



Published in final edited form as:

*Mol Carcinog.* 2017 February ; 56(2): 412–424. doi:10.1002/mc.22504.

## Effects of Supplemental Calcium and Vitamin D on the APC/ $\beta$ -Catenin Pathway in the Normal Colorectal Mucosa of Colorectal Adenoma Patients

Siyu Liu<sup>1</sup>, Elizabeth L. Barry<sup>2</sup>, John A. Baron<sup>2,3,4</sup>, Robin E. Rutherford<sup>5</sup>, March E. Seabrook<sup>6</sup>, and Roberd M. Bostick<sup>1,7</sup>

<sup>1</sup>Department of Epidemiology, Rollins School of Public Health, Emory University, Atlanta, GA

<sup>2</sup>Department of Epidemiology, Geisel School of Medicine at Dartmouth

<sup>3</sup>Department of Medicine, Geisel School of Medicine at Dartmouth

<sup>4</sup>University of North Carolina School of Medicine

<sup>5</sup>Division of Digestive Diseases, Department of Medicine, School of Medicine, Emory University

<sup>6</sup>Consultants in Gastroenterology, West Columbia, SC

<sup>7</sup>Winship Cancer Institute, Emory University

### Abstract

APC/ $\beta$ -catenin pathway malfunction is a common and early event in colorectal carcinogenesis. To assess calcium and vitamin D effects on the APC/ $\beta$ -catenin pathway in the normal-appearing colorectal mucosa of sporadic colorectal adenoma patients, nested within a larger randomized, double-blind, placebo-controlled, partial 2 $\times$ 2 factorial chemoprevention clinical trial of supplemental calcium (1,200 mg daily) and vitamin D (1,000 IU daily), alone and in combination versus placebo, we assessed APC,  $\beta$ -catenin, and E-cadherin expression in colon crypts in normal-appearing rectal mucosa biopsies from 104 participants at baseline and one-year follow up using standardized, automated immunohistochemistry and quantitative image analysis. For vitamin D vs. no vitamin D, the ratio of APC expression to  $\beta$ -catenin expression in the upper 40% (differentiation zone) of crypts (APC/ $\beta$ -catenin score) increased by 28% ( $P = 0.02$ ), for calcium vs. no calcium it increased by 1% ( $P = 0.88$ ), and for vitamin D + calcium vs. calcium by 35% ( $P = 0.01$ ). Total E-cadherin expression increased by 7% ( $P = 0.35$ ) for vitamin D vs. no vitamin D,

---

Corresponding Author: Roberd M. Bostick, MD, MPH. Department of Epidemiology, 1518 Clifton Road NE, Emory University, Atlanta, GA 30322; Phone: 404-727-2671; Fax: 404-727-8737; rmbosti@emory.edu.

**Conflicts of Interest:** None

**Trial Registration ID:** NCT00399607

#### Authors' Contributions:

Conception and design: RM Bostick, JA Baron

Development of methodology: RM Bostick

Acquisition of data: RM Bostick, RE Rutherford, ME Seabrook, JA Baron, EL Barry

Analysis and interpretation of data: S Liu, RM Bostick, JA Baron, EL Barry

Writing, review, and/or revision of the manuscript: S Liu, RM Bostick, JA Baron, EL Barry

Administrative, technical, or material support: RM Bostick, JA Baron, EL Barry, RE Rutherford, ME Seabrook

Study supervision: RM Bostick

8% ( $P = 0.31$ ) for calcium vs. no calcium, and 12% ( $P = 0.21$ ) for vitamin D + calcium vs. calcium. These results support (i) that vitamin D, alone or in combination with calcium, may modify APC,  $\beta$ -catenin, and E-cadherin expression in humans in directions hypothesized to reduce risk for colorectal neoplasms; (ii) vitamin D as a potential chemopreventive agent against colorectal neoplasms; and (iii) the potential of APC,  $\beta$ -catenin, and E-cadherin expression as treatable, pre-neoplastic risk biomarkers for colorectal neoplasms.

## Keywords

Calcium; vitamin D; colorectal neoplasms; biological markers; clinical trial

---

## Introduction

Despite advances in screening and treatment, colorectal cancer (CRC) remains the second leading cause of cancer death in the US (1). CRC incidence varies approximately 20-fold internationally (2), and migrants from lower- to higher-risk countries tend to acquire the CRC incidence rates of their adopted country within 1 – 2 generations (3, 4), emphasizing the importance of environmental exposures—especially diet and lifestyle—in the etiology and preventability of the disease.

There is strong biological plausibility and animal experimental and human observational evidence for protective effects of calcium and vitamin D against CRC (5). Proposed mechanisms of calcium effects against CRC include protection of the colorectal mucosa against free bile and fatty acids, direct effects on the cell cycle, and modulation of the APC colon carcinogenesis pathway (6, 7). Moreover, calcium intake has been consistently, modestly, inversely associated with colorectal neoplasms across numerous epidemiologic observational studies, and in large randomized controlled trials calcium supplementation reduced adenoma recurrence (5, 8). Besides its important role in maintaining calcium balance, vitamin D promotes bile acid degradation, regulates cell cycle events, and modulates growth factor signaling, DNA repair, and more than 200 responsive genes (7). Also, higher serum levels of 25-hydroxyvitamin-D (25[OH]D), the best indicator of total vitamin D exposure, have been consistently inversely associated with colorectal neoplasms, although there have been relatively few studies of this association (5, 9).

An aim of our research group is to develop “treatable” pre-neoplastic biomarkers of risk for colorectal neoplasms: 1) to corroborate the likely relevance of mechanisms discovered *in vitro* and in animal models, in free-living humans; 2) as endpoints for screening for the potential efficacy and safety and optimal doses of dietary and other preventive interventions against colorectal carcinogenesis; and 3) for clinical assessment for risk stratification and preventive treatment, analogously to measuring lipid panels for assessing and managing risk for ischemic heart disease (8). The adenomatous polyposis coli (APC) protein,  $\beta$ -catenin, and E-cadherin are potentially treatable pre-neoplastic biomarkers of risk for colorectal adenomas (10, 11). Impaired APC expression, which occurs in approximately 80% to 90% of sporadic CRCs (12), results in an increased potential of  $\beta$ -catenin to translocate to the nucleus, bind with T-cell factor transcription factors, and activate target genes responsible

for promoting cell proliferation and inhibiting differentiation (12–14). Also,  $\beta$ -catenin, together with  $\alpha$ -catenin, can bind to the cytoplasmic tail of the calcium-dependent cell adhesion protein E-cadherin, linking E-cadherin to actin filaments and promoting cell adhesion and differentiation (12, 14). E-cadherin may antagonize the APC/ $\beta$ -catenin pathway by sequestering  $\beta$ -catenin at cell adhesion junctions (12, 14). In the normal colorectal mucosa, APC,  $\beta$ -catenin, and E-cadherin are all strongly expressed—APC primarily in the cytoplasm, and  $\beta$ -catenin and E-cadherin primarily at the cell membrane. During the adenoma-carcinoma sequence, APC and E-cadherin expression markedly decreases, and  $\beta$ -catenin expression increases and translocates from the membrane to the cytoplasm and eventually into the nucleus (15).

Despite the basic science evidence, there is only one reported human *in vivo* investigation on the effects of calcium and vitamin D on the expression of APC,  $\beta$ -catenin, and E-cadherin in the normal colorectal mucosa (10). Based on the above described biological plausibility and the results of the previous pilot trial, we hypothesized that calcium and vitamin D, alone and in combination, would increase APC and E-cadherin expression, decrease  $\beta$ -catenin expression, and increase the balance of APC relative to  $\beta$ -catenin expression in the normal-appearing colorectal mucosa of sporadic colorectal adenoma patients.

