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Pilot investigation of treatment effect modification by vitamin D binding protein isoforms on calcium and vitamin D effects on biomarkers of risk for colorectal cancer in the normal-appearing colorectal mucosa of colorectal adenoma patients

By

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Master of Science in Public Health

Epidemiology

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Roberd M. Bostick, M.D., M.P.H.

Advisor

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B.S., Georgia College and State University, 2010

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Abstract

Pilot investigation of treatment effect modification by vitamin D binding protein isoforms on calcium and vitamin D effects on biomarkers of risk for colorectal cancer in the normal-appearing colorectal mucosa of colorectal adenoma patients

By Patrick Rago

Vitamin D exposure and/or calcium intake associations with colorectal adenoma, incident colorectal cancer (CRC), and CRC survival, and supplemental vitamin D_3 and/or calcium effects on the expression of COX-2 and 15-PGDH (biomarkers of inflammation) in the morphologically-normal colorectal mucosa of sporadic colorectal adenoma patients were reported to be stronger among individuals with the vitamin D binding protein (VDP)-2 isoform, encoded by *GC* rs4588*CA or AA. The expression of proteins (APC, β-catenin, E-cadherin, MSH2) encoded by colorectal carcinogenesis pathway genes, cell cycle biomarkers (mib-1, p21, bcl-2, bax), and transforming growth factors (TGF-α, TGF-β_1) in the morphologically-normal colorectal mucosa were estimated to be associated with colorectal adenoma and to be modifiable by supplemental vitamin D_3 and/or calcium. However, whether these agents' effects on these biomarkers differ by VDP isoform is unknown. To address this, using mixed linear models, we analyzed data on the expression of these 10 biomarkers (measured using automated immunohistochemistry and quantitative image analysis at baseline and 1-year follow-up) in rectal biopsies from a subset of 62 sporadic colorectal adenoma patients in a previously-conducted, placebo-controlled, chemoprevention trial of supplemental calcium (1,200 mg/day) and/or vitamin D_3 (800 I.U./day) , overall and stratified by VDP isoforms (no VDP-2 [GC rs4588*CC] vs. VDP-2 [GC rs4588*CA or AA]). The findings from this pilot study were not statistically significant; however, for all active treatments relative to placebo, among those with the VDP-2 isoform, whole crypt MSH2, mib-1, bax, and bcl-2 expression was estimated to decrease and the p21/mib-1 ratio (in the upper 40%, or differentiation zone, of crypts) was estimated to increase more, whereas among those without the VDP-2 isoform, whole crypt E-cadherin expression increased more. Estimated changes in whole crypt APC, β-catenin, and TGF-β_1 expression did not substantially differ by VDP isoform. These pilot study results suggest that supplemental vitamin D_3 and/or calcium effects on the expression of multiple biomarkers of risk for colorectal neoplasms in the morphologically-normal colorectal mucosa of colorectal adenoma patients may depend on someone's VDP isoform (with effects generally stronger among VDP-2 isoform individuals), and supports a larger trial to test this hypothesis.

Pilot investigation of treatment effect modification by vitamin D binding protein isoforms on calcium and vitamin D effects on biomarkers of risk for colorectal cancer in the normal-appearing colorectal mucosa of colorectal adenoma patients

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Introduction

Colorectal cancer (CRC) is the second leading cause of cancer death in the United States[1]. Strong biological plausibility and basic science research along with consistent epidemiologic findings support that calcium and vitamin D may reduce risk for colorectal carcinogenesis[2-4]. Hypotheses for the action of calcium supplements reducing risk for colorectal carcinogenesis include that calcium binds to bile and fatty acids in the gut lumen and thereby prevents their toxic effects which promote inflammatory responses responsible for promoting tumor formation[5]. Similarly, vitamin D promotes the degradation of bile acids but also regulates multiple inflammatory pathways that potentially promote colorectal carcinogenesis[5].

Variations in the *GC* gene, which codes for the vitamin D-binding protein (VDP), were associated with plasma 25-OH-vitamin D (25[OH]D) concentrations[6]. Nonsynonymous single nucleotide polymorphisms (SNPs) at rs4588 and rs7041 in exon 11 of the *GC* gene were associated with statistically significant differences in circulating 25[OH]D concentrations[6,7]. Variations in these SNPs code for different protein isoforms of the VDP. GC-1f, GC-1s, GC-2 (also known as VDP-1f, VDP-1s, and VDP-2) are different VDP isoforms, and have different binding affinities for 25(OH)D[6]. The VDB-2 isoform is coded by rs4588*CA and rs4588*AA which yield one or two copies, respectively[7]. The VDP-1 isoform is coded by rs4588*CC[6,7]. Vitamin D exposure and/or calcium intake associations with colorectal adenoma, incident CRC, and CRC survival were reported to be stronger among individuals with the VDP-2 isoform [8-10]. Furthermore, supplemental vitamin D_3 and/or calcium effects on the expression of COX-2 and 15-PGDH (biomarkers of inflammation) in the morphologically-normal colorectal mucosa of sporadic colorectal adenoma patients in a chemoprevention trial were reported to be stronger among individuals with the VDP-2 isoform[11].

Many proteins may become dysregulated during colorectal carcinogenesis[12-15]. The expression of these biomarker proteins can be assessed through immunohistochemical analysis of biopsied colorectal tissue[16,17]. The expression of proteins APC, β-catenin, and E-cadherin (involved in the APC colon carcinogenesis pathway), MSH2 (repairs post-replication DNA mismatches), mib-1 (a biomarker of proliferation), p21 (a biomarker of differentiation), bcl-2 (inhibits apoptosis), bax (promotes apoptosis), TGF-α (promotes cell growth) and TGF-β_1 (inhibits cell growth) in the morphologically-normal colorectal mucosa were estimated to be associated with colorectal adenoma and to be modifiable by supplemental vitamin D_3 and/or calcium[18-28]. However, whether calcium and/or vitamin D_3 supplementations effects on these biomarkers differ by VDP isoform is unknown.

Accordingly, we investigated whether VDP isoforms may modify the effects of supplemental calcium and/or vitamin D_3 on the above-described biomarkers of risk in the normal-appearing rectal mucosa of colorectal adenoma patients enrolled in a chemoprevention trial. We hypothesized that the supplemental calcium and vitamin D_3, separately and combined, would most strongly affect the expression of the 10 biomarkers among those with the VDP-2 isoform.

Methods

Study participants

Participants in this adjunct biomarker study (n=62) were recruited from a larger parent study an 11-center, randomized, double-blind, placebo-controlled, partial 2x2 factorial chemoprevention clinical trial (n=2259) of the effects of supplemental calcium (600 mg of elemental calcium in the form of calcium carbonate twice daily, orally), vitamin D (500 IU of vitamin D_3 twice daily, orally), or both over 3-5 years on colorectal adenoma recurrence[29]. The

parent study also included a 2-arm study of women who were already prescribed supplemental calcium and so were randomized to vitamin D or vitamin D placebo pills. Eligibility criteria for the parent study were previously published[29]. Briefly, participants were 45-75 years of age, had at least one colorectal adenoma removed within 120 days of enrollment with no remaining polyps after a complete colonoscopy, and were scheduled to undergo a 3-year or 5-year follow-up colonoscopy[29]. Participants also must have been in good general health and not have familial colorectal cancer syndromes (e.g., familial adenomatous polyposis) or serious intestinal disease, and have no contraindications to or medical requirements for supplemental calcium or vitamin D[29]. After the parent trial had begun, patients for the adjunct biomarker study were recruited from the parent study, without knowledge of their treatment assignment, from two of the 11 centers (Columbia, SC and Atlanta, GA). Further exclusions for the adjunct biomarker study were medical indications for taking aspirin within 7 days prior to biopsy, history of a bleeding disorder, and current use of an anticoagulant medication. At enrollment, each participant provided baseline information, including medical history, medication/supplement use, demographics, lifestyle, and diet with a Block Brief 2000 food frequency questionnaire (NutritionQuest)[29]. Serum calcium and 25(OH)D concentrations were measured at baseline, and 25(OH)D was measured again at the 1-year follow-up visit[29]. During the trial, participants agreed to refrain from taking additional vitamin D or calcium supplements[29]. Those who desired a multi-vitamin supplement were provided one that did not contain vitamin D or calcium[29].

