

A Randomized Clinical Trial of the Effects of Supplemental Calcium and Vitamin D₃ on Markers of Their Metabolism in Normal Mucosa of Colorectal Adenoma Patients

Thomas U. Ahearn^{1,2}, Marjorie L. McCullough^{1,3}, W. Dana Flanders^{2,4,5}, Qi Long^{2,5}, Eduard Sidelnikov⁴, Veronika Fedirko^{2,4}, Carrie R. Daniel^{1,2}, Robin E. Rutherford⁶, Aasma Shaikat⁷, and Roberd M. Bostick^{2,4}

Abstract

In cancer cell lines and rodent models, calcium and vitamin D favorably modulate cell proliferation, differentiation, and apoptosis in colonic epithelia. These effects may be modulated by local expression of the calcium receptor (CaR), the vitamin D receptor (VDR), and the P450 cytochromes, CYP27B1 and CYP24A1; however, they have yet to be investigated in humans. To address this gap, we conducted a randomized, double-blinded, placebo-controlled 2 × 2 factorial clinical trial. Patients with at least one pathology-confirmed colorectal adenoma were treated with 2 g/d elemental calcium and/or 800 IU/d vitamin D₃ versus placebo over 6 months (*n* = 92; 23 per group). CaR, VDR, CYP27B1, and CYP24A1 expression and distribution in biopsies of normal appearing rectal mucosa were detected by standardized, automated immunohistochemistry and quantified by image analysis. In the calcium-supplemented group, CaR expression increased 27% (*P* = 0.03) and CYP24A1 expression decreased 21% (*P* = 0.79). In the vitamin D₃-supplemented group, CaR expression increased 39% (*P* = 0.01) and CYP27B1 expression increased 159% (*P* = 0.06). In patients supplemented with both calcium and vitamin D₃, VDR expression increased 19% (*P* = 0.13) and CaR expression increased 24% (*P* = 0.05). These results provide mechanistic support for further investigation of calcium and vitamin D₃ as chemopreventive agents against colorectal neoplasms, and CaR, VDR, CYP27B1, and CYP24A1 as modifiable, preneoplastic risk biomarkers for colorectal neoplasms. *Cancer Res*; 71(2): 413–23. ©2010 AACR.

Introduction

Colorectal cancer remains the second leading cause of cancer deaths in the United States, despite advances in cancer screening and treatment (1). The molecular basis for colon carcinogenesis has become clearer (2), aiding in the development of much needed treatable preneoplastic biomarkers of risk for colorectal neoplasms.

There is strong biological plausibility and animal and human observational evidence for protective effects of calcium and vitamin D, acting separately and synergistically

through, at least in part, their respective receptors against colorectal neoplasms. Colonic luminal calcium binding to the calcium receptor (CaR, also referred to as the calcium-sensing receptor) may directly modulate the cell cycle of colonocytes, partly by (i) inhibiting the β-catenin/TCF transcription complex (3, 4), (ii) promoting activation of E-cadherin (3), and (iii) reducing the concentration of 25-hydroxyvitamin D 24-hydroxylase (CYP24A1; ref. 5). Luminal calcium is also hypothesized to bind proinflammatory, secondary bile acids and ionized fatty acids (3, 6). Binding of vitamin D to the vitamin D receptor (VDR) may modulate the cell cycle, partly by (i) competitively binding β-catenin (7), (ii) upregulating p21 (8) and E-cadherin expression (9), and (iii) regulating growth factors (10). The VDR also promotes detoxification of the secondary bile acid lithocholic acid (10). Also, prospective cohort studies have consistently found higher total calcium intake to be associated with reduced risk for colorectal neoplasms (10), calcium supplementation reduces colorectal adenoma recurrence (modified by vitamin D status; ref. 11), and higher circulating concentrations of 25(OH)D are inversely associated with colorectal neoplasms (10, 12, 13).

The CaR is expressed in all parts of the gastrointestinal tract and sporadically expressed in well- to moderately differentiated colon carcinomas; however, little to no expression of the CaR is found in undifferentiated carcinomas (14). Colorectal mucosa expresses the VDR; the 1,25(OH)₂D synthesizing enzyme, 25(OH) D-1α-hydroxylase (CYP27B1); and the catabolizing enzyme

Authors' Affiliations: ¹Nutrition and Health Sciences Program, Graduate Division of Biological and Biomedical Sciences; ²Winship Cancer Institute, Emory University; ³Epidemiology Research Program, American Cancer Society; Departments of ⁴Epidemiology and ⁵Biostatistics and Bioinformatics, Rollins School of Public Health, Emory University; ⁶Division of Digestive Diseases, Emory University School of Medicine, Atlanta, Georgia; and ⁷Department of Medicine, GI Division, University of Minnesota, Minneapolis, Minnesota

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

Corresponding Author: Roberd M. Bostick, Department of Epidemiology, Rollins School of Public Health, Emory University, 1518 Clifton Road, Northeast, Atlanta, GA 30322. Phone: 404-727-2671; Fax: 404-727-8737; E-mail: rmbosti@sph.emory.edu

doi: 10.1158/0008-5472.CAN-10-1560

©2010 American Association for Cancer Research.

CYP24A1, suggesting an autocrine/paracrine function for vitamin D in colorectal mucosa that is separate from its role in systemic calcium homeostasis (15). Expression of the VDR and CYP27B1 was reported to be increased in well- to moderately differentiated colon carcinomas; however, little to no expression was observed in undifferentiated colon carcinomas (16–19). Expression of CYP24A1 was reported to be increased in undifferentiated colon carcinomas relative to moderately differentiated colon carcinomas (17, 19).