## Materials and Methods

### Participant population

The participants in this study (“adjunct biomarker study”) were all participating in a larger 11-center, randomized, placebo-controlled, partial  $2 \times 2$  factorial chemoprevention clinical trial (“parent study”) which was designed to test the efficacy of supplemental calcium and vitamin D, alone and in combination, over 3–5 years on adenoma recurrence in colorectal adenoma patients (16). Eligible participants were 45 to 75 years of age and in general good health; within 4 months of study entry had a complete, clean colonoscopy during which all visible polypoid lesions were removed, at least one of which was a histologically-verified neoplastic polyp  $\geq 2$  mm in diameter; and were scheduled for a follow-up colonoscopy three or five years after their index colonoscopy. Exclusions from participation included invasive carcinoma in any colonic polyp removed, familial colonic polyposis syndromes, inflammatory bowel diseases, malabsorption syndromes, history of large bowel resection, narcotic or alcohol dependence, serum calcium outside normal range, creatinine greater than 20% above the upper limit of normal, serum 25-hydroxy vitamin D levels (25[OH]D)  $< 12$  ng/ml or  $> 90$  ng/ml, history of kidney stones or hyperparathyroidism, and history of osteoporosis or other medical condition that may require supplemental vitamin D or calcium. For participation in the adjunct biomarker study, additional exclusions were being unable to be off aspirin for 7 days, history of a bleeding disorder, or current use of an anticoagulant medication.

### Clinical trial protocol

Details of the parent clinical trial protocol, including the recruitment yields, were previously published (16). Briefly, for the parent study, between May 2004 and July 2008, 19,083 apparently eligible patients were identified through initial screening of colonoscopy and

pathology reports; of these, 2,259 met final eligibility criteria, consented to participate, and were randomized. After the parent study was underway, funding was received for the adjunct biomarker study. For the adjunct biomarker study, near the end of the placebo run-in period, without knowledge of treatment assignment, a total of 231 apparently eligible parent study participants at two clinical centers (South Carolina and Georgia) were offered participation in the biomarker study; of these, 109 met final eligibility, signed consent, and had baseline rectal biopsies taken, and of these, sufficient rectal biopsy tissue for biomarker measurements was obtained at baseline and 1-year follow up on 104. All participants signed a consent form at enrollment; the Institutional Review Boards at each clinical center approved the research.

At enrollment, the coordinator collected information from each parent study participant on medical history, medication and nutritional supplement use, and diet and lifestyle. Diet was assessed using the semi-quantitative Block Brief 2000 food frequency questionnaire (Nutritionquest, Berkeley, CA). After the subsequent placebo run-in period, subjects were randomly assigned to the following 4 treatment groups: placebo, 1,200 mg/day calcium supplementation (as calcium carbonate in equal doses twice daily), 1,000 IU/day vitamin D<sub>3</sub> supplementation (500 IU twice daily), and 1,200 mg/day elemental calcium plus 1,000 IU/day vitamin D<sub>3</sub> supplementation (“full factorial randomization”). Women who declined to forego calcium supplementation were randomized to calcium or calcium plus vitamin D<sub>3</sub> (“2-arm randomization”). Participants agreed to avoid taking vitamin D or calcium supplements outside the trial, although personal supplements up to 1,000 IU vitamin D and/or 400 mg elemental calcium were permitted from April 2008 onwards. Randomization was conducted using computer-generated random numbers with permuted blocks, and stratified by sex, clinical center, scheduled colonoscopic follow-up of 3 or 5 years, and 4- vs. 2-arm participation. Participants and all clinical, coordination, and laboratory staff were blinded to treatment assignment.

During the treatment period, every four months, bottles of study tablets were mailed to participants who were interviewed via telephone every 6 months regarding their adherence to study treatment, illnesses, use of medications and supplements, and colorectal endoscopic or surgical procedures. During the first year of follow-up (the period that is relevant to the adjunct biomarker study) blood levels of calcium, creatinine, 25(OH)D, and 1,25(OH)<sub>2</sub>D were obtained at baseline and 1 year after randomization.

Participants in the adjunct biomarker study underwent “non-prep” (i.e., with no preceding bowel-cleansing preparation or procedure) biopsies of normal-appearing rectal mucosa at baseline and at a year 1 follow-up visit. Six approximately 1 mm thick biopsy specimens were taken from the rectal mucosa 10 cm above the level of the external anal aperture through a short rigid proctoscope using a jumbo cup flexible biopsy forceps mounted on a semi-rigid rod. All biopsies were taken at least 4 cm from any polypoid lesions to avoid possible field effects from them. Biopsies were placed onto a strip of bibulous paper and immediately placed in normal saline, oriented, transferred to 10% normal buffered formalin for 24 hours, and then transferred to 70% ethanol. Then, within a week, the biopsies were processed and embedded in paraffin blocks (2 blocks of 3 biopsies per participant, per

biopsy visit). APC,  $\beta$ -catenin, and E-cadherin, were measured in the biopsies using automated immunohistochemistry with image analysis.

### Immunohistochemistry protocol

Five slides with 3 levels of 3  $\mu$ m-thick biopsy sections taken 40  $\mu$ m apart were prepared for each biomarker, yielding a total of 15 levels for each biomarker. To uncover the epitope, heat-mediated antigen retrieval was used: the slides were placed in a preheated Pretreatment Module (Lab Vision Corp., CA) with 100x Citrate Buffer pH 6.0 (DAKO S1699, DAKO Corp., Carpinteria, CA) and steamed for 40 minutes. Then, the slides were placed in a DakoCytomation Autostainer Plus System automated immunostainer and immunohistochemically processed using a labeled streptavidin-biotin method (LSAB2 Detection System [DAKO K0675]) and a monoclonal antibody to each biomarker (for APC, Oncogene OP80 at a concentration of 1:50; for  $\beta$ -catenin, BD Pharmingen [formerly Transduction Laboratories 610154], at a concentration of 1:300; for E-cadherin, Zymed 33–4000 at a concentration of 1:50). For each participant, baseline and follow-up biopsy slides were stained in the same batch, and each staining batch included a balance of participants from each treatment group. The slides, which were not counterstained, were coverslipped with a Leica CV5000 Coverslipper (Leica Microsystems, Inc., IL). Positive and negative control slides were included in each slide staining batch.

### Protocol for quantifying labeling densities of immunohistochemically-detected biomarkers in normal colon crypts (“scoring”)

A quantitative image analysis method (“scoring”) was used to measure detected levels of the biomarkers in colon crypts, as depicted in Figure 1. The major equipment and software for the image analysis procedures were: Scanscope CS digital scanner (Aperio Technologies, Inc., CA), computer, digital drawing board, MatLab software (MathWorks, Inc., MA), CellularEyes Image Analysis Suite (DivEyes LLC, GA), and MySQL (Sun Microsystems Inc., CA). First, slides were scanned with the Aperio Scanscope CS digital scanner, then, electronic images were reviewed in the CellularEyes program to identify colon crypts acceptable for analysis. A “scorable” crypt was defined as an intact crypt extending from the muscularis mucosa to the colon lumen. Before analysis, images of negative and positive control slides were checked for staining adequacy. Standardized settings were used on all equipment throughout the scoring procedures. The technician, who was blinded to treatment assignment, selected two of three biopsies with 16 to 20 “scorable” hemicypts (one half of the crypt) per biopsy. Using the digital drawing board the borders of each selected hemicypt were traced. The program then divided the outline into equally spaced segments with the average widths of normal colonocytes. Finally, the program measured the background-corrected optical density of the biomarker labeling across the entire hemicypt as well as within each segment. Subcellular locations of the biomarkers, such as nuclear  $\beta$ -catenin, were not measured. All resulting data were automatically transferred into the MySQL database. Then, the technician moved to the next identified hemicypt and repeated all the previously described analysis steps. A reliability control sample previously analyzed by the reader was re-analyzed during the course of the trial to determine intra-reader “scoring” reliability by intra-class correlation coefficient, which was  $> 0.90$  for each biomarker.

## Statistical analysis

Our main analyses were to assess changes in the expression of APC and  $\beta$ -catenin (individually and in relation to one another), and E-cadherin after randomization in the treatment groups that received 1) vitamin D relative to those that did not (“vitamin D vs. no vitamin D”), 2) calcium relative to those that did not (“calcium vs. no calcium”), and 3) in those that received calcium plus vitamin D relative to those that received only calcium (“calcium + vitamin D vs. calcium”). In addition to evaluating biomarker changes in the whole crypts, we evaluated changes within crypt functional zones, including the upper 40% of the crypts (the canonical differentiation zone), the lower 60% of the crypts (the canonical proliferation zone), and the ratio of the upper 40% of crypts to the whole crypt ( $\Phi_h$ ) (17, 18). To assess changes in the balance of APC relative to  $\beta$ -catenin in the differentiation zone, an APC/ $\beta$ -catenin score was calculated by dividing an individual’s APC expression by their  $\beta$ -catenin expression in the upper 40% of crypts.