Biopsy procurement and immunohistochemistry protocol for biomarker detection

Six ~1 mm-thick biopsies obtained from participants, without any preceding bowel-cleansing preparation, at baseline and one-year follow-up were taken from morphologically-normal rectal mucosa 10 cm above the external anal aperture and at least 4 cm from any polypoid lesions. 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. Within a week, biopsies were processed and embedded in paraffin blocks. For each biomarker, five slides with three levels of 3 µm-thick biopsy sections taken 40 µm apart were prepared for each participant, yielding a total of 15 levels. Heat-mediated antigen retrieval was used to break the protein cross-links formed by formalin to uncover the epitope of interest. To accomplish this, slides were placed in a preheated Pretreatment Module (Lab Vision Corp.) with 100 × Citrate Buffer (pH 6.0; DAKO S1699, DAKO Corp.; further called DAKO) and steamed for 40 min. After antigen retrieval, slides were placed in a DAKO Automated Stainer (DAKO) and rinsed with warm Pretreatment Module Buffer. The Autostainer was programmed for each immunohistochemistry run and the reagents used were as follows. For mib-1: antibody ID-AB_2142367; antibody name-Ki-67 antibody; clone ID-mib-1; clonality-monoclonal; target antigen-human recombinant peptide corresponding to a 1002 bp Ki-67 cDNA fragment; vendor/catalog number-DAKO M7240; and host organism-mouse. For p21: antibody ID-AB_2077700; antibody name-p21 antibody; clone ID-SX118; clonality-monoclonal; target antigen-CDKN1A human; vendor/catalog number-DAKO M7202; and host organism-mouse. For bcl-2: antibody ID-AB_626733; antibody name-Bcl-2 (100) antibody; clone ID-100; clonality-monoclonal; target antigen-human bcl-2; vendor/catalog number-Santa Cruz Biotechnology sc-509; and host organism-mouse. For bax: antibody name-rabbit anti-human bax antibody; clonality-polyclonal; target antigen-synthetic peptide corresponding to amino acids 43-61 of the human bax protein. For MHS2: antibody K3438, and TBS buffer (DAKO S1968, DAKO). For TGF-α, we applied anti-TGF-α (Calbiochem GF10) at a concentration of 1:100 in Antibody Diluent (Dako S0809). For TGF-β1, we applied anti-TGF-β1 (Santa Cruz sc-146) at a concentration of 1:75 in Background Reducing Antibody Diluent (Dako S3022). For APC: (Calbiochem, OP80; 1:70 dilution). For β-catenin: (Trans-duction Laboratories 610154; 1:300 dilution), and for E-cadherin

(Zymed 33-4000; 1:50 dilution. The slides were not counterstained. After staining, the slides were cover slipped automatically with a Leica CV5000 Coverslipper (Leica Microsystems, Inc.) and placed in opaque slide folders. In each staining batch of slides, positive and negative control slides were included. A surgical specimen of normal colon tissue was used as a control tissue for these biomarkers. The control tissue was processed in the same manner as the participants' tissue and the negative and the positive control slides were treated identically to the patient's slides except that antibody diluent was used rather than primary antibody on the negative control slide. For each participant, for each biomarker, baseline and 1-year follow-up biopsy slides were included in the same immunohistochemistry batch.

Quantification of detected biomarkers ('scoring' protocol)

Biomarker expression was quantified as the optical density of the labeling of each immunohistochemically-detected protein biomarker using custom-developed quantitative image analysis software, CellularEyes (DivEyes LLC), and a validated scoring protocol described previously[13]. Briefly, a technician blinded to treatment assignment systematically reviewed each slide and selected only "scorable" hemicrypts, defined as one side of a crypt bisected from base to colon lumen and extending intact from the muscularis mucosa to the colon lumen. Each section on the slide was viewed sequentially with the aim of identifying and analyzing at least 16, but no more than 40, scorable hemicrypts per patient per biomarker. The image-analysis program divided the outlined area into 50 equally-spaced segments of approximately average normal colonocyte width and measured the background-corrected biomarker labeling optical density within the entire hemicrypt and within each segment. The resulting data were automatically transferred into a MySQL database (Sun Microsystems, Inc.). Using this protocol, we outlined approximately 2,220 hemicrypts per biomarker (a mean of 18 per patient-visit).

To assess intrareader scoring reliability, slides previously analyzed by the technician were re-scored at intervals during the course of the study. Intraclass correlation coefficients for scoring reliability were >0.90 for each biomarker.

Statistical methods

We summarized and compared the baseline characteristics of the participants across the treatment groups using descriptive statistics and the chi square test for categorical variables and ANOVA for continuous variables (transformed by the natural logarithm to achieve a normal distribution, when indicated).

We estimated treatment effects by comparing changes in biomarker expression from baseline to 1-year follow-up in each treatment group relative to placebo using generalized linear mixed models with an unstructured correlation matrix (PROC MIXED, SAS 9.4). Models included a random intercept and fixed effects for visit (baseline and 1-year follow-up), treatment group, and a treatment-by-visit interaction term. Prior to running these models, 1) for each participant, for each biomarker, we excluded biomarker values on any visit at which the participant had less than six scorable hemicrypts; and 2) we transformed biomarker values that were not normally distributed by the natural logarithm. To assess potential confounding, identified based on biological plausibility and imbalances of participant characteristics across treatment groups at baseline, we ran additional models that included physical activity (metabolic equivalents of task [MET]-minutes/week), dietary fiber per 1000 Kcal consumed per day, and dietary calcium per 1000 Kcal per day as covariates. Adjustment for these potential confounding variables did not materially affect the estimated treatment effects and, thus, were not included in the final models. Finally, we stratified all final models by *GC* rs4588 genotype (CC *vs.* AC/AA; the A allele encodes the functional vitamin D-binding protein 2 [VDP-2] isoform).

For the biomarkers that were not natural logarithm-transformed, we took the absolute treatment effect, 95% confidence intervals (95% CI), and associated *P*-values directly from the SAS Proc Mixed output. We then hand-calculated the relative treatment effect as the mean active treatment value at follow-up divided by the mean active treatment value at baseline divided by the mean placebo value at follow-up divided by the mean placebo value at baseline. The interpretation of the relative effect is like that of an odds ratio: for example, a value of 0.8 could be interpreted as a 20% decrease in biomarker expression in the active treatment group relative to the placebo group after 1 year.

For the biomarkers that were natural logarithm-transformed, for the relative treatment effect, we exponentiated the values directly from the SAS Proc Mixed output. The 95% CI and the *P*-values were taken directly from the output and are associated with the relative treatment effect for these biomarkers. We then hand-calculated the absolute treatment effect as the average value in the active treatment group at follow-up minus the average value in the active treatment group at baseline minus the average value in the placebo group at follow-up minus the average value in the placebo group at baseline.

Results

Baseline characteristics

The baseline characteristics of the 62 study participants, by treatment arm, are summarized in **Table 1**. The average age of the participants was 59 years, and 77% were male and 82% were White. The participant characteristics were balanced across treatment groups except for physical activity and dietary fiber intake, both of which were, on average, lower in the placebo group.