CaR, VDR, CYP27B1, and CYP24A1 are appealing potential modifiable biomarkers of risk for colorectal neoplasms given the reported differences in their expression between cancerous and normal mucosa and their functional importance in modulating the disease-reducing actions of calcium and vitamin D. We know of no other reported human studies in which the effects of calcium and vitamin D supplementation on the expression of these markers in normal human colorectal mucosa were evaluated. To address this, as reported herein, we conducted a pilot, randomized, double-blind, placebo-controlled 2 × 2 factorial chemoprevention clinical trial of supplemental calcium and vitamin D₃, alone and in combination versus placebo over 6 months, to estimate the efficacy of these agents on markers of calcium and vitamin D metabolism in the normal colorectal mucosa.

Study Participants and Methods

Participant population

A detailed description of the study protocol for recruitment procedures and detailed specific exclusions was published previously (20). Briefly, eligible participants were 30 to 75 years of age, in general good health, and had a history of at least one pathology-confirmed adenomatous colorectal polyp within the past 36 months. Exclusions from participation included contraindications to calcium or vitamin D supplementation or rectal biopsy procedures, and medical conditions, habits, or medication usage that potentially could interfere with the study. Participants were recruited from patients attending the Digestive Diseases Clinic at the Emory Clinic, Emory University.

Clinical trial protocol

Between April 2005 and January 2006, 522 potentially eligible patients were identified through initial screening of electronic medical records; of these, 244 (43%) were contacted, and of these, 105 (47%) were eligible to be interviewed and subsequently signed a consent form, completed questionnaires, and provided blood samples (20). Diet was assessed using a semi-quantitative Willett Food Frequency Questionnaire (21). Medical and pathology records were reviewed. Following a 30-day placebo run-in trial, 92 (88%) participants with no significant perceived side effects and who took at least 80% of their assigned tablets underwent a baseline rectal biopsy, and, with additional consent, were randomly assigned to the following 4 treatment groups: placebo ($n = 23$), 2.0 g elemental calcium supplementation (as calcium carbonate in equal doses twice daily; $n = 23$), 800 IU vitamin D₃ supplementation (400 IU twice daily; $n = 23$), and 2.0 g elemental calcium plus 800 IU vitamin D₃ supplementation ($n = 23$). Additional details and rationale

for the doses and forms of calcium and vitamin D₃ supplements were previously published (20). Participants were instructed to maintain their usual diet and not take any new nutritional supplements that they were not taking at the time of entry into the study. All aspects of the trial were approved by the Institutional Review Board of Emory University.

During the 6-month treatment period, participants attended follow-up visits 2 and 6 months after randomization. At follow-up visits, participants completed questionnaires and were interviewed about adherence and adverse events. At the 6-month follow-up, participants again underwent a venipuncture and rectal biopsy. All visits for a given participant were scheduled for the same time of day to control for potential circadian variation. Dietary, lifestyle, and other factors hypothesized to modify biomarker expression in normal colon mucosa were assessed at baseline and at 6-months follow-up. Participants were asked to abstain from aspirin use 7 days prior to each biopsy. Participants were not required to be fasting for their visits and did not take a bowel cleansing preparation or enema.

Six, approximately, 1-mm thick biopsy specimens were taken from the normal appearing rectal mucosa 10 cm above the level of the external anal aperture through a short rigid sigmoidoscope using a jumbo cup flexible biopsy forceps mounted on a semirigid rod. No biopsies were taken within 4.0 cm of a polypoid lesion. Biopsies were placed onto a strip of bibulous paper and immediately placed in PBS, 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).

Immunohistochemistry protocol

Five slides with 4 levels of 3- μ m thick biopsy sections taken 40 μ m apart were prepared for each antigen, yielding a total of 20 levels for each antigen. Antigen retrieval was performed by placing the slides in a preheated Pretreatment Module (Lab Vision Corp.) with 100 \times citrate buffer (pH 6.0; DAKO S1699, DAKO Corp.) and steaming them for 40 minutes. Following antigen retrieval, slides were immunohistochemically processed in a DAKO Automated Immunostainer (DAKO Corp.) using a labeled streptavidin-biotin method for CaR, VDR, CYP27B1, and CYP24A1 (Table 1, Fig. 1, and Supplementary Material Fig. 1 and References). No slides were counterstained. After processing, slides were coverslipped with a Leica CV5000 Coverslipper (Leica Microsystems, Inc.). Each staining batch contained positive and negative slides; normal kidney was the positive control tissue for CYP27B1 and CYP24A1, and normal colon tissue was the positive control for CaR and VDR. The negative and positive control slides were treated identically to the patients' slides except that antibody diluent was used rather than primary antibody on the negative slides.

Protocol for quantifying labeling densities of immunohistochemically detected biomarkers in normal colon crypts ("scoring")

A detailed description of the protocol used to quantify biomarker labeling optical densities ("biomarker expression") in normal colon crypts was previously described (20). Briefly, a

Table 1. Summary of biomarker immunohistochemical protocols for calcium receptor, vitamin D receptor, CYP27B1, and CYP24A1

Antibody	Manufacturer	Catalog no.	Dilution	Detection kit ^a
CaR	Sigma-Aldrich Co.	C0493	1:200	LSAB2
VDR	Santa Cruz Inc.	SC-13133	1:7,500	LSAB2
CYP27B1	Santa Cruz Inc.	SC-49642	1:100	LSAB ⁺
CYP24A1	Santa Cruz Inc.	SC-32166	1:50	LSAB ⁺

^aDAKO Corp.

scorable crypt was defined as an intact crypt extending from the muscularis mucosa to colon lumen (22). Prior to "scoring", the negative and positive control slides were checked for staining adequacy. The major equipment and software for the image analysis procedures included: personal computer, light microscope (Olympus BX40, Olympus Corporation) with appropriate filters and attached digital light microscope camera (Polaroid DMC Digital Light Microscope Camera, Polaroid Corporation), digital drawing board, ImagePro Plus image analysis software (Media Cybernetics, Inc.), our in-house developed plug-in software for colorectal crypt analysis, and Microsoft Access 2003 relational database software (Microsoft Corporation).