Treatment groups were assessed for comparability of characteristics at baseline and at final follow-up by chi square test for categorical variables and ANOVA or t-test for continuous variables. Treatment effects were evaluated by assessing the differences in APC,  $\beta$ -catenin, and E-cadherin expression from baseline to the final follow-up between participants in the treatment group of interest and those in the comparison group using a general MIXED linear model. The model included the intercept, follow-up visit effects, time, treatment group, and the interaction of treatment with time. The calcium analyses included only participants randomized to calcium (i.e., none of the 2-arm study participants were included). Potential confounders, selected because of imbalances in their distributions across treatment groups at baseline, included current smoking status, non-aspirin non-steroidal anti-inflammatory drug (NSAID) use, multivitamin use, physical activity measured as metabolic equivalent of task (MET)-minutes, and dietary fiber intake. Exploratory analyses to assess potential treatment effect modification were conducted by stratifying the above analyses on sex, body mass index (BMI), age, NSAID use, and total fat intake. For these analyses, NSAID use was categorized as < vs. once a week) and BMI, age, and total fat intake were categorized as below and above the sex-specific medians. Because all biomarker measurements were in optical density, to provide perspective on the magnitudes of the estimated treatment effects, relative treatment effects were calculated (relative effect = [(treatment group follow-up) / (treatment group baseline)] / [(control group follow-up) / (control group baseline)]). The interpretation of a relative effect is similar to that for an odds ratio; for example, a relative effect of 1.3 would indicate that a biomarker increased about 30% more in the treatment group of interest relative to the control group. In all analyses of randomized treatments, participants were retained in their originally assigned treatment group, regardless of adherence to study treatment and procedures. All statistical analyses were conducted using SAS 9.4 statistical software (SAS Institute Inc.). A P value  $\leq 0.05$  (2-sided) was considered statistically significant.

## Results

Selected baseline characteristics of the adjunct biomarker study participants are presented in Table 1. The mean age of study participants was 59 years, 46% were men, and 79% were



white. Most participants were high school graduates, non-current smokers, and overweight. There were significant differences in physical activity and dietary fiber intake among the treatment groups.

For the adjunct biomarker study, during the first year after randomization, 76% of participants reported taking 80% or more of their study tablets. There was a mean increase in serum 25(OH)D of 10.87 (SD = 9.57) ng/ml at year 1 in subjects randomized to vitamin D relative to those who were not.

The estimated effects of the study interventions on the expression of the three biomarkers are presented in Table 2 and described below. Adjustment for factors on which the treatment groups differed at baseline did not materially affect the estimated treatment effects, so only the unadjusted results are presented.

### APC

Following 1 year of treatment, for vitamin D vs. no vitamin D, APC expression increased by an estimated 12% (P = 0.21) in the full length of crypts, 21% (P = 0.03) in the upper 40% of crypts, 5% (P = 0.65) in the lower 60% of crypts, and 4% (P = 0.16) in the  $\Phi$ h of crypts (Table 2). For calcium vs. no calcium, there were minimal non-statistically significant estimated increases in APC expression in all of the crypt parameters. For vitamin D + calcium vs. calcium, APC increased by 19% (P = 0.12) in the full length of crypts, 27% (P = 0.03) in the upper 40% of crypts, 13% (P = 0.32) in the lower 60% of crypts, and 4% (P = 0.31) in the  $\Phi$ h of crypts.

### $\beta$ -catenin

For vitamin D vs. no vitamin D,  $\beta$ -catenin expression decreased by an estimated 3% (P = 0.41), 4% (P = 0.28), and 2% (P = 0.58) in the full length, the upper 40%, and the lower 60% of the crypts, respectively (Table 2). The estimated treatment effects for vitamin D + calcium vs. calcium on the three crypt parameters were identical to those for vitamin D vs. no vitamin D. For calcium vs. no calcium there were non-statistically significant increases in  $\beta$ -catenin expression of 6 – 7% in the three crypt parameters. None of the treatments appeared to materially affect the  $\Phi$ h of crypts.

### APC/ $\beta$ -catenin score

For vitamin D vs. no vitamin D, the APC/ $\beta$ -catenin score increased by 28% (P = 0.02), for calcium vs. no calcium it increased by 1% (P = 0.88), and for vitamin D + calcium vs. calcium by 35% (P = 0.01) (Table 2).

### E-cadherin

For vitamin D vs. no vitamin D, E-cadherin expression increased by an estimated 7% (P = 0.35) in the full length of crypts, 3% (P = 0.66) in the upper 40% of crypts, and 10% (P = 0.22) in the lower 60% of crypts (Table 2). For vitamin D + calcium vs. calcium, E-cadherin expression increased by 12% (P = 0.21) in the full length of crypts, 9% (P = 0.38) in the upper 40% of crypts, and 15% (P = 0.14) in the lower 60% of crypts. For calcium vs. no calcium, E-cadherin expression increased by 8% (P = 0.31) in the full length of crypts, 12%

( $P = 0.14$ ) in the upper 40% of crypts, and 5% ( $P = 0.55$ ) in the lower 60% of crypts. None of the treatments appeared to materially affect the  $\Phi$ h of crypts.

### Stratified analyses

For vitamin D vs. no vitamin D and vitamin D + calcium vs. calcium, APC expression and the APC/ $\beta$ -catenin score tended to be higher among subjects who took a non-aspirin NSAID 1/week and among those with a higher total fat intake (Table 3).

### Discussion

Our findings suggest that supplemental vitamin D, alone or in combination with calcium, may increase APC (especially in relation to  $\beta$ -catenin, and especially in the crypt differentiation zone) and, to a lesser extent, E-cadherin, expression in the normal appearing colorectal mucosa of sporadic colorectal adenoma patients. Our findings also suggest that calcium may modestly increase E-cadherin expression. These possible effects are in directions hypothesized to reduce risk for colorectal neoplasms. Our findings also suggest that calcium may not materially affect APC or  $\beta$ -catenin expression.

APC,  $\beta$ -catenin, and E-cadherin are appealing candidates as treatable pre-neoplastic biomarkers of risk for colorectal adenomas because malfunctioning of the APC/ $\beta$ -catenin pathway is a common and early event in colorectal carcinogenesis (12). In the normal colorectal mucosa, APC protein functions to degrade  $\beta$ -catenin, the effector of the WNT signaling pathway that controls the coordinated expansion and differentiation of the intestinal crypt stem cell (12). The WNT signaling pathway is normally inactive, but impaired APC expression can result in WNT signaling through stabilization of nuclear  $\beta$ -catenin, thus promoting cell proliferation and inhibiting differentiation (12). E-cadherin may antagonize the APC/ $\beta$ -catenin pathway by sequestering  $\beta$ -catenin at cell adhesion junctions (15). In the normal colorectal mucosa, APC,  $\beta$ -catenin, and E-cadherin are all strongly expressed; during the adenoma-carcinoma sequence, APC and E-cadherin expression markedly decreases and  $\beta$ -catenin expression increases (15). An APC/ $\beta$ -catenin score was suggested to be a modifiable predictor of risk for colorectal adenomas because it may represent the potential of  $\beta$ -catenin to translocate to the nucleus and promote proliferative signaling (11). It was found that an APC/ $\beta$ -catenin score was statistically significantly lower in the normal colorectal mucosa of sporadic colorectal adenoma patients than in the normal colorectal mucosa of healthy controls (11), and APC,  $\beta$ -catenin, and E-cadherin expression and the APC/ $\beta$ -catenin score were associated with lifestyle and dietary risk factors for colorectal neoplasms (10), suggesting that they may be modifiable pre-neoplastic biomarkers of risk for colorectal neoplasms.

The etiology of CRC is heavily influenced by modifiable dietary and lifestyle factors, and vitamin D and calcium are two promising chemopreventive agents against colorectal adenomas. Findings from CRC cell line studies indicate that calcium and  $1,25(\text{OH})_2\text{D}$  upregulate E-cadherin production and promote a shift in  $\beta$ -catenin distribution from the nucleus and cytoplasm to the cell membrane (19–22). The typical “Western” diet was found to induce increased  $\beta$ -catenin expression and decreased APC expression when fed to wild-



type mice; however, intake of a “Western” diet with increased dietary calcium and vitamin D decreased  $\beta$ -catenin expression, but had no significant effect on APC expression (23).

