(61.67-69.5)	5.6	
Obese (≥ 30)	40	61.3	(57.3-65.2)	-1.4	*0.23*
Number of adenomas					
1 polyp	76	63.0	(59.6-66.4)		
> 1 polyp	29	63.1	(58.5-67.8)	0.2	*0.96*
Had advanced adenomas					
No	83	64.1	(60.8-67.3)		
Yes	19	58.7	(53.4-64.0)	-8.4	*0.07*
Had serrated adenomas					
No	79	63.8	(60.4-67.1)		
Yes	26	61.2	(56.5-65.9)	-4.0	*0.33*

Table S.4. Categorical Baseline Predictors of TLR5 Expression in ϕh at Baseline[a] *(Cont.)*

		ϕh[b]			
Baseline Characteristics	n	Mean	95% CI	Diff [c]	*p value*[d]
Total energy intake (kcal/d)[t]*					
Low	35	63.9	(59.7-68.0)		
Medium	36	62.1	(57.5-66.8)	-2.7	
High	34	62.9	(58.7-67.1)	-1.5	*0.69*
Percent daily calories from fat[t]*					
Low	35	64.4	(60.0-68.8)		
Moderate	35	62.7	(57.9-67.5)	-2.6	
High	35	62.0	(57.9-66.1)	-3.7	*0.40*
Dietary fiber (g/1000kcal/d)[t]*					
Low	35	62.6	(58.5-66.8)		
Moderate	35	63.4	(59.0-67.9)	1.3	
High	35	63.1	(58.4-67.8)	0.7	*0.88*
Red and processed meat, servings/d					
< 0.5	37	65.0	(61.0-69.1)		
0.5-1	30	64.1	(59.3-68.9)	-1.4	
≥ 1	38	60.0	(55.7-64.3)	-7.7	*0.08*
Achieved daily recommended intake of fruit and vegetables (≥ 5 servings/d)[t]*					
No	66	61.8	(58.4-65.3)		
Yes	39	65.1	(60.8-69.3)	5.2	*0.19*
Alcohol intake					
None	36	62.6	(58.1-67.1)		
≤ 1 drink/wk	46	61.9	(57.5-66.3)	-1.1	
> 1 drink/wk	23	65.1	(59.7-70.6)	4.1	*0.50*
Vitamin D Baseline Serum Levels (Tertiles)					
Low (12.90-17.90)	35	62.9	(58.7-67.1)		
Medium (17.91-26.91)	36	62.3	(57.9-66.8)	-0.8	
High (26.92-68.75)	34	64.2	(59.5-68.9)	2.2	*0.61*
Baseline Calcium Intake (Tertiles)					
Tertile 1	34	62.2	(58.2-66.2)		
Tertile 2	36	63.4	(58.8-67.9)	1.9	
Tertile 3	35	64.0	(59.2-68.9)	3.0	*0.51*

Abbreviations: Diff= proportional difference; CRC= colorectal cancer; HRT= hormone replacement therapy; NSAID= non-steroidal anti-inflammatory drug; BMI= body mass index; MET= metabolic equivalent of task; g= grams; IU= international units; kcal= kilocalories; d= day; wk= week

[a] The effect of the baseline characteristics on TLR5 expression was modeled using PROC GLM in SAS 9.4 (Cary, NC), controlling for age (continuous), gender (by study arm), study center and batch number, where appropriate.

[b] Defined as the percent expression in the upper 40% of stromal region to expression in whole stromal region. Values are means (95% CI) of the optical density (not geometric means)

[c] Proportional Difference= (Comparison mean- reference mean)/reference mean*100%

[d] Reported p values are the Type III SS of the baseline characteristic. For baseline characteristics with more than two categories, the variables were treated as continuous and the reported p-value are of the overall trend

[t] Categorized by tertiles

*Missing values were replaced with treatment group- and sex-specific means

Figure S.1. Distribution of Mean TLR5 Biomarker Optical Density Across Stroma in Calcium vs. Placebo Groups