Mib-1

Overall, mib-1 expression in whole crypts was not estimated to change substantially in any of the active treatment groups relative to placebo (**Supplemental Table 1**). However, as shown in **Table 2**, among VDP2 individuals, mib-1 expression was estimated to decrease in all three active treatment groups relative to placebo, whereas among non-VDP2 individuals it did not ($P_{interaction}$ = 0.21). More specifically, among VDP2 individuals, mib-1 expression was estimated to decrease 17% (*P*=0.46), 22% (*P*=0.33), and 37% (*P*=0.08) in the calcium, vitamin D, and calcium + vitamin D groups, respectively, relative to the placebo group. The corresponding findings for those who did not have a VDP2 isoform were for estimated increases of 13%, 6%, and 39%; none of these estimates was statistically significant.

Bcl-2

Overall, bcl-2 expression in whole crypts was estimated to decrease 24% (*P*=0.18) in the calcium group relative to placebo, but it was not estimated to decrease substantially in the vitamin D and calcium + vitamin D groups (Supplemental Table 1). However, as shown in Table 2, among VDP2 individuals, bcl-2 expression was estimated to decrease in all three active treatment groups relative to placebo, whereas among non-VDP2 individuals the estimated changes were closer to null ($P_{interaction}$ = 0.86). More specifically, among VDP2 individuals, bcl-2 expression was estimated to decrease 38% (*P*=0.27), 28% (*P*=0.42), and 15% (*P*=0.70) in the calcium, vitamin D, and calcium + vitamin D groups, respectively, relative to the placebo group. Among those who did not have a VDP2 isoform, bcl-2 expression was estimated to decrease of 17% and 7% in the calcium and calcium + vitamin D groups, respectively, but increase 11% in the vitamin D group; none of these estimates was statistically significant.

Bax

Overall, bax expression in whole crypts was estimated to decrease in all active treatment groups relative to placebo (Supplemental Table 1). However, as shown in Table 2, the estimated decreases were stronger among VDP2 individuals ($P_{interaction}$ = 0.33). More specifically, among VDP2 individuals, bax expression was estimated to decrease 78% (*P*=0.02), 77% (*P*=0.02), and 58% (*P*=0.15) in the calcium, vitamin D, and calcium + vitamin D groups, respectively, relative to the placebo group. The corresponding findings for those who did not have a VDP2 isoform were for estimated decreases of 21% (*P*=0.37), 27% (*P*=0.31), and 30% (*P*=0.40).

p21/mib-1 ratio

Overall, the ratio of the expression of p21 to mib-1 in the upper 40% (differentiation zone) of crypts was estimated to decrease in the calcium and vitamin D groups relative to placebo, but not change substantially in the calcium + vitamin D group (Supplemental Table 1). However, as shown in Table 2, among VDP2 individuals, the p21/mib-1 ratio expression ratio was estimated to increase in all three active treatment groups relative to placebo, whereas among non-VDP2 individuals it was estimated to decrease in all three treatment groups ($P_{interaction}$ = 0.07). More specifically, among VDP2 individuals, the p21/mib-1 ratio expression ratio was estimated to increase 19% (*P*=0.85), 60% (*P*=0.60), and 331% (*P*=0.12) in the calcium, vitamin D, and calcium + vitamin D groups, respectively, relative to the placebo group. The corresponding findings for those who did not have a VDP2 isoform were for estimated decreases of 55%, 41%, and 45%; none of these estimates was statistically significant.

TGF-α

Overall, TGF-α expression in whole crypts was not estimated to change substantially in any of the active treatment groups relative to placebo (Supplemental Table 1). However, as shown in Table 2, among those with the VDP2 isoform, TGF-α expression was estimated to decrease in all three active treatment groups relative to placebo, but not estimated to change substantially among non-VDP2 individuals ($P_{interaction}$ = 0.06). More specifically, TGF-α expression among VDP2 individuals decreased 24% (*P*=0.09), 36% (*P*=0.01), and 19% (*P*=0.17) in the calcium, vitamin D, and calcium + vitamin D groups respectively, relative to the placebo group.

TGF-β_1

The results for TGF-β expression in whole crypts, overall (Supplemental Table 1) and by VDP isoform (Table 2) were mixed. More specifically, among those with the VDP2 isoform, TGF-β_1 expression was estimated to increase 30% (*P*=0.75) in the calcium group, decrease 28% (*P*=0.68) in the vitamin D group, and not change substantially in the calcium + vitamin D group. Among those without the VDB2 isoform, TGF-β_1 expression was estimated to decrease 43% (*P*=0.29) and 26% (*P*=0.59) in the calcium and calcium + vitamin D groups, respectively, but increase 98% (*P*=0.24) in the vitamin D group relative to the placebo group.

APC

Overall, APC expression in whole crypts was not estimated to change substantially in any of the active treatment groups relative to placebo (**Supplemental Table 2**). In the analyses stratified by VDP isoform, the findings, none of which were statistically significant, were mixed (**Table 3**) ($P_{interaction}$ = 0.30). The estimated APC changes in the calcium group were close to null regardless of VDP isoform. For the vitamin D group, APC expression was estimated to decrease 20% (*P*=0.64) among those with the VDP2 isoform but increase 26% (*P*=0.14) among those

without the VDP2 isoform. For the calcium + vitamin D group, APC expression was estimated to increase 17% (*P*=0.72) among those with the VDP2 isoform, but not change among those without the VDP2 isoform.

β-catenin

β-catenin expression in whole crypts was not estimated to change substantially in any of the active treatment groups relative to placebo overall (Supplemental Table 2) or by VDP isoform (Table 3).

E-cadherin

E-cadherin expression in whole crypts was not estimated to change substantially in any of the active treatment groups relative to placebo overall (Supplemental Table 2), with the possible exception of an estimated increase of 11% (*P*=0.35) in the calcium + vitamin D group. However, as shown in Table 3, among VDP2 individuals, E-cadherin expression was estimated to decrease in the calcium and vitamin D groups, but not change substantially in the calcium + vitamin D group relative to placebo, whereas among non-VDP2 individuals it was estimated to increase in all three treatment groups ($P_{\text{interaction}}$ = 0.47). More specifically, among VDP2 individuals, E-cadherin expression was estimated to decrease 20% (*P*=0.37) and 22% (*P*=0.24) in the calcium, and vitamin D groups respectively, relative to the placebo group. Among those who did not have a VDP2 isoform, E-cadherin expression was estimated to increase 17% (*P*=0.23), 16% (*P*=0.28), and 17% (*P*=0.22) in the calcium, vitamin D, and calcium + vitamin D groups, respectively.

MSH2

MSH2 expression in whole crypts was estimated to decrease comparable amounts (20 – 26%) overall (Supplemental Table 2) and by VDP isoform (Table 3) in the calcium group relative to placebo. MSH2 expression was also estimated to decrease modestly (11%) in the vitamin D group overall, more strongly (25%) among those with the VDP2 isoform, but not change substantially among those without the VDP2 isoform. Among those in the calcium + vitamin D group, MSH2 expression was estimated to decrease (16%) among those with the VDB2 isoform, but not change substantially overall or among those without the VDP2 isoforms. Only the estimates for a 21% decrease in the calcium group overall, a 26% decrease in the calcium group among those without the VDP2 isoform, and a 25% decrease in the vitamin D group among those with the VDP2 isoform were statistically significant.