Our results are consistent with the hypothesis that calcium and vitamin D reduce cell proliferation and promote differentiation in the colorectal mucosa. We previously proposed that the APC/ $\beta$ -catenin score may represent the potential of  $\beta$ -catenin to translocate to the nucleus and promote proliferative signaling (11). In the present study, we observed increased APC expression, particularly in the upper 40%, or differentiation zone, of crypts, and an increased APC/ $\beta$ -catenin score in the vitamin D supplementation groups, suggesting that supplemental vitamin D may decrease the potential of  $\beta$ -catenin to promote proliferative signaling. As reviewed in detail elsewhere (8), in one of our previous trials in colorectal adenoma patients we found that supplemental calcium statistically significantly reduced the proportion of proliferating cells in the crypts that were in the upper 40% of the crypts (the  $\Phi$ h of crypts) (24), and in a second calcium and vitamin D statistically significantly increased p21 expression (as a marker of differentiation) (25).

In the only previously reported clinical trial of the effects of calcium and/or vitamin D on the APC/ $\beta$ -catenin pathway (10), 92 sporadic colorectal adenoma patients were randomized to calcium 2,000 mg/day and/or vitamin D 800 IU/day over six months. In the vitamin D<sub>3</sub>-supplemented group relative to placebo, the proportion of APC in the upper 40% of crypts ( $\Phi$ h APC) increased 21% (p=0.01),  $\beta$ -catenin decreased 12% (p=0.18), E-cadherin increased 72% (p=0.03), and the APC/ $\beta$ -catenin score increased 31% (p=0.02). In the calcium-supplemented group  $\Phi$ h APC increased 10% (p=0.12),  $\beta$ -catenin decreased 15% (p=0.08), and the APC/ $\beta$ -catenin score increased 41% (p=0.01). In the calcium/vitamin D<sub>3</sub> supplemented group  $\beta$ -catenin decreased 11% (p=0.20), E-cadherin increased 51% (p=0.08), and the APC/ $\beta$ -catenin score increased 16% (p=0.26). As can be seen, the results for the effects of vitamin D and/or calcium on APC, E-cadherin, and the APC/ $\beta$ -catenin score in the two trials were similar in most, but not all, respects. In the present trial, the estimated treatment effects on  $\beta$ -catenin alone were closer to the null. In addition, in the present trial, unlike in the previous trial but consistent with our hypothesis and other reports (26, 27), the estimated treatment effects of vitamin D plus calcium appeared greater than those of either the calcium or vitamin D alone in increasing APC and the APC/ $\beta$ -catenin score. It was also previously reported that the effects of vitamin D plus calcium may be stronger than those of either agent alone on colorectal mucosa markers of apoptosis and differentiation (25). The reason(s) for the differences in findings between the two studies in relation to  $\beta$ -catenin are unclear, but could be due to the different intervention agent doses, study durations, and study populations, and, considering the small sample sizes, may have been due to chance.

In our stratified analyses, we found that the estimated effects of supplemental vitamin D, alone or in combination with calcium, on APC expression and the APC/ $\beta$ -catenin score tended to be a little stronger among participants who regularly took an NSAID or who had higher intakes of total fat. The sample size for the stratified analyses was small and the results were not statistically significant and thus may have been strictly due to chance. However, there is some plausibility to the findings. NSAID use is consistently reported to reduce risk of colorectal neoplasms, presumably mostly via reducing COX-2 expression, which impacts the APC/ $\beta$ -catenin pathway (28). When vitamin D binds to its receptor, it can

upregulate CYP3A4, which catabolizes the secondary bile acid, lithocholic acid, which can prevent its cytotoxicity and thus a secondary inflammatory response (29). This suggests that vitamin D and NSAIDs together may reduce inflammation and increase APC expression. Higher intakes of total fat are directly associated with risk of colorectal neoplasms, presumably via increased production of cytotoxic, mitogenic bile acids (30). This suggests that vitamin D may be most effective in circumstances in which fat intake is sufficiently high to produce the bile acids that vitamin D can affect via the mechanism described above.

Notably, the parent trial to this study found no evidence that calcium and/or vitamin D reduced adenoma recurrence over 3–5 years (16), despite previous clinical trial evidence that calcium supplementation did so (31). Given the relatively long period of time it takes to develop an adenoma and that a substantial proportion of persons diagnosed with adenomas get subsequent adenomas (in the parent trial population about 40% did), would indicate that such persons have fairly committed clones of cells in the intestinal mucosa about to become grossly visibly manifest as adenomas. This suggests that if a preventive agent would be most effective early in the process (for example, years 1 – 16 of development) and not later (for example, years 17 – 20), then we would not be able to see an effect on preventing new adenomas in documented adenoma formers until after a substantial number of years of treatment (i.e., during, for example, years 1 – 5 of follow-up), the committed clones are still playing out, and after that time (for example, after 5 years) we may be able to begin to see a treatment effect. The pattern of 3 vs. 5 year findings in the parent trial, and the long-term follow up of participants in the previous calcium and adenoma recurrence trial (stronger estimates 5 years post trial vs. at trial end [OR 0.63 vs. 0.85]) also are consistent with this (32).

This study had several limitations and strengths. The primary limitation of this study was the small sample size, which increased the role of chance observations. Despite our limited sample size, we found statistically significant effects of vitamin D, with and without calcium, on APC expression and the APC/ $\beta$ -catenin score. We only examined the rectal mucosa and therefore treatment effects on other parts of the colon are unknown. We only assessed the protein expression of selected biomarkers but not the protein activity and therefore could not correlate changes in expression with changes in protein activity. Also, we were unable to measure subcellular localization of  $\beta$ -catenin; however, our previous findings (11) suggested that sporadic colorectal adenoma cases relative to normal controls may have greater total  $\beta$ -catenin expression in the normal colorectal mucosa. We previously proposed that the APC/ $\beta$  catenin score may represent the potential of  $\beta$ -catenin to promote proliferative signaling, and needs to be investigated in basic science studies (10). The strengths of the study include: (i) the high protocol adherence by study participants, and (ii) the automated immunostaining and novel image analysis software to quantify crypt biomarker distributions and the consequent high biomarker measurement reliability.

In summary, the results of this chemoprevention trial provide human *in vivo* evidence that supplemental vitamin D, alone or in combination with calcium, may modify APC,  $\beta$ -catenin, and E-cadherin expression, and, to a lesser extent, that supplemental calcium may modify E-cadherin expression, in directions hypothesized to reduce risk for colorectal neoplasms. These results provide further support that APC and  $\beta$ -catenin expression, the APC/ $\beta$ -catenin

score, and E-cadherin expression may be modifiable pre-neoplastic biomarkers of risk for colorectal neoplasms and that further, larger investigations are warranted. Our results also support further investigation of vitamin D as a chemopreventive agent against colorectal neoplasms.

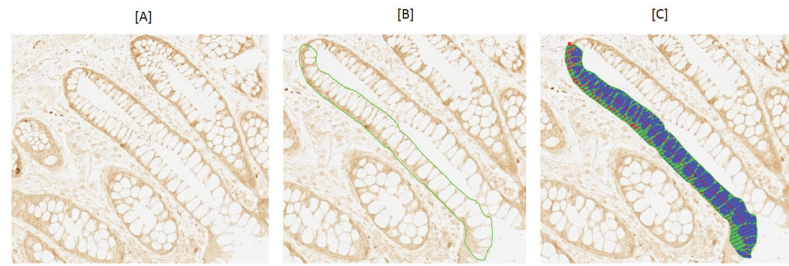
## Acknowledgments

**Grant support:** National Cancer Institute, National Institutes of Health (R01 CA114456 to RMB and R01 CA098286 to JAB); Georgia Cancer Coalition Distinguished Scholar award (to RMB); the Franklin Foundation (to RMB). Pfizer Consumer Healthcare provided the study agents. The National Cancer Institute, the Franklin Foundation, and Pfizer Consumer Healthcare had no influence on the design of this study; the collection, analysis, and interpretation of the data; the decision to submit the manuscript for publication; or the writing of the manuscript.