Evaluation of biomarker expression consisted of the same technician cleaning all slides, selecting the 2 of the 3 biopsies with the most scorable crypts per biopsy, creating background correction images for each slide scored, capturing 16-bit grayscale images of crypts at 200 \times magnification and tracing the border of the "hemicypt" (one half of the crypt). The program then divided the outlined hemicypt into equally spaced segments that corresponded to the average width of colonocytes and measured the optical density of the labeling across the entire hemicypt and within each segment, adjusting for the background. The technician then repeated this process for the adjacent hemicypt and proceeded to the next crypt, level, biopsy, and/or slide. The goal was to score 16 to 20 hemicypts per biopsy visit for each biomarker (Fig. 2).

Reliability control was performed by selecting the samples of previously analyzed slides to be reanalyzed by the technician. The technician was blinded to the selection. Intra-reader reliability for CaR, VDR, CYP27B1, and CYP24A1 was between 0.95 and 0.99 throughout.

Protocol for measuring serum 25-OH-vitamin D and 1,25-(OH)₂-vitamin D levels

Serum 25-OH-vitamin D and 1,25-(OH)₂-vitamin D were measured by Dr. Bruce W. Hollis at the Medical University of South Carolina using a RIA (radioimmunoassay) method as previously described (23, 24). Serum samples for baseline and follow-up visits for all subjects were assayed together, ordered randomly, and labeled to mask treatment group, follow-up visit, and quality control replicates. The average intra-assay coefficient of variation was 2.3% and 6.2% for serum 25-OH-vitamin D and for 1,25-(OH)₂-vitamin D, respectively.

Statistical analysis

Treatment groups were assessed for comparability of characteristics at baseline and at final follow-up by the Fisher's exact test for categorical variables and ANOVA for continuous variables. Slide-scoring reliability was analyzed using intra-class correlation coefficients.

The mean-labeling optical density expression of each biomarker on each study participant, at baseline and 6-month follow-up, was calculated by summing the biomarker's expression for all analyzed crypts and dividing by the total number of analyzed crypts. Biomarker expression was transformed to adjust for possible staining batch effects by dividing an individual's mean biomarker expression by their batch mean biomarker expression (20). Measures of crypt biomarker distribution selected *a priori* were the upper 40% of the crypts (differentiation zone) and the lower 60% of the crypts (proliferation zone), to represent distinct functional zones of colon crypts. In a sensitivity analysis, we also transformed biomarker expression by subtracting from an individual's

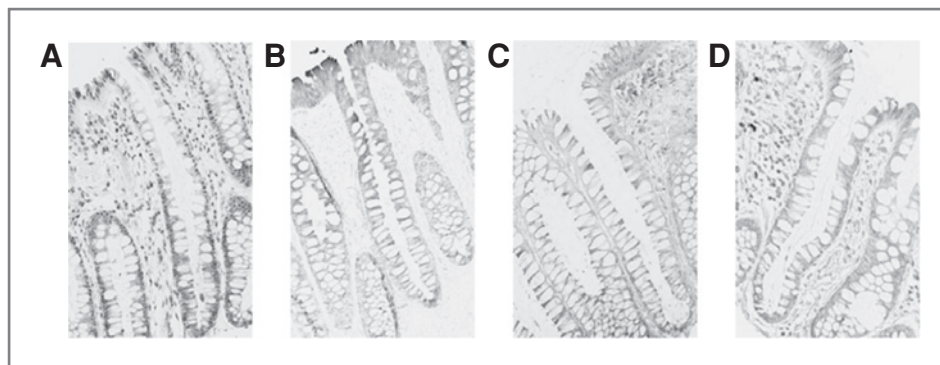


Figure 1. Biomarker immunohistochemical images of colon crypts (200 \times). A, calcium receptor; B, vitamin D receptor; C, CYP27B1; and D, CYP24A1.

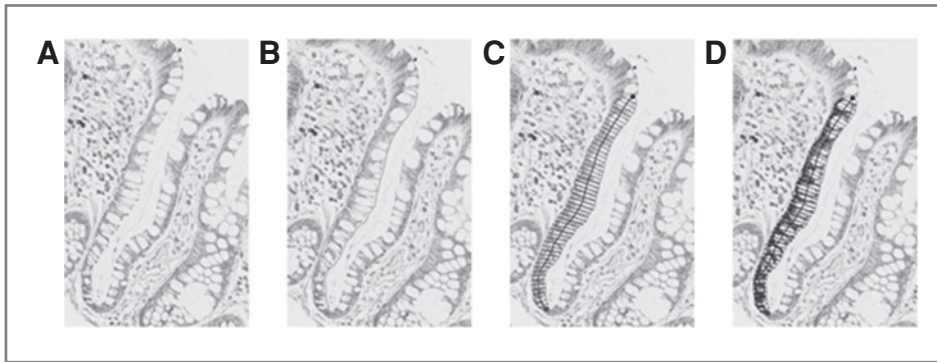


Figure 2. Quantitative image analysis. A, finding scorable crypts; B, tracing the hemicrypt; C, automated sectioning of the trace; and D, automated quantification of CYP24A1 labeling optical density.

mean biomarker expression their batch mean expression; the results did not materially differ from those reported.

Primary analyses were based on assigned treatment at the time of randomization, regardless of adherence status (intent-to-treat analysis). Treatment effects were evaluated by assessing the differences in the transformed biomarker expression from baseline to the 6-month follow-up between participants in the active treatment groups and those in the placebo group by a repeated-measures linear mixed-effects model. The model included the intercept, follow-up visit effects (baseline and follow-up), and interactions between treatment groups and the follow-up visit effect. Because optical density is measured in arbitrary units, to provide perspective on the magnitude of the treatment effects, we also calculated the relative effect. The relative effect was calculated as the (treatment group at follow-up/treatment group at baseline)/(placebo group at follow-up/placebo group at baseline). The relative effect provides a conservative estimate of the proportional change in the treatment group relative to that in the placebo group, and its interpretation is somewhat analogous to that of an OR (e.g., a relative effect of 2.0 would mean that the proportional change in the treatment group was two times that in the placebo group).