Discussion

The results from this pilot study suggest that the effects of calcium and/or vitamin D on the expression of seven of the 10 investigated biomarkers of risk for colorectal neoplasms in the morphologically-normal colorectal mucosa of sporadic colorectal adenoma patients may be modified by VDP isoform. Of the seven for which potential treatment effect modification was suggested, the estimated effects of the study agents were stronger among patients with the VDP2 isoform on six of the biomarkers: mib-1, bcl-2, bax, the p21/mib-1 ratio, MSH2, and TGF-α. Few of the results of this small, preliminary study were statistically significant; however, the pattern of stronger estimated treatment effects on most of the biomarkers among those with the VDP2 isoform is consistent with previous findings of stronger calcium and/or vitamin D treatment effects on COX-2 and 15-HPDG expression in the morphologically normal colorectal mucosa of sporadic adenoma patients, and stronger associations of calcium and vitamin D with

colorectal adenoma, incident colorectal cancer, and colorectal cancer survival among those with the VDP2 isoform. Thus, as further discussed below, our pilot results support further investigation, in larger studies, of the effects of calcium and/or vitamin D on biomarkers of risk for colorectal neoplasms.

Plausibility for calcium and vitamin D as chemopreventive agents against colorectal carcinogenesis

As reviewed elsewhere, considerable biological plausibility, basic science, and epidemiologic evidence supports that calcium and vitamin D reduce risk for colorectal neoplasms[17]. Briefly, a proposed mechanism by which calcium may prevent colorectal carcinogenesis is by binding to unconjugated bile acids and free fatty acids, thus diminishing their toxic effects on the colorectum[5,17,30]. Biomarker studies suggest that calcium may also influence different cell signaling pathways and reduce cell proliferation and promote cell differentiation[17,31]. Experimental models and in vitro studies suggest that 1,25-dihydroxyvitamin D_3 may control cell growth by reducing proliferation and inducing differentiation and apoptosis[17,32]. Vitamin D may also improve innate and acquired immune function, inhibit angiogenesis, reduce inflammation, and regulate microRNA expression[17,32].

Epidemiologic evidence for calcium and vitamin D in reducing risk for colorectal neoplasms

Data from numerous observational studies—especially from prospective cohort studies—solidly supports the hypotheses that higher calcium intakes reduce risk for colorectal cancer. Of 20 cohort studies, 18 (90%) found inverse associations of which eight were statistically significant, and two found direct associations of which none were statistically significant [33]. A pooled analysis of 10 cohort studies from five countries found statistically

significant 22% lower risk for incident colorectal cancer among those who consumed the highest *vs.* the lowest levels of calcium [34].

There have been seven clinical trials of calcium and adenoma recurrence, two of which had large sample sizes, and one major trial of colorectal cancer prevention [33]. In a US multi-center, randomized, double-blind, placebo-controlled clinical trial (n = 913) of calcium supplementation (1,200 mg of elemental calcium daily) and adenoma recurrence (the Calcium Polyp Prevention Study), the relative risk (RR) for any recurrence of adenoma was 0.85 (95% confidence interval [95% CI] 0.74-0.98) and for advanced adenomas, 0.46 (95% CI 0.26-0.83) [35]. In a smaller trial (n = 665), the European Cancer Prevention Organization Intervention Study, there was a statistically non-significant reduction in adenoma recurrence (RR 0.66, 95% CI 0.38-1.17) among those randomized to 2,000 mg of elemental calcium daily relative to placebo [36]. When the results of all seven adenoma recurrence trials were combined in a meta-analysis, the summary RR was 0.80 (95% CI 0.68-0.93) [37]. Finally, in a Women's Health Initiative randomized, double-blind, placebo-controlled clinical trial, 36,282 postmenopausal women were randomized to 1,000 mg of elemental calcium plus 400 IU (10 µg) of vitamin D *vs*. placebo over an average of seven years. There was no evidence for a reduction in the incidence of invasive colorectal cancers (RR 1.08, 95% CI 0.86-1.34) [38]. However, because of the low pill-taking adherence in the active treatment group (only 60% took 80% or more of their pills) and the high rate of drop-in in the placebo group (69% took calcium and vitamin D supplements on their own resulting in intakes twice that of the national averages), the low doses administered, and the short length of follow-up for such a downstream endpoint, the interpretation of these results is problematic.

An important advance in investigating an association of vitamin D exposure with risk for colorectal neoplasms was the recognition of 25-OH-vitamin D blood concentrations as the most accurate indicator of total vitamin D exposure. Circulating 25-OH-vitamin D concentrations

reflect vitamin D from sunlight exposure, which provides 90 – 95% of vitamin D in most people, plus dietary and supplemental intakes [33]. The results from observational epidemiologic studies of colorectal cancer or adenomas that estimated vitamin D exposure from circulating 25-OH-vitamin D concentrations are quite consistent with there being an inverse association of vitamin D exposure with colorectal neoplasms. In a pooled analysis of three case-control studies (pooled n = 616 cases and 770 controls) of incident, sporadic colorectal adenoma, those in the highest quartile of circulating 25-OH-vitamin D_3 concentrations were at a statistically significant, approximately 40% lower risk of adenoma [39]. Of seven other observational studies of 25-OH-vitamin D and colorectal adenoma, six found inverse associations, among which three were statistically significant [40]. Of nine prospective cohort studies that investigated associations of circulating 25-OH-vitamin D and incident colorectal cancer, seven found inverse associations, of which two were statistically significant; when the data from all seven were combined in a meta-analysis the estimated relative risk for those in the upper relative to the lower quantiles of 25-OH-vitamin D was 0.66 (95% CI 0.54 – 0.81) [41]. This consistency for the 25-OH-vitamin D blood concentration studies may be remarkable given the low 25-OH-vitamin D blood concentrations in the studies (mean concentrations in controls were generally less than 32 ng/ml (80 nmol/L)—now considered by many to be the lower limit for vitamin D sufficiency [42-44]). However, given the relatively small number of studies that assessed 25-OH-vitamin D blood concentrations, the "consistency" among the studies should still be viewed as only suggestive. Currently, the only published peer-reviewed report of clinical trials of vitamin D and colon cancer is that of the Women's Health Initiative trial discussed in the previous paragraph.

Plausibility of VDP isoforms as a potential modifier of calcium and/or vitamin D effects

VDP isoforms as a potential modifier of calcium and/or vitamin D effects or associations with colorectal cancer-related outcomes has been previously reported[11]. Relative to the VDP1

isoform, the VDP2 isoform was found to have approximately 2- to 4- fold lower binding affinity for 25(OH)D, which leads to higher circulating 25(OH)D concentrations among those with the VDP2 isoforms[45]. Because those with the VDP2 isoform have lower circulating VDP concentrations, there is a smaller reservoir of stored 25(OH)D; therefore, individuals with this isoform require consistent and high intakes of vitamin D to maintain concentrations 25(OH)D that are bioavailable and bioactive[10]. Interactions between calcium supplementation and VDP2 have not been reported. However, those with VDP2 isoforms have lower VDP concentrations, which may increase parathyroid hormone secretion and calcium absorption, which may lead to stronger calcium effects on biomarkers among individuals with this isoform[11].