## References

1. American Cancer Society. Cancer Facts & Figures 2015. Atlanta: American Cancer Society; 2015.
2. American Cancer Society. Colorectal Cancer Facts & Figures 2014–2016. Atlanta: American Cancer Society; 2014.
3. World Cancer Research Fund / American Institute for Cancer Research. Food, Nutrition, Physical Activity, and the Prevention of Cancer: a Global Perspective. Washington DC: AICR; 2007.
4. Potter JD. Colorectal cancer: molecules and populations. *J Natl Cancer Inst.* 1999; 91(11):916–32. [PubMed: 10359544]
5. Zhang X, Giovannucci E. Calcium, vitamin D and colorectal cancer chemoprevention. *Best Pract Res Clin Gastroenterol.* 2011; 25(4–5):485–94. [PubMed: 22122765]
6. Newmark HL, Lipkin M. Calcium, vitamin D, and colon cancer. *Cancer Res.* 1992; 52(7 Suppl): 2067s–2070s. [PubMed: 1544142]
7. Lamprecht SA, Lipkin M. Chemoprevention of colon cancer by calcium, vitamin D and folate: molecular mechanisms. *Nat Rev Cancer.* 2003; 3(8):601–14. [PubMed: 12894248]
8. Bostick RM. Effects of supplemental vitamin D and calcium on normal colon tissue and circulating biomarkers of risk for colorectal neoplasms. *J Steroid Biochem Mol Biol.* 2015; 148:86–95. [PubMed: 25597952]
9. Kang W, Lee S, Jeon E, Yun YR, Kim KH, Jang JH. Emerging role of vitamin D in colorectal cancer. *World J Gastrointest Oncol.* 2011; 3(8):123–7. [PubMed: 22007275]
10. Ahearn TU, Shaikat A, Flanders WD, Rutherford RE, Bostick RM. A randomized clinical trial of the effects of supplemental calcium and vitamin D3 on the APC/beta-catenin pathway in the normal mucosa of colorectal adenoma patients. *Cancer Prev Res (Phila).* 2012; 5(10):1247–56. [PubMed: 22964475]
11. Ahearn TU, Shaikat A, Flanders WD, Seabrook ME, Bostick RM. Markers of the APC/beta-catenin signaling pathway as potential treatable, preneoplastic biomarkers of risk for colorectal neoplasms. *Cancer Epidemiol Biomarkers Prev.* 2012; 21(6):969–79. [PubMed: 22539608]
12. Goss KH, Groden J. Biology of the adenomatous polyposis coli tumor suppressor. *J Clin Oncol.* 2000; 18(9):1967–79. [PubMed: 10784639]
13. Markowitz SD, Bertagnolli MM. Molecular origins of cancer: Molecular basis of colorectal cancer. *N Engl J Med.* 2009; 361(25):2449–60. [PubMed: 20018966]
14. Willert K, Jones KA. Wnt signaling: is the party in the nucleus? *Genes Dev.* 2006; 20(11):1394–404. [PubMed: 16751178]
15. Zoratto F, Rossi L, Verrico M, Papa A, Basso E, Zullo A, et al. Focus on genetic and epigenetic events of colorectal cancer pathogenesis: implications for molecular diagnosis. *Tumour Biol.* 2014; 35(7):6195–206. [PubMed: 25051912]
16. Baron JA, Barry EL, Mott LA, Rees JR, Sandler RS, Snover DC, et al. A Trial of Calcium and Vitamin D for the Prevention of Colorectal Adenomas. *N Engl J Med.* 2015; 373(16):1519–30. [PubMed: 26465985]

17. Bostick RM, Fosdick L, Lillemoe TJ, Overn P, Wood JR, Grambsch P, et al. Methodological findings and considerations in measuring colorectal epithelial cell proliferation in humans. *Cancer Epidemiol Biomarkers Prev.* 1997; 6(11):931–42. [PubMed: 9367067]
18. Bostick RM. Human studies of calcium supplementation and colorectal epithelial cell proliferation. *Cancer Epidemiol Biomarkers Prev.* 1997; 6(11):971–80. [PubMed: 9367073]
19. Bhagavathula N, Hanosh AW, Nerusu KC, Appelman H, Chakrabarty S, Varani J. Regulation of E-cadherin and beta-catenin by Ca<sup>2+</sup> in colon carcinoma is dependent on calcium-sensing receptor expression and function. *Int J Cancer.* 2007; 121(7):1455–62. [PubMed: 17557293]
20. Chakrabarty S, Radjendirane V, Appelman H, Varani J. Extracellular calcium and calcium sensing receptor function in human colon carcinomas: promotion of E-cadherin expression and suppression of beta-catenin/TCF activation. *Cancer Res.* 2003; 63(1):67–71. [PubMed: 12517779]
21. Chakrabarty S, Wang H, Canaff L, Hendy GN, Appelman H, Varani J. Calcium sensing receptor in human colon carcinoma: interaction with Ca(2+) and 1,25-dihydroxyvitamin D(3). *Cancer Res.* 2005; 65(2):493–8. [PubMed: 15695391]
22. Palmer HG, Gonzalez-Sancho JM, Espada J, Berciano MT, Puig I, Baulida J, et al. Vitamin D(3) promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of beta-catenin signaling. *J Cell Biol.* 2001; 154(2):369–87. [PubMed: 11470825]
23. Yang K, Kurihara N, Fan K, Newmark H, Rigas B, Bancroft L, et al. Dietary induction of colonic tumors in a mouse model of sporadic colon cancer. *Cancer Res.* 2008; 68(19):7803–10. [PubMed: 18829535]
24. Bostick RM, Fosdick L, Wood JR, Grambsch P, Grandits GA, Lillemoe TJ, et al. Calcium and colorectal epithelial cell proliferation in sporadic adenoma patients: a randomized, double-blinded, placebo-controlled clinical trial. *J Natl Cancer Inst.* 1995; 87(17):1307–15. [PubMed: 7658483]
25. Fedirko V, Bostick RM, Flanders WD, Long Q, Sidelnikov E, Shaikat A, et al. Effects of vitamin D and calcium on proliferation and differentiation in normal colon mucosa: a randomized clinical trial. *Cancer Epidemiol Biomarkers Prev.* 2009; 18(11):2933–41. [PubMed: 19861511]
26. Grau MV, Baron JA, Sandler RS, Haile RW, Beach ML, Church TR, et al. Vitamin D, calcium supplementation, and colorectal adenomas: results of a randomized trial. *J Natl Cancer Inst.* 2003; 95(23):1765–71. [PubMed: 14652238]
27. Lappe JM, Travers-Gustafson D, Davies KM, Recker RR, Heaney RP. Vitamin D and calcium supplementation reduces cancer risk: results of a randomized trial. *Am J Clin Nutr.* 2007; 85(6):1586–91. [PubMed: 17556697]
28. Potter JD, Slattery ML, Bostick RM, Gapstur SM. Colon cancer: a review of the epidemiology. *Epidemiol Rev.* 1993; 15(2):499–545. [PubMed: 8174669]
29. Cheng J, Fang ZZ, Kim JH, Krausz KW, Tanaka N, Chiang JY, et al. Intestinal CYP3A4 protects against lithocholic acid-induced hepatotoxicity in intestine-specific VDR-deficient mice. *J Lipid Res.* 2014; 55(3):455–65. [PubMed: 24343899]
30. Ruder EH, Thiebaut AC, Thompson FE, Potischman N, Subar AF, Park Y, et al. Adolescent and mid-life diet: risk of colorectal cancer in the NIH-AARP Diet and Health Study. *Am J Clin Nutr.* 2011; 94(6):1607–19. [PubMed: 22071715]
31. Baron JA, Beach M, Mandel JS, van Stolk RU, Haile RW, Sandler RS, et al. Calcium supplements for the prevention of colorectal adenomas. Calcium Polyp Prevention Study Group. *N Engl J Med.* 1999; 340(2):101–7. [PubMed: 9887161]
32. Grau MV, Baron JA, Sandler RS, Wallace K, Haile RW, Church TR, et al. Prolonged effect of calcium supplementation on risk of colorectal adenomas in a randomized trial. *J Natl Cancer Inst.* 2007; 99(2):129–36. [PubMed: 17227996]



**Figure 1.** Measurement of biomarker expression (in this figure, APC) in crypts of normal appearing rectal mucosa using custom-designed quantitative image analysis software. A, finding a full length hemicypt; B, tracing the hemicypt; C, automated sectioning and quantification of APC labeling optical density, overall and within each section of the hemicypt.