The distributions of batch-standardized CaR, VDR, CYP27B1, and CYP24A1 labeling optical densities along the full length of the crypts were graphically plotted and evaluated using the LOESS procedure. First, each hemicrypt was standardized to 50 sections. Then, the average of each section across all crypts was predicted by the LOESS model separately for each patient and then for each treatment group by visit. The results were graphically plotted along with the smoothing lines. Although the plots illustrate the distribution of expression, they do not provide a complete analysis of treatment effects because they do not account for changes in the placebo group.

Statistical analyses were performed using SAS 9.2 statistical software (SAS Institute Inc.). A P value ≤ 0.05 (2-sided) was considered statistically significant.

Results

Characteristics of study participants

Baseline characteristics of study participants did not significantly differ by treatment group (Table 2). The mean age of study participants was 61 years, 70% were men, 71% were white, and 20% had a family history of colorectal cancer in a

first-degree relative. Most participants were nonsmokers, college graduates, and overweight.

Adherence to visit attendance averaged 92% and did not significantly differ among the 4 treatment groups. On average, at least 80% of pills were taken by 93% of participants at the first follow-up visit and by 84% of participants at the final follow-up visit. No adverse events were attributed to study procedures or treatments. Seven participants (8%) were lost to follow-up. Dropouts included 1 person from the vitamin D₃ supplementation group and 2 from each of the other 3 groups.

Baseline serum 25-OH-vitamin D and 1,25-(OH)₂-vitamin D levels did not differ between the 4 treatment groups. At the conclusion of the study, serum 25-OH-vitamin D levels had increased 60% ($P < 0.0001$) and 56% ($P < 0.0001$) in the vitamin D₃ and calcium/vitamin D₃ groups, respectively, relative to placebo; however, mean serum 25-OH-vitamin D concentrations remained below 32 ng/mL in all treatment groups (20). There was no evidence of treatment effect modification by obesity status (body mass index, BMI, ≥ 30).

Calcium receptor

CaR expression along the full lengths of colorectal crypts appeared highest at the base of the crypts and gradually decreased toward the crypt apex (Fig. 3A). After 6 months of treatment, CaR expression statistically significantly increased along the full length of crypts by 27% ($P = 0.03$), 39% ($P = 0.01$), and 24% ($P = 0.05$) in the calcium, vitamin D₃, and calcium/vitamin D₃ groups, respectively, relative to the placebo group. In the upper 40% of crypts, the CaR expression increased 21% ($P = 0.13$), 38% ($P = 0.02$), and 26% ($P = 0.07$) in the calcium, vitamin D₃, and calcium/vitamin D₃ groups, respectively, relative to the placebo group. In the lower 60% of crypts, CaR expression increased 17% ($P = 0.14$), 26% ($P = 0.06$), and 17% ($P = 0.15$) in the calcium, vitamin D₃, and calcium/vitamin D₃ groups, respectively, relative to the placebo group (Table 3A).

Vitamin D receptor

VDR expression along the full lengths of colorectal crypts appeared lowest at the base of crypts and gradually increased toward the crypt apex (Fig. 3B). After 6 months of treatment, VDR expression increased in the calcium/vitamin D₃ group 19% ($P = 0.13$) along the full length of crypts, 19% ($P = 0.09$) in the upper 40% of crypts, and 31% ($P = 0.10$) in the lower 60% of

Table 2. Selected baseline characteristics of the study participants^a (*n* = 92)

Characteristics	Treatment group				P-value ^b
	Placebo (<i>n</i> = 23)	Calcium (<i>n</i> = 23)	Vitamin D (<i>n</i> = 23)	Calcium + vitamin D (<i>n</i> = 23)	
Demographics, medical history, habits, anthropometrics					
Age, y	58.5 (8.2)	61.9 (8.2)	60.2 (8.1)	62.1 (7.5)	0.39
Men, %	70	70	70	70	1.00
White, %	74	83	65	61	0.39
College graduate, %	65	61	57	44	0.53
History of colorectal cancer in first-degree relative, %	17	30	17	13	0.60
Take NSAID regularly ^c , %	22	13	9	22	0.60
If woman (<i>n</i> = 28), taking estrogens, %	4	9	4	4	1.00
Current smoker, %	9	4	0	0	0.61
Take multivitamin, %	30	30	26	39	0.86
BMI, kg/m ²	30.6 (7.2)	29.4 (5.5)	28.9 (5.56)	31.6 (6.0)	0.44
Mean dietary intakes ^d					
Total energy intake, kcal/d	1,596 (528)	1,788 (691)	1,848 (821)	1,845 (752)	0.59
Total ^e calcium, mg/d	625	678	753	733	0.75
Total ^e vitamin D, IU/d	279	326	348	401	0.50
Total fat, gm/d	66	66	61	65	0.59
Dietary fiber, gm/d	15	16	16	15	0.97
Alcohol intake, gm/d	8	10	13	9	0.84
Total serum vitamin D					
25-OH-vitamin D, ng/mL	20.4 (7.6)	25.7 (7.6)	21.0 (8.3)	20.9 (9.7)	0.12

Abbreviation: NSAID, nonsteroidal anti-inflammatory drug.

^aData are given as means (SD) unless otherwise specified.

^bBy Fisher's exact χ^2 test for categorical variables, and ANOVA for continuous variables.

^cAt least once a week.

^dAll nutrients energy adjusted using residual method (49).

^eDiet plus supplements.

crypts, relative to the placebo group. In the calcium and vitamin D₃ groups, there were also estimated increases in VDR expression, but they were of lower magnitude and not statistically significant (Table 3B).