Epidemiologic evidence for VDP isoforms as a potential modifier of calcium and/or vitamin D in relation to colorectal neoplasms

VDP isoforms have been reported to modify associations of calcium and/or vitamin D with colorectal adenoma, incident colorectal cancer, and colorectal cancer survival. In a case-control study of incident, sporadic colorectal adenoma, among individuals of European ancestry, 25(OH)D concentrations of 50 or greater relative to less than 50 nmol/L were inversely associated with colorectal adenoma among those with VDP2 (OR = 0.51, CI = 0.33 – 0.81), but not among those without VDP2 (OR = 1.11, CI = 0.68 – 1.92) ($P_{\text{interaction}} = 0.05$)[10]. In a pooled analysis of three prospective cohort studies, the association of 25(OH)D concentrations with CRC risk differed by VDP2: 25(OH)D concentrations considered sufficient (≥50 nmol/L), relative to deficient (<30 nmol/L), were associated with a 53% lower CRC risk among individuals with the VDP2 isoform (RR = 0.47, 95% CI: 0.33 to 0.67), but a non-statistically significant 12% lower risk among individuals without it (RR = 0.88, 95% CI: 0.61 to 1.27) ($P_{heterogeneity} = 0.01$)[9]. Associations of pre-diagnostic 25(OH)D concentrations with mortality according to VDP2 isoform were investigated among 1,281 CRC cases (635 deaths, 483 from CRC) from two large prospective

cohorts conducted in the United States (Cancer Prevention Study-II) and Europe (European Prospective Investigation into Cancer and Nutrition)[46]. In the pooled analysis, multivariable-adjusted hazard ratios (HRs) for CRC-specific mortality associated with deficient relative to sufficient 25(OH)D concentrations were 2.24 (95% CI 1.44–3.49) among cases with the VDP2 isoform, and 0.94 (95% CI 0.68–1.22) among cases without VDP2 ($P_{interaction}$ = 0.0002)[46]. The corresponding HRs for all-cause mortality were 1.80 (95% CI 1.24–2.60) among those with VDP2, and 1.12 (95% CI 0.84–1.51) among those without VDP2 ($P_{interaction}$ = 0.004)[46].

VDP isoforms have also been reported to modify the effects of calcium and/or vitamin D on 25(OH)D concentrations, colorectal adenoma recurrence, and two pre-neoplastic biomarkers of risk for colorectal neoplasms[11,47,48]. In the parent randomized clinical trial (n=2,259) to the present study, the effects of vitamin D supplementation on increasing 25(OH) vitamin D blood concentrations and the effect of reducing colorectal adenoma recurrence risk were stronger and in a dose-response pattern for each VDP2-encoding *GC* rs4588*A allele inherited[47,48]. Specifically, the effect of vitamin D supplementation on adenoma risk was statistically significantly lower by 18% with each rs4588*A allele inherited (interaction relative risk = 0.82, 95% CI 0.69-0.98, $P_{\text{interaction}}$ = 0.03)[48]. Finally, in the same chemoprevention trial population as in the present study, among all 62 participants after 1 year of treatment, the mean COX-2/15-HPGD expression ratio in full-length crypts proportionately decreased 47% in the vitamin D group (*P* = 0.001), 46% in the calcium group (*P* = 0.002), and 34% in the calcium + vitamin D group (*P* = 0.03), relative to the placebo group[11]. However, among individuals with the functional vitamin D-binding protein isoform VDP2 (*GC* rs4588*A), the COX-2/15-HPDG ratio decreased 70% (*P* = 0.0006), 75% (*P* = 0.0002), and 60% (*P* = 0.006) in the vitamin D, calcium, and combined supplementation groups, respectively, relative to placebo[11].

Evidence for the plausibility and validity of the 10 investigated biomarkers of risk for colorectal neoplasms

The plausibility and validity of the 10 colorectal tissue biomarkers as biomarkers of risk for colorectal neoplasms was previously reported[19,31,49-53]. Briefly, the "APC Pathway" of colon carcinogenesis involves genetic silencing or inactivation of the "pathway gatekeeper" ***APC*** tumor suppressor gene[17,54,55]. This ***APC*** gene's protein product acts by degrading β-catenin, which functions as a both pro-proliferative and by regulating ***E-cadherin***[17,56]***.*** The protein product of the gene ***E-cadherin*** functions as a calcium-dependent cell adhesion molecule that is necessary for colon crypt structure and function[17,57]. When β-catenin is allowed to build-up due to inactivation of ***APC*** there are downstream effects that inevitably promote colonocytes to enter the proliferative phase of the cell cycle, thus increasing proliferation as a direct effect and decreasing differentiation and apoptosis as an indirect effect[17,58]. Another colon carcinogenesis pathway is the "Mismatch Repair (MMR) Pathway"[17,50]. This pathway is triggered by the mutation or epigenetic silencing of ***MSH2*** or ***MLH1***[17,28]. MMR genes maintain DNA strands by repairing mismatches after DNA replicates in preparation for cell division[17]. The result of the inactivation of the MMR pathway is impaired function of the genes for TGF-β_1 and bax, leading to increased proliferation and decreased differentiation and apoptosis[17].

There are other protein biomarkers of the cell cycle, including biomarkers of cell proliferation, differentiation, and apoptosis[17,23]. Mib-1 is a marker that a cell is in a proliferative phase[17,22]. A marker that a cell is no longer able to proliferate and is differentiated is p21[17,59]. Apoptosis is inhibited by bcl-2 and promoted by bax in colonocytes of the normal colon[17,26,60,61]. TGF-α and TGF-β_1 are autocrine and paracrine growth factors that are potent promoters and inhibitors of growth, respectively[62] and are involved in colon carcinogenesis[17]. TGF-β_1 was found to have contextually-related tumor suppressor and pro-oncogenic activities, and colon cancers

typically have increased TGF-β_1 expression[17]. TGF-β_1 was also found to induce p21, and its growth suppressor activity was also found to be inhibited by β-catenin[17,24]. TGF-α expression is associated with the distribution of proliferating cells[17,62,63]. Conversely TGF-β_1 tends to be located in the non-proliferative (upper 40%) zone of the colon crypt[17]. *In vitro*, TGF-β_1 is potently induced by vitamin D_3 which promotes apoptosis and inhibits proliferation[17,24].

As reported in detail elsewhere[64-68], the potential validity of the above-discussed biomarkers as biomarkers of risk for colorectal neoplasms was investigated in a colonoscopy-based case-control study (Markers of Adenomatous Polyps II, or MAP II) of incident, sporadic colorectal adenoma (n=49 cases, 152 controls). Briefly, biomarker expression in cases relative to controls was reported as 14% lower for an APC/ β-catenin ratio, 49% lower for MSH2, 26% lower for a bax/bcl-2 ratio, 49% higher for TGF-α, and 7% lower for TGF-β_1[17].

Previous evidence that calcium and/or vitamin D modulate the 10 investigated biomarkers of risk for colorectal neoplasms

The effects of calcium and/or vitamin D on the 10 biomarkers in randomized, double-blind, placebo-controlled trials were also previously reported[17,29]. In one trial (n=193), participants were randomized to placebo, 1.0 g of elemental calcium, or 2.0 g of elemental calcium taken twice daily with food over six months[17]. The main, statistically significant finding of this study was that calcium shifted the zone of proliferation downward into the lower 60%, or normal proliferation zone, of the crypt[69]. In the Calcium and Vitamin D vs. Markers of Adenomatous Polyps trial (CaDvMAP)[19,31,49-53,70], 92 colorectal adenoma patients were randomized to vitamin D_3 (800 IU), elemental calcium (2.0 g), both, or placebo. Changes in participants' serum 25(OH)D concentrations and 10 tissue biomarkers were reported[17]. Briefly, statistically significant increases in serum 25(OH)D concentrations were found among the