**Table 1** Selected baseline characteristics of the adjunct biomarker study participants (n = 104), according to treatment assignment<sup>a</sup>

Characteristics	Treatment assignment						P value <sup>c</sup>	
	Randomization to vitamin D and to calcium (4-arm)			Randomization to vitamin D only (2-arm)				
	Placebo (n = 12)	Calcium (n = 16)	Vitamin D (n = 17)	Calcium + Vitamin D (n = 17)	P value <sup>b</sup>	Placebo (n = 23)		Vitamin D (n = 19)
<b>Demographics, medical history, habits, anthropometrics</b>								
Age, years	59.9 (7.2)	59.9 (6.5)	59.2 (7.8)	57.7 (7.1)	0.79	58.2 (5.3)	59.2 (7.3)	0.60
Male (%)	75	81	71	82	0.87	0	0	--
White (%)	83	75	71	94	0.42	70	84	0.57
College (%)	92	63	88	82	0.24	91	74	0.21
Take non-aspirin NSAID <sup>d</sup> regularly <sup>e</sup>	33	44	24	29	0.69	26	32	0.74
Current smoker (%)	25	6	0	6	0.11	0	16	0.16
Alcohol intake, drinks/day	0.7 (0.7)	0.8 (1.0)	0.9 (1.0)	0.9 (0.9)	0.92	0.5 (1.0)	0.3 (0.5)	0.40
Take multivitamin (%)	42	81	47	65	0.11	70	89	0.15
Physical activity, MET <sup>f</sup> -mins./wk. <sup>g</sup>	1,620 (1,195)	2,128 (2,378)	2,782 (2,764)	3,875 (2,424)	0.06	1,458 (1,235)	3,021 (3,469)	0.05
BMI, kg/m <sup>2</sup>	29.4 (4.9)	32.3 (7.6)	28.7 (5.5)	30.0 (4.5)	0.32	29.7 (5.6)	27.5 (4.7)	0.18
<b>Dietary intakes</b>								
Total energy intake, kcal/d <sup>h</sup>	1,314 (381)	1,737 (556)	1,437 (527)	1,613 (550)	0.18	1,254 (549)	1,429 (595)	0.39
Total fat, gm/d <sup>h</sup>	57.1 (22.3)	68.9 (25.6)	60.5 (27.3)	62.6 (27.2)	0.70	50.3 (25.9)	61.5 (36.1)	0.22
Dietary fiber, gm/d <sup>h</sup>	9.5 (4.1)	15.8 (5.6)	13.7 (6.2)	15.6 (5.5)	0.03	13.8 (5.4)	17.2 (5.0)	0.07
Total calcium <sup>i</sup> , mg/d <sup>j</sup>	715.3 (455.4)	894.5 (263.9)	671.3 (278.3)	667.1 (254.7)	0.14	995.6 (97.6)	1,232.3 (562.9)	0.34
<b>Serum levels</b>								
25-OH-vitamin D, ng/ml	22.4 (8.2)	24.5 (13.4)	23.1 (8.7)	22.5 (6.5)	0.93	24.8 (8.9)	26.5 (9.6)	0.54

<sup>a</sup>Data are given as means (SD) unless otherwise specified

<sup>b</sup>By Fisher's Exact test for categorical variables, and ANOVA for continuous variables

<sup>c</sup>By Fisher's Exact test for categorical variables, and t-test for continuous variables

<sup>d</sup>Nonsteroidal anti-inflammatory drug



Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

<sup>e</sup>At least once a week

<sup>f</sup>Metabolic equivalent of task

<sup>g</sup>One missing value in the vitamin D group, 2-arm

<sup>h</sup>Two missing values in the placebo group, 4-arm; 1 missing value in the calcium group, 4-arm

<sup>i</sup>Dietary plus supplemental calcium intake

<sup>j</sup>Two missing values in the placebo group, 4-arm; 1 missing value in the calcium group, 4-arm; 1 missing value in the vitamin D group, 4-arm; 6 missing values in the placebo group, 2-arm; 1 missing value in the vitamin D group, 2-arm

**Table 2**

Expression of APC,  $\beta$ -catenin, E-cadherin, and the APC/ $\beta$ -catenin score<sup>d</sup> in the normal-appearing colorectal mucosa of the adjunct biomarker study participants (n = 104)

Treatment group	Baseline			1-year follow-up			Absolute Rx effect					
	n	Mean	SE	P	n	Mean	SE	P	Rx effect <sup>b</sup>	SE	P <sup>c</sup>	Relative effect <sup>d</sup>
<b>APC (OD)</b>												
<i>Whole crypts</i>												
No vitamin D	51	2,607	194		51	2,098	149					
Vitamin D	53	2,419	173	0.47	53	2,177	145	0.71	266	209	0.21	1.12
No calcium	29	2,583	244		29	2,008	194					
Calcium	33	2,858	212	0.40	33	2,333	148	0.18	49	265	0.85	1.05
Calcium	39	2,524	224		39	2,041	164					
Vitamin D + calcium	36	2,440	210	0.79	36	2,348	183	0.21	391	249	0.12	1.19
<i>Upper 40% of crypts</i>												
No vitamin D	51	1,106	89		51	867	68					
Vitamin D	53	929	72	0.13	53	881	73	0.89	190	86	0.03	1.21
No calcium	29	1,040	120		29	813	100					
Calcium	33	1,216	92	0.24	33	986	70	0.15	-2	115	0.99	1.04
Calcium	39	1,050	97		39	835	73					
Vitamin D + calcium	36	959	88	0.49	36	966	88	0.25	221	101	0.03	1.27
<i>Lower 60% of crypts</i>												
No vitamin D	51	1,339	110		51	1,101	88					
Vitamin D	53	1,356	108	0.91	53	1,171	77	0.55	53.3	118	0.65	1.05
No calcium	29	1,395	140		29	1,073	104					
Calcium	33	1,462	131	0.73	33	1,201	88	0.35	60.9	145	0.68	1.07
Calcium	39	1,322	131		39	1,084	99					
Vitamin D + calcium	36	1,338	131	0.93	36	1,244	99	0.26	144.5	143	0.32	1.13
<i><math>\phi/\psi^e</math></i>												
No vitamin D	51	0.42	0.02		51	0.41	0.02					
Vitamin D	53	0.37	0.02	0.03	53	0.38	0.02	0.16	0.02	0.01	0.16	1.04
No calcium	29	0.38	0.02		29	0.37	0.03					

Treatment group	Baseline			1-year follow-up					Absolute Rx effect			Relative effect <sup>d</sup>
	n	Mean	SE	n	Mean	SE	P	Rx effect <sup>b</sup>	SE	p <sup>c</sup>		
Calcium	33	0.43	0.02	0.07	0.43	0.02	0.05	0.01	0.02	0.49	1.03	
Calcium	39	0.41	0.02		0.42	0.02						
Vitamin D + calcium	36	0.38	0.02	0.15	0.39	0.02	0.34	0.01	0.01	0.31	1.04	
<b><math>\beta</math>-catenin<sup>f</sup> (OD)</b>												
<i>Whole crypts</i>												
No vitamin D	51	10,517	341		10,990	356						
Vitamin D	52	10,728	366	0.68	10,868	370	0.81	-332	402	0.41	0.97	
No calcium	29	10,882	489		10,872	427						
Calcium	32	10,922	420	0.95	11,591	514	0.29	680	588	0.25	1.06	
Calcium	39	10,477	376		11,080	398						
Vitamin D + calcium	35	10,572	454	0.87	10,807	504	0.67	-368	469	0.43	0.97	
<i>Upper 40% of crypts</i>												
No vitamin D	51	3,805	128		4,011	139						
Vitamin D	52	3,915	140	0.56	3,955	137	0.78	-166	153	0.28	0.96	
No calcium	29	3,900	188		3,941	165						
Calcium	32	4,007	173	0.68	4,318	201	0.16	269	227	0.24	1.07	
Calcium	39	3,818	140		4,056	154						
Vitamin D + calcium	35	3,875	175	0.80	3,935	186	0.62	-178	185	0.34	0.96	
<i>Lower 60% of crypts</i>												
No vitamin D	51	6,355	207		6,585	212						
Vitamin D	52	6,423	219	0.82	6,514	227	0.82	-138	248	0.58	0.98	
No calcium	29	6,616	295		6,533	261						
Calcium	32	6,520	237	0.80	6,840	302	0.45	403	359	0.27	1.06	
Calcium	39	6,298	229		6,631	241						
Vitamin D + calcium	35	6,303	269	0.99	6,473	304	0.68	-164	283	0.56	0.98	
<i>Diff<sup>e</sup></i>												
No vitamin D	51	0.36	0.00		0.36	0.00						
Vitamin D	52	0.36	0.00	0.55	0.36	0.00	0.89	0.00	0.01	0.65	0.99	
No calcium	29	0.36	0.01		0.36	0.01						