CYP27B1

CYP27B1 expression along the full lengths of colorectal crypts at baseline tended to decrease from the base to the mid-third of the crypt where it remained level and then tended to increase slightly from there to the crypt apex (Fig. 3C). After 6 months of treatment, CYP27B1 expression increased in the vitamin D₃ group 159% (*P* = 0.06) along the full length of crypts, 111% (*P* = 0.04) in the upper 40% of crypts, and 110% (*P* = 0.04) in the lower 60% of crypts, relative to the placebo group. In the calcium and the calcium/vitamin D₃ groups, there were also estimated increases in CYP27B1 expression, similar in both treatment groups, but of much lower magnitude than in the vitamin D₃ alone group and not statistically significant (Table 4A).

CYP24A1

CYP24A1 expression along the full lengths of colorectal crypts appeared highest at the base of crypts and sharply

declined toward the upper 40% of the crypts, followed by a leveling off of expression throughout the upper parts of crypts (Fig. 3D). After 6 months of treatment, CYP24A1 expression in the calcium group tended to decrease, particularly in the upper 40% of the crypts, whereas in the vitamin D₃ groups it tended to increase throughout the crypts (Table 4B); however, despite the large, estimated treatment effects none of these findings was statistically significant.

Discussion

The results of this pilot randomized, controlled clinical trial provide the first evidence that supplemental calcium and vitamin D₃, alone or in combination, modify CaR, VDR, CYP27B1, and CYP24A1 expression in the normal colorectal mucosa of sporadic adenoma patients. Following 6 months of treatment, 2.0 g of calcium daily significantly increased CaR expression, but did not substantially change VDR expression. Although not statistically significant, the results also suggested that calcium substantially increased CYP27B1 and decreased CYP24A1 expression. Treatment with 800 IU of vitamin D₃ daily significantly increased expression of the CaR (slightly more than did calcium) and CYP27B1. Although

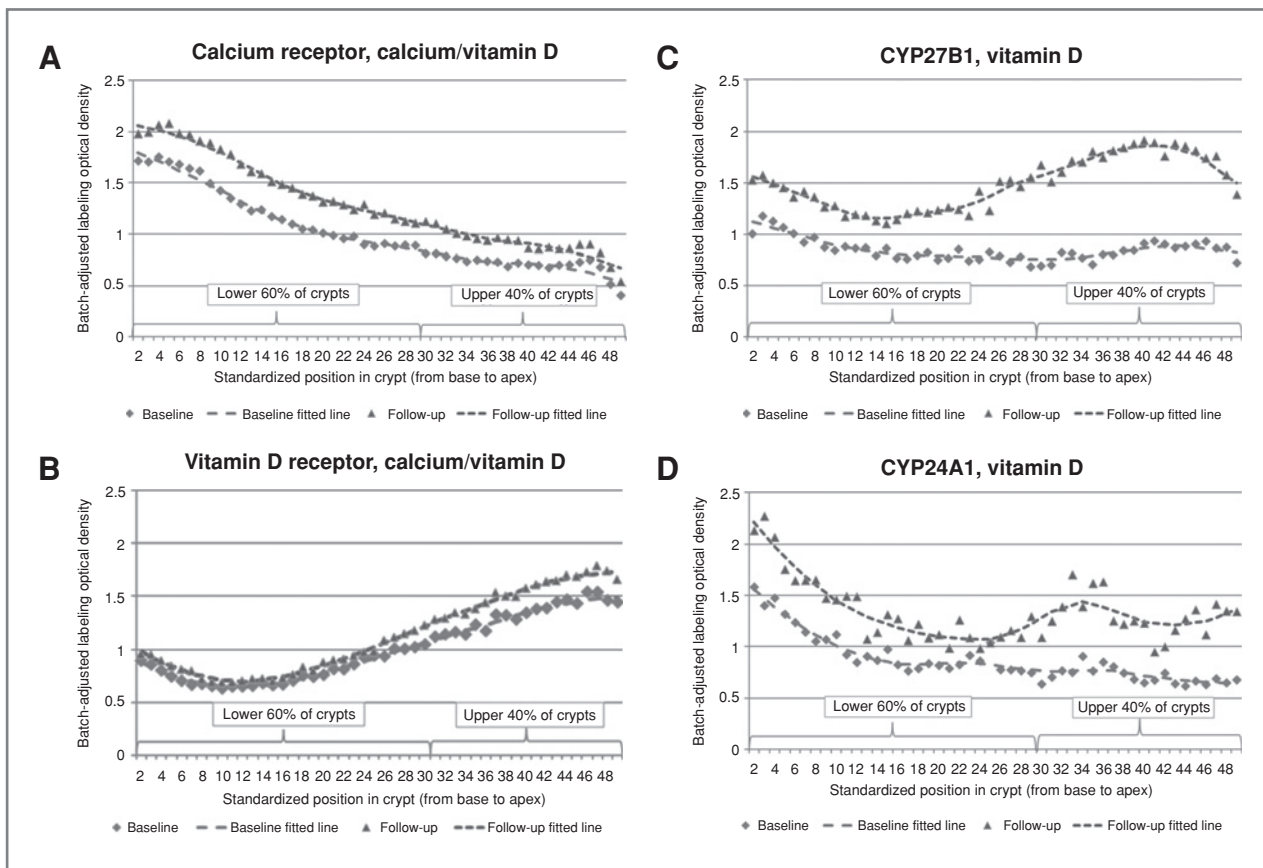


Figure 3. Representative examples of labeling expression distribution (distribution plots represent labeling optical density in colon crypts) of the (A) calcium receptor and (B) VDR (representative example from the calcium/vitamin D₃ treatment group), (C) CYP27B and (D) CYP24A1 (representative example from the vitamin D₃ treatment group) along normal colorectal crypts by treatment group at baseline and 6-month follow-up.

not statistically significant, the results also suggested that vitamin D₃ increased VDR and CYP24A1 expression. The combined calcium and vitamin D₃ treatment statistically significantly increased CaR expression to about the same degree as calcium treatment alone, and thus less than vitamin D₃ alone. Although not statistically significant, the results also suggested that the combined treatment increased expression of the VDR (more than did calcium or vitamin D₃ alone), CYP27B1 (about the same as did calcium alone, but less than vitamin D₃ alone), and CYP24A1 (about the same as did vitamin D₃ alone). The findings from this study are relevant because there is substantial epidemiologic (11, 25, 26) and basic science (19, 27) evidence that calcium and vitamin D₃ may reduce risk of incident or recurrent colorectal neoplasms, and that any protective effects of calcium and vitamin D₃ may, at least in part, operate through the CaR, the VDR, and the vitamin D-metabolizing enzymes, CYP27B1 and CYP24A1.