vitamin D and the calcium + vitamin D groups[17]. APC expression in the differentiation zone increased in the vitamin D group, β-catenin expression in the whole crypt decreased modestly in all groups, E-cadherin expression in the differentiation zone statistically significantly increased in the vitamin D_3 groups, MSH2 expression in the differentiation zone increased in all groups, p21 expression in the whole crypt increased in calcium and vitamin D groups but to a lesser degree in the calcium + vitamin D group, bax expression in the whole crypt increased in all groups, TGF-α expression in the differentiation and proliferative zones decreased in all groups, and TGF-β_1 expression in the whole crypt increased in all groups, relative to the placebo group[17]. In a second trial, an adjunct biomarker study (encompassing the same study population as for the present study) to a larger randomized, double-blind, placebo-controlled trial of colorectal adenoma recurrence, in the vitamin D plus calcium group relative to control, in the crypt differentiation zone (upper 40% of crypts), mib-1 expression decreased 24% (P=0.28); p21 expression alone and relative to mib-1 expression increased 29% (P=0.06) and 73% (P=0.06), respectively; and bax expression relative to mib-1 expression increased 58% (P=0.21)[71]. The estimated vitamin D alone treatment effects were similar but of lesser magnitudes, and those for calcium alone were mixed[71]. All estimated treatment effects on bcl-2 expression were close to the null[71]. Also, in the vitamin D_3 and vitamin D_3 plus calcium groups, relative to their reference groups, in the upper 40% (differentiation zone) of crypts, it was estimated that, respectively, the MSH2/mib-1 ratio increased by 47% (p=0.14) and 62% (p=0.08), TGFβ_1 expression increased by 41% (p=0.25) and 78% (p=0.14), and the TGFα/TGFβ_1 ratio decreased by 25% (p=0.31) and 44% (p=0.13)[72]. Finally, for vitamin D vs. no vitamin D, the ratio of APC expression to β-catenin expression in the upper 40% (differentiation zone) of crypts (APC/β-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)[13]. Total E-cadherin expression increased by 7% (P = 0.35) for vitamin D vs. no

vitamin D, 8% (P = 0.31) for calcium vs. no calcium, and 12% (P = 0.21) for vitamin D + calcium vs. calcium[13].

Strengths and Limitations

The primary limitation of this pilot study was its small sample size, especially among VDP2 individuals in the placebo group. Thus, we could only investigate patterns of findings in context with biological plausibility and previous literature in order to assess whether or not a larger, more definitive study would be supported. Also, we assessed the expression of the biomarkers only in the normal rectal mucosa; thus, treatment effects in other parts of the colon are unknown. In addition, we did not quantify biomarker expression beyond 1 year of follow-up or in tumor tissue, warranting future studies to investigate long-term vitamin D and calcium effects on the biomarkers as well as potential differences in effects in normal versus neoplastic tissue. Last, since most of our study participants were white, our results may not be generalizable to other races.

Strengths of this study include that it is the first to investigate potential calcium and/or vitamin D treatment effect modification on colorectal tissue biomarkers of the cell cycle, colorectal carcinogenesis pathways, and transforming growth factors according to VDP isoform. Other strengths are that we quantified biomarker expression using automated immunohistochemistry and precise image analysis, and analyzed a large number of crypts per patient, thereby reducing measurement error and bias due to outcome misclassification. Our powerful image analysis tool also allowed us to quantify biomarker expression in different functional zones of the colorectal crypts. Additional strengths include the high adherence to study protocol, complete follow-up, high biomarker scorer reliability, and 2x2 factorial

randomization allowing us to assess treatment effects of calcium and vitamin D alone and in combination.

Conclusions

In conclusion, the findings from our pilot study, in context with previous literature and considering the strengths and limitations of the present study, support further investigation of calcium and/or vitamin D effects on pre-neoplastic biomarkers of risk for colorectal neoplasms according to VDP isoforms. Our findings also add to the growing literature on VDP isoforms as potential modifiers of calcium and vitamin D in relation to risk for colorectal neoplasms. Such knowledge could help inform more personalized recommendations for reducing colorectal cancer risk.

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Tables

Table 1. Selected baseline characteristics of the trial participants, by treatment group assignment (n = 62)[a]

Baseline characteristics	Treatment groups				*P*- value[b]
	Placebo (n = 12)	Calcium (n = 16)	Vitamin D (n = 17)	Calcium + Vit D (n = 17)	
Age, years	59.9 (7.2)	59.9 (6.5)	59.2 (7.8)	57.6 (7.1)	0.79
Men, %	75.0	81.3	70.6	82.4	0.83
White, %	83.3	75.0	70.6	94.1	0.49
College graduate[c], %	66.7	37.5	64.7	53.0	0.35
Family history of CRC[d], %	0.0	12.5	20.0	5.9	0.33
Regularly[e] take NSAID, %	33.3	43.8	23.5	29.4	0.65
Regularly[e] take aspirin, %	58.3	75.0	58.3	64.7	0.75
Multivitamin user, %	41.7	81.3	47.1	64.7	0.11
Current smoker, %	25.0	6.3	0.0	5.9	0.12
High alcohol use[f], %	16.7	12.5	23.5	18.2	0.71
Physical activity, MET-mins./wk.[g,h]	80 (480)	840 (1,360)	300 (960)	480 (2,400)	0.003
Body mass index, kg/m^2	29.4 (4.9)	32.3 (7.6)	28.7 (5.5)	30.0 (4.5)	0.32
VDP2[i], %	25.0	43.8	56.3	50.0	0.12
Dietary intakes					
Total energy, Kcal/day	1,314 (371)	1,737 (546)	1,437 (519)	1,613 (541)	0.18
Total fat, % kcal	38.9 (7.8)	35.8 (6.8)	37.2 (6.5)	34.4 (7.5)	0.13
Dietary fiber, (g/1,000 Kcal/day)	7.2 (2.3)	9.5 (2.9)	9.8 (3.9)	9.8 (2.6)	0.01
Dietary calcium (mg/1,000 Kcal/day)	564 (324)	549 (173)	489 (169)	434 (177)	0.07
Dietary vitamin D (IU/ 1,000 Kcal/day)	261 (202)	310 (172)	218 (212)	278 (208)	0.37
Serum concentrations					
Calcium, mg/dL	9.2 (0.2)	9.3 (0.3)	9.3 (0.3)	9.4 (0.3)	0.22
25-hydroxyvitamin D, ng/mL	22.4 (8.2)	24.5 (13.4)	23.1 (8.7)	22.5 (6.5)	0.93

Abbreviations: CRC, colorectal cancer; Vit D, Vitamin D; MET, metabolic equivalents of task; NSAID, nonsteroidal anti-inflammatory drug; VDP2, vitamin D binding protein isoform 2

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

[b] From chi square test for categorical variables, and ANOVA for continuous variables (transformed by the natural logarithm when indicated to achieve a normal distribution)

[c] Received a Bachelor's degree or higher

[d] In a first degree relative

[e] At least four times a week

[f] High alcohol use is defined as > 1 drink per day for women and > 2 drinks per day for men

[g] Median and interquartile range

[n] Median (IQR) is presented because of the right-skewed distribution of the physical activity variable; the variable was natural logarithm-transformed, yielding a normal distribution, prior to the ANOVA test

[i] Percentage of participants with *GC* rs4588*CA or AA

Table 2. Estimated effects[a] of calcium and/or vitamin D supplementation on the expression[b] of cell cycle and growth factor biomarkers in the crypts of normal-appearing colorectal mucosa among adjunct biomarker study participants, stratified by vitamin D binding protein isoform (n = 60)