Treatment group	Baseline			1-year follow-up			Absolute Rx effect			Relative effect <sup>d</sup>		
	n	Mean	SE	P	n	Mean	SE	P	Rx effect <sup>b</sup>		SE	p <sup>c</sup>
Calcium	32	0.37	0.00	0.26	32	0.37	0.00	0.15	0.00	0.01	0.77	1.01
Calcium	39	0.36	0.00		39	0.37	0.00					
Vitamin D + calcium	35	0.37	0.00	0.88	35	0.36	0.00	0.84	0.00	0.01	0.77	1.00
<b>APC/<math>\beta</math>-catenin score<sup>a,e,f</sup></b>												
No vitamin D	51	0.30	0.03		51	0.19	0.01					
Vitamin D	52	0.26	0.02	0.19	52	0.21	0.02	0.66	0.06	0.03	0.02	1.28
No calcium	29	0.29	0.04		29	0.22	0.03					
Calcium	32	0.32	0.03	0.60	32	0.24	0.02	0.56	-0.01	0.03	0.88	1.01
Calcium	39	0.28	0.03		39	0.21	0.02					
Vitamin D + calcium	35	0.27	0.03	0.71	35	0.26	0.03	0.07	0.07	0.03	0.01	1.35
<b>E-cadherin<sup>g</sup> (OD)</b>												
<i>Whole crypts</i>												
No vitamin D	46	4,700	210		46	4,603	193					
Vitamin D	50	4,665	264	0.92	50	4,888	275	0.41	320	343	0.35	1.07
No calcium	27	5,165	398		27	4,753	254					
Calcium	31	4,568	233	0.19	31	4,533	238	0.53	376	365	0.31	1.08
Calcium	34	4,571	217		34	4,562	229					
Vitamin D + calcium	35	4,417	273	0.66	35	4,933	364	0.39	525	411	0.21	1.12
<i>Upper 40% of crypts</i>												
No vitamin D	46	1,727	83		46	1,771	74					
Vitamin D	50	1,740	91	0.92	50	1,842	105	0.59	59	133	0.66	1.03
No calcium	27	1,918	139		27	1,794	88					
Calcium	31	1,708	89	0.20	31	1,784	100	0.94	200	134	0.14	1.12
Calcium	34	1,697	91		34	1,776	89					
Vitamin D + calcium	35	1,627	92	0.59	35	1,850	142	0.67	144	163	0.38	1.09
<i>Lower 60% of crypts</i>												
No vitamin D	46	2,792	126		46	2,641	117					
Vitamin D	50	2,734	169	0.79	50	2,840	162	0.33	257	209	0.22	1.10
No calcium	27	3,043	255		27	2,770	168					

Treatment group	Baseline			1-year follow-up			Absolute Rx effect			Relative effect <sup>d</sup>		
	n	Mean	SE	P	n	Mean	SE	P	Rx effect <sup>b</sup>		SE	p <sup>c</sup>
Calcium	31	2,678	141	0.20	31	2,550	134	0.31	144	240	0.55	1.05
Calcium	34	2,694	124		34	2,590	134					
Vitamin D + calcium	35	2,611	176	0.70	35	2,875	209	0.26	368	244	0.14	1.15
<i>φ</i> <sup>e</sup>												
No vitamin D	46	0.37	0.01		46	0.39	0.01					
Vitamin D	50	0.38	0.01	0.14	50	0.38	0.01	0.18	-0.02	0.01	0.03	0.95
No calcium	27	0.37	0.01		27	0.38	0.01					
Calcium	31	0.37	0.01	0.99	31	0.39	0.01	0.20	0.01	0.01	0.33	1.03
Calcium	34	0.37	0.01		34	0.39	0.01					
Vitamin D + calcium	35	0.37	0.01	0.66	35	0.37	0.01	0.03	-0.02	0.01	0.08	0.95

Abbreviations: SE, standard error; OD, optical density

<sup>a</sup>APC/β-catenin score = APC expression in the upper 40% of crypts/β-catenin expression in the upper 40% of crypts

<sup>b</sup>Rx effect (treatment effect) = [(treatment group follow-up) - (treatment group baseline)] - [(placebo group follow-up) - (placebo group baseline)]

<sup>c</sup>P value for difference between each active treatment group and placebo group from repeated-measures MIXED model

<sup>d</sup>Relative effect = [(treatment group follow-up) / (treatment group baseline)] / [(placebo group follow-up) / (placebo group baseline)]; interpretation similar to that for an odds ratio

<sup>e</sup>φ = proportion of expression in the distribution zone (i.e., ratio of expression in upper 40% to expression in whole crypt)

<sup>f</sup>One subject excluded due to missing values for the measurement of β-catenin expression

<sup>g</sup>Eight subjects excluded due to missing values for the measurement of E-cadherin expression

Expression of APC,  $\beta$ -catenin, E-cadherin, and the APC/ $\beta$ -catenin score<sup>d</sup> in the normal-appearing colorectal mucosa of the adjunct biomarker study participants according to categories of selected risk factors (n = 104)

Table 3

Treatment group	Baseline			1-year follow-up			Absolute Rx effect					
	n	Mean	SE	P	n	Mean	SE	P	Rx effect <sup>b</sup>	SE	P <sup>c</sup>	Relative effect <sup>d</sup>
<b>Non-aspirin NSAID<sup>e</sup> use &lt; 1/week</b>												
<b>APC (OD)</b>												
<i>Upper 40% of crypts</i>												
No vitamin D	34	1,127	104		34	891	73					
Vitamin D	38	946	82	0.17	38	832	89	0.61	122	103	0.24	1.11
No calcium	21	894	127		21	618	91					
Calcium	21	1,347	89	0.01	21	1,106	74	0.00	36	137	0.80	1.19
Calcium	26	1,136	115		26	925	84					
Vitamin D + calcium	25	1,038	102	0.53	25	995	110	0.62	167	123	0.18	1.18
<b><math>\beta</math>-catenin (OD)</b>												
<i>Upper 40% of crypts</i>												
No vitamin D	34	3,715	165		34	4,000	189					
Vitamin D	37	3,801	150	0.70	37	3,956	175	0.86	-130	174	0.46	0.97
No calcium	21	3,634	181		21	3,797	206					
Calcium	20	3,986	201	0.20	20	4,451	282	0.07	302	258	0.25	1.07
Calcium	26	3,834	187		26	4,100	206					
Vitamin D + calcium	24	3,791	208	0.88	24	4,003	248	0.76	-54	226	0.81	0.99
<b>APC/<math>\beta</math>-catenin score<sup>d</sup></b>												
No vitamin D	34	0.32	0.04		34	0.23	0.02					
Vitamin D	37	0.27	0.03	0.29	37	0.23	0.03	0.87	0.04	0.03	0.16	1.15
No calcium	21	0.28	0.05		21	0.18	0.04					
Calcium	20	0.36	0.03	0.19	20	0.27	0.02	0.05	0.01	0.04	0.87	1.15
Calcium	26	0.30	0.03		26	0.23	0.02					
Vitamin D + calcium	24	0.30	0.04	1.00	24	0.28	0.03	0.22	0.05	0.03	0.17	1.21
<b>E-cadherin (OD)</b>												