Expression of the CaR was reported in normal appearing mucosa in humans (28–32) and rats (33), and in differentiated carcinomas (28–32); however, sporadic to no expression was reported in undifferentiated carcinomas (28–32). It was proposed that in normal colon crypts, there is a calcium gradient with low calcium concentrations at the base of the crypts and high concentrations at the crypt apex (34). Decreased calcium

concentrations were reported to increase cellular proliferation (4, 32, 35, 36), whereas increased calcium concentrations were reported to decrease proliferation and promote differentiation (4, 28–30, 32, 36). Loss of function of the CaR disrupts the signaling actions from increased calcium concentrations, such as activating E-cadherin and downregulating β -catenin/TCF signaling (28).

There are no reported human randomized clinical trials on the effects of calcium and vitamin D on CaR expression in normal colon mucosa; however, these effects have been evaluated in colon cancer cell lines. In cell culture, incubation with calcium and/or 1,25(OH)₂D₃ increased CaR expression (30, 36); calcium and 1,25(OH)₂D₃ combined increased CaR expression more than did calcium or 1,25(OH)₂D₃ alone (30). We, therefore, hypothesized that supplementation with calcium and vitamin D₃ alone and combined would increase CaR expression in the normal colon mucosa. Our hypothesis was further supported by the presence of vitamin D response elements in both the P1 and P2 promoters of the CaR gene (37). As hypothesized, we observed increased CaR expression in the calcium, vitamin D, and the combination calcium/vitamin D₃ supplementation groups. Vitamin D₃ supplementation alone appeared to have had the largest treatment effect, whereas calcium alone and in combination with vitamin D₃

Table 3. Expression of calcium receptor and VDR in the normal appearing colorectal mucosa during the clinical trial

Treatment group	Baseline				6-mo follow-up				Absolute Rx effect			Relative effect ^c
	<i>n</i>	Mean	SE	<i>P</i>	<i>n</i>	Mean	SE	<i>P</i>	Rx effect ^a	SE	<i>P</i> ^b	
A. CaR												
Whole crypts												
Placebo	20	1.11	0.07		19	1.00	0.07		0.00	.	.	1.00
Calcium	22	1.01	0.06	0.28	20	1.16	0.07	0.09	0.26	0.12	0.03	1.27
Vitamin D	23	0.87	0.06	0.01	19	1.09	0.07	0.37	0.33	0.12	0.01	1.39
Ca + vitamin D	22	1.04	0.06	0.45	20	1.16	0.07	0.10	0.23	0.12	0.05	1.24
Upper 40% of crypts												
Placebo	20	0.34	0.02		19	0.32	0.02		0.00	.	.	1.00
Calcium	22	0.31	0.02	0.37	20	0.35	0.02	0.33	0.06	0.04	0.13	1.21
Vitamin D	23	0.25	0.02	0.01	19	0.33	0.02	0.89	0.09	0.04	0.02	1.38
Ca + vitamin D	22	0.29	0.02	0.13	20	0.35	0.02	0.45	0.07	0.04	0.07	1.26
Lower 60% of crypts												
Placebo	20	0.76	0.05		19	0.74	0.05		0.00	.	.	1.00
Calcium	22	0.73	0.04	0.63	20	0.84	0.05	0.16	0.12	0.08	0.14	1.17
Vitamin D	23	0.63	0.04	0.04	19	0.77	0.05	0.66	0.16	0.08	0.06	1.26
Ca + vitamin D	22	0.73	0.04	0.57	20	0.83	0.05	0.18	0.12	0.08	0.15	1.17
B. VDR												
Whole crypts												
Placebo	21	1.06	0.06		18	1.00	0.07		0.00	.	.	1.00
Calcium	21	0.95	0.06	0.19	21	0.93	0.06	0.46	0.05	0.12	0.67	1.05
Vitamin D	22	0.93	0.06	0.14	17	0.97	0.07	0.70	0.09	0.12	0.43	1.10
Ca + vitamin D	21	1.06	0.06	0.92	18	1.18	0.07	0.07	0.18	0.12	0.13	1.19
Upper 40% of crypts												
Placebo	21	0.58	0.03		18	0.59	0.04		0.00	.	.	1.00
Calcium	21	0.52	0.03	0.19	21	0.56	0.03	0.47	0.03	0.06	0.66	1.05
Vitamin D	22	0.49	0.03	0.04	17	0.55	0.04	0.38	0.05	0.06	0.41	1.11
Ca + vitamin D	21	0.54	0.03	0.42	18	0.66	0.04	0.19	0.10	0.06	0.09	1.19
Lower 60% of crypts												
Placebo	21	0.47	0.04		18	0.41	0.04		0.00	.	.	1.00
Calcium	21	0.46	0.04	0.81	21	0.41	0.04	0.92	0.01	0.07	0.92	1.01
Vitamin D	22	0.45	0.04	0.58	17	0.44	0.04	0.58	0.06	0.08	0.44	1.14
Ca + vitamin D	21	0.45	0.04	0.69	18	0.52	0.04	0.06	0.13	0.08	0.10	1.31

^aRx effect = [(treatment group follow-up) – (treatment group baseline)] – [(placebo group follow-up) – (placebo group baseline)].

^b*P* value for difference between each active treatment group and placebo group from repeated-measures mixed model.