Biomarkers and treatment groups	No VDB2 (*GC* rs4588*CC)					VDB2 (*GC* rs4588*CA or AA)					$P_{interaction}$[e]
		Relative Rx effect[c]			Absolute Rx effect[d]		Relative Rx effect[c]			Absolute Rx effect[d]	
	n	Rx effect	(95% CI)	*P*-value		n	Rx effect	(95% CI)	*P*-value		
Mib-1 (whole crypts)											
Placebo	9					3					
Calcium	9	1.13	(0.72 - 1.8)	0.58	214	7	0.83	(0.49 - 1.39)	0.46	-271	
Vitamin D	7	1.06	(0.65 - 1.74)	0.80	150	9	0.78	(0.47 - 1.3)	0.33	-311	
Calcium + vit. D	8	1.39	(0.86 - 2.22)	0.17	482	8	0.63	(0.37 - 1.06)	0.08	-577	*0.21*
Bcl-2 (whole crypts)											
Placebo	9					3					
Calcium	9	0.83	(0.53 - 1.31)	0.41	-131	7	0.62	(0.26 - 1.48)	0.27	-336	
Vitamin D	7	1.11	(0.68 - 1.8)	0.68	50	9	0.72	(0.31 - 1.66)	0.42	-221	
Calcium + vit. D	8	0.93	(0.58 - 1.49)	0.76	-55	8	0.85	(0.36 - 2.03)	0.70	-89	*0.86*
Bax (whole crypts)											
Placebo	9					3					
Calcium	9	0.69	(0.3 - 1.59)	0.37	-145	7	0.22	(0.06 - 0.73)	0.02	-493	
Vitamin D	7	0.63	(0.26 - 1.56)	0.31	-172	9	0.23	(0.07 - 0.74)	0.02	-493	
Calcium + vit. D	8	0.70	(0.29 - 1.66)	0.40	-109	8	0.42	(0.13 - 1.4)	0.15	-295	*0.33*
p21/mib-1 (differentiation zone)[f]											
Placebo	9					3					
Calcium	9	0.45	(0.1 - 1.95)	0.27	-4.4	7	1.19	(0.18 - 7.93)	0.85	1.3	
Vitamin D	7	0.59	(0.12 - 2.87)	0.50	-2.4	9	1.60	(0.26 - 9.96)	0.60	4.4	
Calcium + vit. D	8	0.55	(0.12 - 2.52)	0.43	-2.9	8	4.31	(0.66 - 28.11)	0.12	12.9	*0.07*
TGF-α (whole crypts)											
Placebo	9					3					
Calcium	9	0.94	(0.74 - 1.2)	0.61	-267	7	0.76	(0.55 - 1.04)	0.09	-2,141	
Vitamin D	7	1.07	(0.83 - 1.39)	0.58	458	9	0.66	(0.49 - 0.89)	0.01	-3,058	

Calcium + vit. D	8	1.00	(0.77 - 1.3)	0.99	2	8	0.81	(0.59 - 1.1)	0.17	-1,825	*0.06*
TGF-β (whole crypts)											
Placebo	9					3					
Calcium	9	0.57	(0.19 - 1.68)	0.29	-101	7	1.30	(0.24 - 7.1)	0.75	348	
Vitamin D	7	1.98	(0.62 - 6.32)	0.24	225	9	0.72	(0.14 - 3.68)	0.68	37	
Calcium + vit. D	8	0.74	(0.24 - 2.27)	0.59	57	8	1.08	(0.2 - 5.67)	0.93	315	*0.22*

Abbreviations: CI, confidence interval; Rx, treatment; VDB, vitamin D-binding protein; vit., vitamin

[a] The results are from mixed linear models implemented in SAS v.9.4 PROC MIXED; biomarker labeling density distributions were normalized with natural log-transformation, and the relative effects[c], 95% CIs, and *P*-values were taken directly from the mixed linear models' output, and the absolute effects[d] were hand calculated from the geometric means

[b] Biomarkers measured using automated immunohistochemistry and quantitative image analysis; all biomarker measurements are in optical density

[c] Relative effect = [(treatment group follow-up) / (treatment group baseline)] / [(placebo group follow-up) / (placebo group baseline)]; interpretation is similar to that for an odds ratio (e.g., a value of 1.50 would be interpreted as a 50% increase in biomarker expression in the treatment group relative to the placebo group after 1 year)

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

[e] $P_{interaction}$ by VDB2 isoform calculated using a likelihood ratio test in SAS 9.4 (Cary, NC)

[f] Ratio of mean p21 to mean mib-1 in the upper 40% (differentiation zone) of crypts

Table 3. Estimated effects[a] of calcium and/or vitamin D supplementation on the expression[b] of colorectal carcinogenesis pathway proteins in whole crypts of normal-appearing colorectal mucosa among adjunct biomarker study participants, stratified by vitamin D binding protein isoform (n = 60)

Biomarkers and treatment groups	No VDB2 (*GC* rs4588*CC)					VDB2 (*GC* rs4588*CA or AA)					$P_{interaction}$[e]
	n	Relative Rx effect[c]	Absolute Rx effect[d]			n	Relative Rx effect[c]	Absolute Rx effect[d]			
			Rx effect	(95% CI)	*P*-value			Rx effect	(95% CI)	*P*-value	
APC											
Placebo	9					3					
Calcium	9	0.91	-309	(-1,108 - 489)	0.44	7	1.03	104	(-1,665 - 1,873)	0.90	
Vitamin D	7	1.26	626	(-228 - 1,479)	0.14	9	0.80	-392	(-2,101 - 1,316)	0.64	
Calcium + vit. D	8	1.00	-78	(-901 - 745)	0.85	8	1.17	302	(-1,434 - 2,037)	0.72	*0.30*
β-catenin											
Placebo	9					3					
Calcium	9	1.05	597	(-1,831 - 3,025)	0.62	7	1.11	1115	(-2,122 - 4,353)	0.48	
Vitamin D	7	0.99	-152	(-2,748 - 2,443)	0.91	9	0.97	-414	(-3,542 - 2,713)	0.79	
Calcium + vit. D	8	1.05	556	(-1,947 - 3,059)	0.65	8	1.04	404	(-2,834 - 3,641)	0.80	*0.99*
E-cadherin											
Placebo	9					3					
Calcium	9	1.17	750	(-491 - 1,992)	0.23	7	0.80	-976	(-3,204 - 1,252)	0.37	
Vitamin D	7	1.16	704	(-598 - 2,007)	0.28	9	0.78	-1290	(-3,493 - 913)	0.24	
Calcium + vit. D	8	1.17	765	(-491 - 2,021)	0.22	8	0.98	-185	(-2,391 - 2,020)	0.86	*0.47*
MSH2											
Placebo	9					3					
Calcium	9	0.74	-541	(-1,012 - -71)	0.03	7	0.80	-577	(-1,241 - 86)	0.08	
Vitamin D	7	1.01	-28	(-531 - 475)	0.91	9	0.75	-698	(-1,338 - -57)	0.03	
Calcium + vit. D	8	0.97	-58	(-557 - 441)	0.81	8	0.84	-501	(-1,192 - 190)	0.15	*0.27*

Abbreviations: CI, confidence interval; VDB2, vitamin D-binding protein isoform 2; Rx, treatment; vit., vitamin

[a] The results are from mixed linear models implemented in SAS v.9.4 PROC MIXED; since the biomarker labeling densities were normally distributed, the optical density crude means are presented, the absolute effects were taken directly from the mixed linear models' output, and the relative effects were hand calculated from the crude means

[b] Biomarkers measured using automated immunohistochemistry and quantitative image analysis; all biomarker measurements are in optical density

[c] Relative effect = [(treatment group follow-up) / (treatment group baseline)] / [(placebo group follow-up) / (placebo group baseline)]; interpretation is similar to that for an odds ratio (e.g., a value of 1.50 would be interpreted as a 50% increase in biomarker expression in the treatment group relative to the placebo group after 1 year)

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

[e] $P_{\text{interaction}}$ by VDB2 isoform calculated using a likelihood ratio test in SAS 9.4 (Cary, NC)

Supplemental Table 1. Estimated effects[a] of calcium and/or vitamin D supplementation on the expression[b] of cell cycle and growth factor biomarkers in the crypts of normal-appearing colorectal mucosa among adjunct biomarker study participants (n = 62)