Treatment group	Baseline			1-year follow-up				Absolute Rx effect			Relative effect <sup>d</sup>
	n	Mean	SE	n	Mean	SE	P	Rx effect <sup>b</sup>	SE	p <sup>c</sup>	
<i>Whole crypts</i>											
No vitamin D	30	4,551	258	30	4,614	256					
Vitamin D	35	4,777	287	35	5,057	370	0.34	218	439	0.62	1.04
No calcium	19	4,900	383	19	4,461	280					
Calcium	20	4,725	302	20	4,831	310	0.38	545	402	0.18	1.12
Calcium	22	4,578	284	22	4,702	325					
Vitamin D + calcium	24	4,579	351	24	5,300	503	0.33	597	560	0.29	1.13
<b>Non-aspirin NSAID<sup>e</sup> use I/week</b>											
<b>APC (OD)</b>											
<i>Upper 40% of crypts</i>											
No vitamin D	17	1,063	174	17	819	145					
Vitamin D	15	887	154	15	1,005	122	0.34	362	160	0.03	1.47
No calcium	8	1,424	243	8	1,323	177					
Calcium	12	987	187	12	776	123	0.02	-110	221	0.62	0.85
Calcium	13	878	176	13	655	131					
Vitamin D + calcium	11	779	169	11	900	148	0.23	345	184	0.07	1.55
<b>β-catenin (OD)</b>											
<i>Upper 40% of crypts</i>											
No vitamin D	17	3,983	198	17	4,032	185					
Vitamin D	15	4,197	309	15	3,952	208	0.78	-294	298	0.33	0.93
No calcium	8	4,598	411	8	4,320	221					
Calcium	12	4,043	330	12	4,096	261	0.55	331	448	0.47	1.08
Calcium	13	3,787	201	13	3,969	219					
Vitamin D + calcium	11	4,060	330	11	3,787	250	0.59	-455	314	0.16	0.89
<b>APC/β-catenin score<sup>d</sup></b>											
No vitamin D	17	0.27	0.04	17	0.20	0.03					
Vitamin D	15	0.22	0.04	15	0.26	0.03	0.28	0.11	0.04	0.02	1.57
No calcium	8	0.34	0.06	8	0.32	0.05					
Calcium	12	0.25	0.05	12	0.19	0.02	0.02	-0.04	0.06	0.47	0.79

Treatment group	Baseline			1-year follow-up			Absolute Rx effect			Relative effect <sup>d</sup>	
	n	Mean	SE	P	n	Mean	SE	P	Rx effect <sup>b</sup>		SE
Calcium	13	0.24	0.05		13	0.16	0.03				
Vitamin D + calcium	11	0.19	0.03	0.41	11	0.24	0.04	0.15	0.13	0.05	0.01
<b>E-cadherin (OD)</b>											
<i>Whole crypts</i>											
No vitamin D	16	4,980	359		16	4,582	290				
Vitamin D	15	4,405	584	0.40	15	4,493	296	0.83	485	542	0.38
No calcium	8	5,794	1,005		8	5,446	482				
Calcium	11	4,283	362	0.13	11	3,991	314	0.02	55	782	0.94
Calcium	12	4,558	343		12	4,306	257				
Vitamin D + calcium	11	4,064	415	0.37	11	4,133	267	0.65	321	516	0.54
<b>Total fat intake &lt; median<sup>f</sup></b>											
<b>APC (OD)</b>											
<i>Upper 40% of crypts</i>											
No vitamin D	26	1,124	124		26	898	96				
Vitamin D	24	959	105	0.32	24	746	95	0.27	13	110	0.91
No calcium	15	1,097	164		15	748	139				
Calcium	14	1,325	112	0.27	14	1,044	96	0.10	68	147	0.65
Calcium	19	1,030	131		19	875	102				
Vitamin D + calcium	16	1,012	143	0.93	16	838	120	0.82	-19	136	0.89
<b>β-catenin (OD)</b>											
<i>Upper 40% of crypts</i>											
No vitamin D	26	3,705	186		26	4,000	219				
Vitamin D	23	3,883	140	0.46	23	4,148	231	0.64	-30	244	0.90
No calcium	15	3,541	228		15	3,755	268				
Calcium	13	4,160	182	0.05	13	4,874	351	0.02	500	357	0.17
Calcium	19	3,728	208		19	4,081	250				
Vitamin D + calcium	15	4,111	145	0.16	15	4,370	302	0.46	-94	328	0.78
<b>APC/β-catenin score<sup>g</sup></b>											
No vitamin D	26	0.33	0.05		26	0.24	0.03				

Treatment group	Baseline			1-year follow-up			Absolute Rx effect			Relative effect <sup>d</sup>		
	n	Mean	SE	P	n	Mean	SE	P	Rx effect <sup>b</sup>		SE	P <sup>c</sup>
Vitamin D	23	0.25	0.03	0.14	23	0.19	0.02	0.17	0.03	0.04	0.44	1.02
No calcium	15	0.35	0.07		15	0.22	0.05					
Calcium	13	0.33	0.03	0.82	13	0.24	0.03	0.80	0.03	0.05	0.47	1.13
Calcium	19	0.29	0.04		19	0.22	0.03					
Vitamin D + calcium	15	0.24	0.04	0.40	15	0.20	0.03	0.68	0.03	0.04	0.45	1.10
<b>E-cadherin (OD)</b>												
<i>Whole crypts</i>												
No vitamin D	24	4,412	308		24	4,615	254					
Vitamin D	22	4,771	341	0.44	22	4,862	260	0.50	-112	379	0.77	0.97
No calcium	13	5,071	614		13	4,923	438					
Calcium	14	4,302	234	0.24	14	4,689	334	0.67	534	488	0.28	1.12
Calcium	17	4,219	270		17	4,419	275					
Vitamin D + calcium	16	4,575	315	0.40	16	4,913	245	0.19	138	409	0.74	1.03
<b>Total fat intake median<sup>f</sup></b>												
<b>APC (OD)</b>												
<i>Upper 40% of crypts</i>												
No vitamin D	22	1,077	139		22	805	96					
Vitamin D	29	905	101	0.31	29	993	104	0.20	360	137	0.01	1.47
No calcium	12	961	187		12	868	142					
Calcium	18	1,127	144	0.48	18	927	103	0.74	-108	193	0.58	0.91
Calcium	19	1,057	154		19	774	110					
Vitamin D + calcium	20	916	113	0.46	20	1,069	124	0.08	435	145	0.00	1.59
<b>β-catenin (OD)</b>												
<i>Upper 40% of crypts</i>												
No vitamin D	22	3,836	187		22	3,999	192					
Vitamin D	29	3,941	227	0.73	29	3,801	162	0.43	-303	192	0.12	0.93
No calcium	12	4,247	295		12	4,172	199					
Calcium	18	3,879	279	0.39	18	3,897	211	0.38	92	297	0.76	1.02
Calcium	19	3,881	201		19	4,000	199					

Treatment group	Baseline			1-year follow-up			Absolute Rx effect			Relative effect <sup>d</sup>		
	n	Mean	SE	P	n	Mean	SE	P	Rx effect <sup>b</sup>		SE	P <sup>c</sup>
Vitamin D + calcium	20	3,698	283	0.60	20	3,608	213	0.19	-209	205	0.31	0.95
<b>APC/<math>\beta</math>-catenin score<sup>a</sup></b>												
No vitamin D	22	0.28	0.03		22	0.20	0.02					
Vitamin D	29	0.26	0.04	0.76	29	0.28	0.03	0.07	0.09	0.04	0.02	1.47
No calcium	12	0.24	0.05		12	0.22	0.04					
Calcium	18	0.31	0.05	0.28	18	0.24	0.03	0.63	-0.05	0.05	0.33	0.85
Calcium	19	0.27	0.04		19	0.19	0.02					
Vitamin D + calcium	20	0.27	0.05	0.82	20	0.31	0.04	0.01	0.11	0.04	0.01	1.57
<b>E-cadherin (OD)</b>												
<i>Whole crypts</i>												
No vitamin D	19	5,007	315		19	4,569	341					
Vitamin D	28	4,582	393	0.44	28	4,908	451	0.58	764	603	0.21	1.17
No calcium	12	5,325	626		12	4,633	320					
Calcium	16	4,739	398	0.42	16	4,341	356	0.56	293	580	0.62	1.05
Calcium	16	4,883	343		16	4,661	392					
Vitamin D + calcium	19	4,284	434	0.30	19	4,950	647	0.72	888	722	0.23	1.21

Abbreviations: SE, standard error; OD, optical density

<sup>a</sup>APC/ $\beta$ -catenin score = APC expression in the upper 40% of crypts/ $\beta$ -catenin expression in the upper 40% of crypts

<sup>b</sup>Rx effect (treatment effect) = [(treatment group follow-up) - (treatment group baseline)] - [(placebo group follow-up) - (placebo group baseline)]

<sup>c</sup>P value for difference between each active treatment group and placebo group from repeated-measures MIXED model.

<sup>d</sup>Relative effect = [(treatment group follow-up) / (treatment group baseline)] / [(placebo group follow-up) / (placebo group baseline)]; interpretation similar to that for an odds ratio

<sup>e</sup>Nonsteroidal anti-inflammatory drug

<sup>f</sup>Sex-specific median for total fat intake: 61.25 g/d for men, and 47.00 g/d for women