^cRelative effect = [(treatment group follow-up)/(treatment group baseline)]/[(placebo follow-up)/(placebo baseline)]; interpretation similar to that for an odds ratio (e.g., a relative effect of 1.7 indicates a 70% proportional increase in the treatment group relative to that in the placebo group).

appeared to yield lesser, approximately equal treatment effects. We expected that calcium plus vitamin D₃ supplementation would have the greatest treatment effect. There are several plausible explanations for why this was not observed, the first being that our findings may have been due to chance. At least 1 experiment in rodents reported that calcium and vitamin D individually suppressed tumorigenesis, but the combination of the 2 was ineffective (38). However, some studies reported stronger combined effects of calcium and vitamin D₃ in reducing adenoma and colorectal cancer risk (39, 40). Some of the strongest evidence comes from a large

clinical trial of colorectal adenoma recurrence that suggested that calcium supplementation was primarily effective among those with 25(OH)D concentrations greater than the median in the study population (29.1 ng/mL; ref. 11). In our trial, only the vitamin D₃ supplementation group reached 25(OH)D concentrations greater than 29.1 ng/mL (20), suggesting the possibility of a threshold effect.

There is little consensus on the distribution of the CaR in the normal colon crypt (28, 30, 31, 33). Our study, the largest to investigate this thus far, used disease free colon tissue rather than normal appearing sections of colon mucosa adjacent to

Table 4. Expression of CYP27B1 and CYP24A1 in the normal-appearing colorectal mucosa during the clinical trial

Treatment group	Baseline				6-mo follow-up				Absolute Rx effect			Relative effect ^c
	<i>n</i>	Mean	SE	<i>P</i>	<i>n</i>	Mean	SE	<i>P</i>	Rx effect ^a	SE	<i>P</i> ^b	
A. CYP27B1												
Whole crypts												
Placebo	20	1.07	0.18		20	1.00	0.09		0.00	.	.	1.00
Calcium	21	0.89	0.10	0.38	16	1.11	0.36	0.76	0.29	0.42	0.49	1.34
Vitamin D	22	0.85	0.12	0.31	18	2.06	0.61	0.10	1.28	0.66	0.06	2.59
Ca + vitamin D	22	1.20	0.15	0.56	19	1.46	0.41	0.29	0.32	0.48	0.50	1.30
Upper 40% of crypts												
Placebo	20	0.40			20	0.45	0.10	0.10	0.00	.	.	1.00
Calcium	21	0.38	0.10	0.86	16	0.51	0.11	0.70	0.08	0.21	0.69	1.21
Vitamin D	22	0.35	0.10	0.73	18	0.84	0.11	0.01	0.44	0.20	0.04	2.11
Ca + vitamin D	22	0.46	0.10	0.67	19	0.57	0.10	0.43	0.05	0.20	0.79	1.09
Lower 60% of crypts												
Placebo	20	0.65	0.13		20	0.58	0.13		0.00	.	.	1.00
Calcium	21	0.52	0.13	0.47	16	0.60	0.14	0.92	0.15	0.25	0.55	1.30
Vitamin D	22	0.51	0.13	0.44	18	0.96	0.14	0.05	0.52	0.25	0.04	2.10
Ca + vitamin D	22	0.67	0.13	0.95	19	0.72	0.13	0.43	0.14	0.25	0.59	1.23
B. CYP24A1												
Whole crypts												
Placebo	21	1.04	0.21		18	1.00	0.23		0.00	.	.	1.00
Calcium	20	1.05	0.21	0.96	20	0.80	0.21	0.52	-0.22	0.43	0.62	0.79
Vitamin D	21	0.85	0.21	0.52	19	1.34	0.22	0.28	0.53	0.43	0.22	1.65
Ca + vitamin D	22	1.06	0.20	0.94	17	1.54	0.23	0.10	0.52	0.43	0.24	1.51
Upper 40% of crypts												
Placebo	21	0.37	0.09		18	0.40	0.10		0.00	.	.	1.00
Calcium	20	0.42	0.09	0.70	20	0.27	0.09	0.35	-0.18	0.19	0.35	0.59
Vitamin D	21	0.31	0.09	0.68	19	0.51	0.10	0.40	0.17	0.19	0.37	1.51
Ca + vitamin D	22	0.40	0.09	0.81	17	0.59	0.10	0.17	0.16	0.19	0.39	1.38
Lower 60% of crypts												
Placebo	21	0.69	0.13		18	0.63	0.14		0.00	.	.	1.00
Calcium	20	0.66	0.13	0.89	20	0.58	0.13	0.79	-0.03	0.25	0.92	0.96
Vitamin D	21	0.61	0.13	0.67	19	0.78	0.14	0.46	0.22	0.25	0.39	1.38
Ca + vitamin D	22	0.74	0.13	0.78	17	0.89	0.14	0.21	0.21	0.26	0.43	1.31

Abbreviations: CYP27B1, cytochrome P450 family 27B1; CYP24A1, cytochrome P450 family 24A1.

^aRx effect = [(treatment group follow-up) – (treatment group baseline)] – [(placebo group follow-up) – (placebo group baseline)].

^b*P* value for difference between each active treatment group and placebo group from repeated-measures mixed model.

^cRelative effect = [(treatment group follow-up)/(treatment group baseline)]/[(placebo follow-up)/(placebo baseline)];

interpretation similar to that for an odds ratio (e.g., a relative effect of 1.7 indicates a 70% proportional increase in the treatment group relative to that in the placebo group).

neoplastic tissue and, to the best of our knowledge, is the first to use image analysis software to analyze CaR distribution in the normal colon mucosa. We found CaR expression to be highest at the base of the crypts, amongst the most proliferative cells and to gradually decline in the differentiation zone toward the crypt apex. These results suggest that CaR expression may undergo negative feedback regulation by luminal calcium.