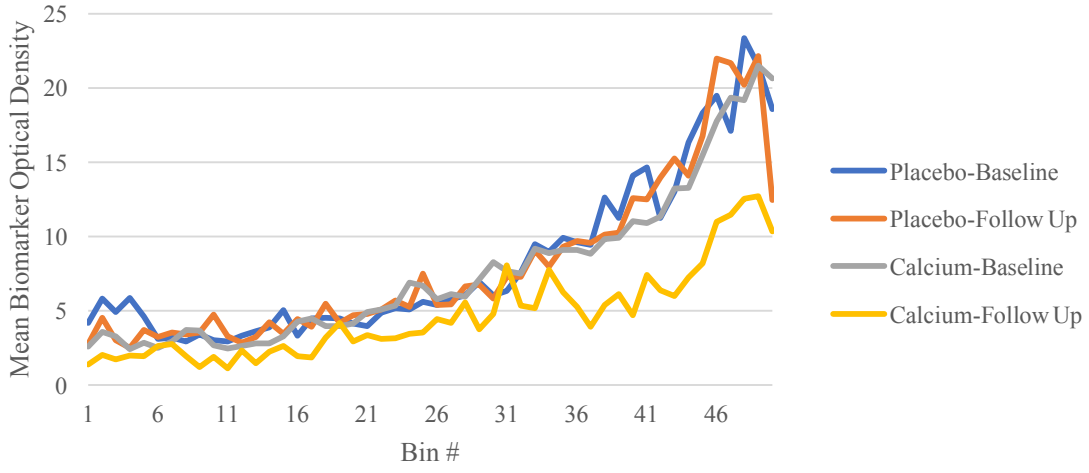

Figure S.1. Mean TLR5 biomarker optical density across the stroma in calcium *versus* placebo groups was graphed at baseline and 1- year follow-up.

Figure S.2. Distribution of Mean TLR5 Biomarker Optical Density Across Stroma in Vitamin D vs. Placebo Groups

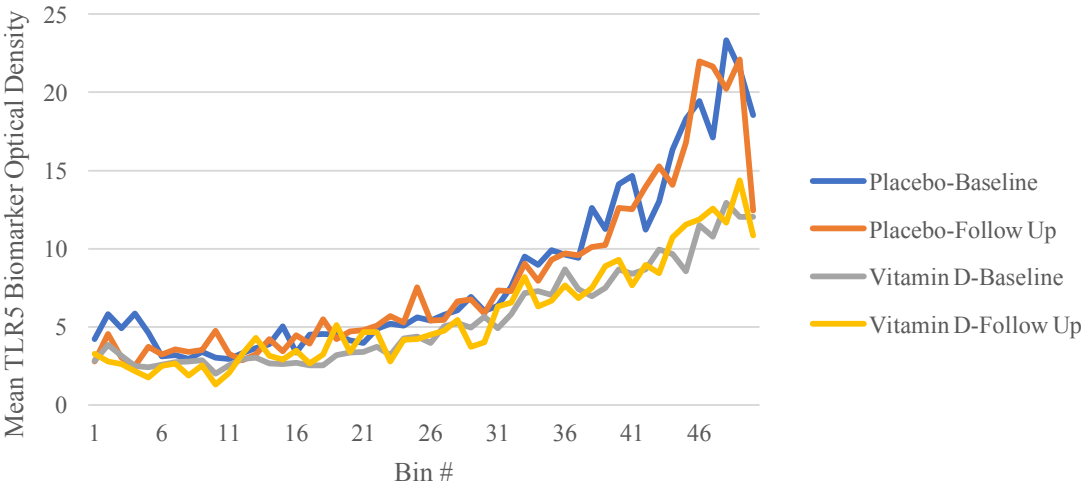

Figure S.2. Mean TLR5 biomarker optical density across the stroma in vitamin D *versus* placebo groups was graphed at baseline and 1-year follow-up.