Biomarker and treatment group	Baseline			1-Yr. follow-up			Relative Rx effect[c]			Absolute Rx effect[d]
	n	Mean	(95% CI)	n	Mean	(95% CI)	Rx effect	(95% CI)	*P*-value	
Mib-1 (whole crypts)										
Placebo	12	1,491	(1,182 - 1,882)	12	1,391	(1,103 - 1,755)				
Calcium	16	1,098	(898 - 1,344)	16	1,113	(910 - 1,362)	1.09	(0.78 - 1.51)	0.62	115
Vitamin D	17	1,213	(998 - 1,475)	17	1,184	(974 - 1,439)	1.05	(0.75 - 1.45)	0.79	71
Calcium + vit. D	17	1,330	(1,089 - 1,625)	17	1,351	(1,111 - 1,642)	1.09	(0.78 - 1.52)	0.61	121
Bcl-2 (whole crypts)										
Placebo	12	671	(477 - 943)	12	779	(581 - 1,045)				
Calcium	16	757	(564 - 1,017)	16	671	(520 - 865)	0.76	(0.51 - 1.13)	0.18	-195
Vitamin D	17	644	(484 - 858)	17	680	(531 - 870)	0.91	(0.61 - 1.34)	0.63	-72
Calcium + vit. D	17	650	(484 - 872)	17	687	(533 - 886)	0.91	(0.61 - 1.35)	0.64	-70
Bax (whole crypts)										
Placebo	12	229	(140 - 375)	12	507	(306 - 841)				
Calcium	16	274	(179 - 419)	16	291	(188 - 451)	0.48	(0.24 - 0.95)	0.04	-261
Vitamin D	17	356	(236 - 539)	17	368	(241 - 563)	0.47	(0.24 - 0.92)	0.03	-267
Calcium + vit. D	17	304	(201 - 459)	17	389	(255 - 595)	0.58	(0.29 - 1.14)	0.11	-193
p21/mib-1 (differentiation zone)[e]										
Placebo	12	4.7	(2.7- 8.2)	12	5.7	(2.7 - 12.2)				
Calcium	16	8.9	(5.5 - 14.4)	16	5.8	(3.0 - 11.3)	0.54	(0.18 - 1.59)	0.26	-4.07
Vitamin D	17	8.3	(5.2 - 13.2)	17	6.8	(3.6 - 12.9)	0.68	(0.23 - 1.96)	0.46	-2.49
Calcium + vit D	17	6.9	(4.3 - 11.1)	17	8.8	(4.7 - 16.7)	1.05	(0.36 - 3.07)	0.93	0.91
TGF-α (whole crypts)										
Placebo	12	6,678	(5,537 - 8,055)	12	7,098	(5,882 - 8,565)				
Calcium	16	5,434	(4,636 - 6,369)	16	5,240	(4,441 - 6,182)	0.91	(0.75 - 1.1)	0.32	-614
Vitamin D	17	6,966	(5,972 - 8,127)	17	6,723	(5,741 - 7,873)	0.91	(0.75 - 1.1)	0.31	-662
Calcium + vit. D	17	6,100	(5,178 - 7,187)	17	6,219	(5,257 - 7,358)	0.96	(0.79 - 1.16)	0.67	-300
TGF-β (whole crypts)										
Placebo	12	651	(209 - 2,030)	12	457	(145 - 1,444)				
Calcium	16	483	(180 - 1,294)	16	285	(105 - 771)	0.84	(0.35 - 2.04)	0.69	-5

Vitamin D	17	420	(161 - 1,092)	17	330	(126 - 866)	1.12	(0.47 - 2.68)	0.80	103
Calcium + vit. D	17	179	(69 - 467)	17	121	(46 - 319)	0.96	(0.4 - 2.31)	0.93	135

Abbreviations: CI, confidence interval; Rx, treatment; vit., vitamin

[a] The results are from mixed linear models implemented in SAS v.9.4 PROC MIXED; biomarker labeling density distributions were normalized with natural log-transformation, and the relative effects[c], 95% CIs, and *P* -values were taken directly from the mixed linear models' output, and the absolute effects[d] were hand calculated from the geometric means

[b] Biomarkers measured using automated immunohistochemistry and quantitative image analysis; all biomarker measurements are in optical density

[c] Relative effect = [(treatment group follow-up) / (treatment group baseline)] / [(placebo group follow-up) / (placebo group baseline)]; interpretation is similar to that for an odds ratio (e.g., a value of 1.50 would be interpreted as a 50% increase in biomarker expression in the treatment group relative to the placebo group after 1 year)

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

[e] Ratio of mean p21 to mean mib-1 in the upper 40% (differentiation zone) of crypts

Supplemental Table 2. Estimated effects[a] of calcium and/or vitamin D supplementation on the expression[b] of colorectal carcinogenesis pathway proteins in whole crypts of normal-appearing colorectal mucosa among adjunct biomarker study participants (n = 62)

Biomarker and treatment group	Baseline			1-Yr. follow-up			Relative Rx effect[c]	Absolute Rx effect[d]		
	n	Mean	(95% CI)	n	Mean	(95% CI)		Rx effect	(95% CI)	*P*-value
APC										
Placebo	12	2,876	(2,139 - 3,612)	12	2,284	(1,743 - 2,824)				
Calcium	16	2,849	(2,211 - 3,487)	16	2,142	(1,674 - 2,610)	0.95	-115	(-919 - 690)	0.78
Vitamin D	17	2,375	(1,757 - 2,994)	17	1,814	(1,360 - 2,268)	0.96	31	(-764 - 825)	0.94
Calcium + vit. D	17	2,866	(2,247 - 3,484)	17	2,512	(2,058 - 2,966)	1.10	238	(-556 - 1,033)	0.55
β-catenin										
Placebo	12	10,648	(9,184 - 12,111)	12	10,698	(9,148 - 12,247)				
Calcium	16	10,633	(9,366 - 11,900)	16	11,543	(10,201 - 12,885)	1.08	860	(-921 - 2,640)	0.34
Vitamin D	17	11,048	(9,818 - 12,277)	17	10,995	(9,693 - 12,296)	0.99	-103	(-1,861 - 1,654)	0.91
Calcium + vit. D	17	11,210	(9,943 - 12,478)	17	11,640	(10,298 - 12,981)	1.03	379	(-1,401 - 2,159)	0.67
E-cadherin										
Placebo	12	5,065	(4,082 - 6,048)	12	4,716	(3,955 - 5,477)				
Calcium	16	4,379	(3,528 - 5,231)	16	4,213	(3,540 - 4,886)	1.03	183	(-890 - 1,255)	0.73
Vitamin D	17	5,227	(4,384 - 6,069)	17	4,752	(4,100 - 5,405)	0.98	-125	(-1,197 - 946)	0.82
Calcium + vit. D	17	4,639	(3,814 - 5,465)	17	4,783	(4,131 - 5,435)	1.11	493	(-565 - 1,551)	0.35
MSH2										
Placebo	12	2,006	(1,581 - 2,431)	12	2,295	(1,912 - 2,678)				
Calcium	16	1,886	(1,518 - 2,254)	16	1,698	(1,366 - 2,030)	0.79	-476	(-852 - -101)	0.01
Vitamin D	17	1,856	(1,499 - 2,213)	17	1,879	(1,557 - 2,201)	0.89	-265	(-636 - 105)	0.16
Calcium + vit. D	17	1,752	(1,390 - 2,114)	17	1,918	(1,581 - 2,254)	0.96	-123	(-512 - 266)	0.53

Abbreviations: CI, confidence interval; Rx, treatment; vit D, vitamin D

[a] The results are from mixed linear models implemented in SAS v.9.4 PROC MIXED; since the biomarker labeling densities were normally distributed, the optical density crude means are presented, the absolute effects were taken directly from the mixed linear models' output, and the relative effects were hand calculated from the crude means

[b] Biomarkers measured using automated immunohistochemistry and quantitative image analysis; all biomarker measurements are in optical density

[c] Relative effect = [(treatment group follow-up) / (treatment group baseline)] / [(placebo group follow-up) / (placebo group baseline)]; interpretation is similar to that for an odds ratio (e.g., a value of 1.50 would be interpreted as a 50% increase in biomarker expression in the treatment group relative to the placebo group after 1 year)

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