Figure S.3. Distribution of Mean TLR5 Biomarker Optical Density Across Stroma in Calcium + Vitamin D vs. Placebo Group

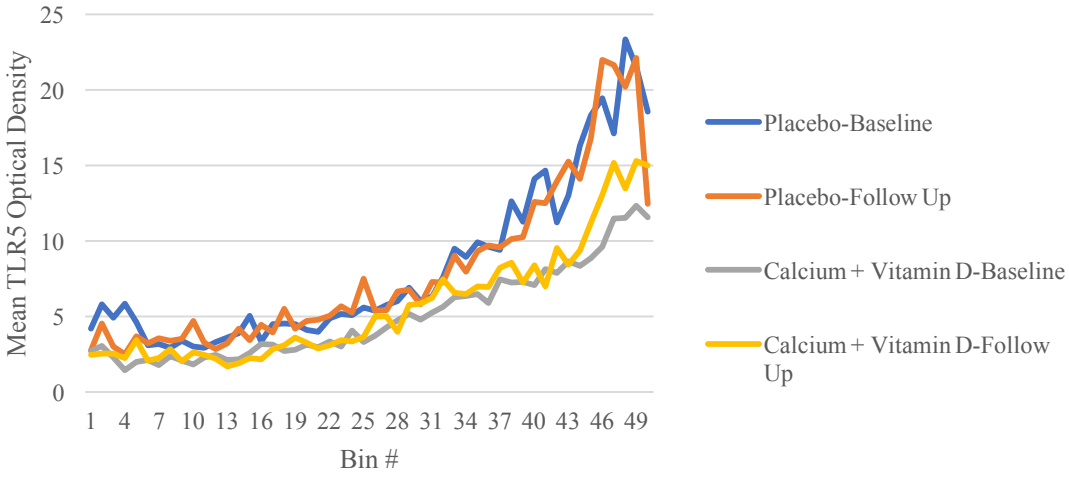

Figure S.3. Mean TLR5 biomarker optical density across the stroma in calcium + vitamin D *versus* placebo groups was graphed at baseline and 1-year follow-up.

Figure S.4. Distribution of Mean TLR5 Biomarker Optical Density Across Stroma in 2-arm Vitamin D vs. Placebo Group

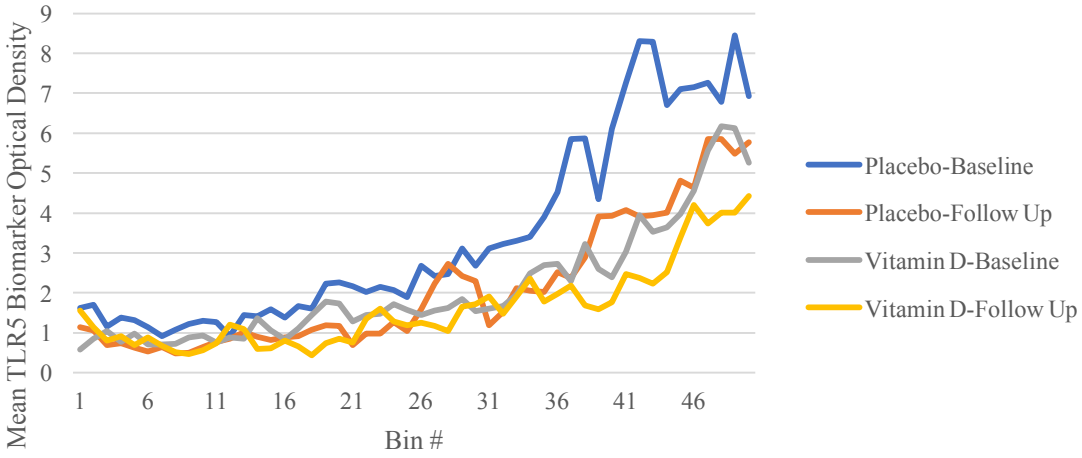

Figure S.4. Mean TLR5 biomarker optical density across the stroma in the 2-arm vitamin D *versus* placebo groups was graphed at baseline and 1-year follow-up.