An autocrine/paracrine function for vitamin D in the normal colon mucosa is suggested by the expression of the VDR and the vitamin D-metabolizing enzymes CYP27B1 and

CYP24A1 in the colon mucosa (15). The ability of colon cells to produce the active vitamin D metabolite, 1,25(OH)₂D, was recently demonstrated (41–43). Evidence from animal models suggests that the colonic vitamin D system is regulated separately from the renal vitamin D system (5). It was reported that renal synthesis of 1,25(OH)₂D does not produce 1,25(OH)₂D in the nanomolar concentrations needed to halt proliferation and induce apoptosis, suggesting that colonic metabolism of 1,25(OH)₂D is needed to explain vitamin D's possible anti-colon carcinogenesis effects (19). The expression of the VDR and CYP27B1 was reported to increase in the

early stages of cancer development, but to be greatly reduced in undifferentiated cancers; this suggests that the VDR and CYP27B1 act as tumor suppressors, presumably by maximizing 1,25(OH)₂D signaling to normalize cell-cycle regulation (16–18). CYP24A1 expression was reported to be increased in undifferentiated cancers, presumably mitigating the effects of 1,25(OH)₂D signaling (17, 41). Our observation of increased VDR and CYP27B1 expression in the active treatment groups is in agreement with previous reports, which suggests that the antiproliferative and proapoptotic effects of supplemental vitamin D₃ may, in part, be due to increased VDR and CYP27B1 expression. We also observed an insignificant increase in CYP24A1 expression in the vitamin D and calcium plus vitamin D treatment groups, which may minimize the effects of increased VDR and CYP27B1 expression. However, the apparent decrease of CYP24A1 and increase in CYP27B1 in the calcium treatment group may lead to increased vitamin D signaling despite no increase in VDR expression; however, these estimated treatment effects were not statistically significant. The distribution of the VDR and CYP27B1 in the upper 40% of crypts provides additional evidence of the importance of vitamin D signaling for promoting differentiation and apoptosis. CYP24A1 also appeared to be expressed most strongly in the bottom half of the colon crypts, presumably lessening the effects of 1,25(OH)₂D and thus favoring proliferation.

There are only a few cell culture and animal studies and no large randomized human trials on the effects of calcium and/or vitamin D on the expression of the VDR, CYP27B1, or CYP24A1 in the normal human colon mucosa. The effects of 25(OH)D₃ or 1,25(OH)₂D₃ on VDR and CYP27B1 expression in colon cancer cells appear to vary depending upon the differentiation status of the cells; however, CYP24A1 expression consistently increases in response to 25(OH)D₃ or 1,25(OH)₂D₃ treatment (42, 44, 45). Two separate studies in mice found that low calcium diets increased CYP24A1 mRNA expression, especially in male mice and in the right colon. The low calcium diet also increased VDR and CYP27B1 mRNA expression in the proximal colon and reduced VDR expression in the distal colon; however, this was only observed in female mice. No significant change in VDR or CYP27B1 expression was observed in either the left or right colon in male mice fed a low calcium diet (46, 47).

We previously reported that calcium and vitamin D₃ supplementation in this same trial favorably modified expression of markers of proliferation (8), differentiation (8), apoptosis (20), and oxidative DNA damage (48) in the normal human colorectal mucosa. Previous reports in cancer cell lines (49) and human studies (48) suggest that some of the chemopreventive effects of supplemental calcium and vitamin D₃ may depend on the expression of the CaR and the VDR. Our results provide *in vivo* human evidence that supplemental calcium and vitamin D₃ modify the expression of the CaR, the VDR, CYP27B1, and CYP24A1 in the normal colorectal epithelium, which may then promote calcium and vitamin D antineoplastic signaling pathways.

Our study had several limitations. First, it was a pilot study with a relatively small sample size, increasing the role of

chance observations and limiting our ability to perform stratified analyses. Also, we only examined the rectal mucosa and therefore treatment effects in other parts of the colon remain unknown. Another limitation is that we measured protein expression but not protein activity and, therefore, could not correlate changes in expression with changes in protein activity. Finally, these markers are not proven biomarkers of risk and we do not yet know whether changes in CaR, VDR, CYP27B1, or CYP24A1 affect risk for colorectal neoplasms in humans.

The strengths of this study include (i) that it is, to our knowledge, the first randomized, double-blind, placebo-controlled clinical trial to test the effects of supplemental calcium and vitamin D₃, alone and in combination, on components of the calcium and vitamin D-metabolizing system in the normal colorectal epithelium in sporadic adenoma patients; (ii) the high protocol adherence by study participants; and (iii) the automated immunostaining and newly designed image analysis software to quantify the crypt distribution of the expression of the CaR, the VDR, CYP27B1, and CYP24A1, resulting in high biomarker measurement reliability.

In summary, the results of this pilot clinical trial indicate that calcium and vitamin D₃ supplementation, alone and in combination, may increase expression of the CaR, the VDR, and CYP27B1, and that vitamin D₃ supplementation, alone and in combination with calcium supplementation, may increase CYP24A1 expression in the normal colorectal mucosa of sporadic colorectal adenoma patients. The anticarcinogenesis effects of supplemental calcium and vitamin D₃ may, in part, depend on the ability of these agents to favorably modulate the expression of the CaR, the VDR, CYP27B1, and CYP24A1 in the colorectal mucosa. These results, taken together with previous reports (8, 20, 48), suggest that calcium and vitamin D₃ supplementation modulate the colorectal mucosa molecular phenotype to inhibit proliferation and promote cellular differentiation and apoptosis. Our results also suggest that the CaR, the VDR, CYP27B1, and CYP24A1 may be treatable biomarkers of risk for colorectal neoplasms and warrant further investigation. Finally, our results support further investigation of calcium and vitamin D₃ as chemopreventive agents against colorectal neoplasms.

Disclosure of Potential Conflicts of Interest

The National Cancer Institute, the Georgia Cancer Coalition, and the Franklin Foundation 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.

Grant Support

National Cancer Institute, National Institutes of Health (R01 CA104637 and R03 CA121873 to R.M. Bostick); Georgia Cancer Coalition Distinguished Scholar award (R.M. Bostick); the Franklin Foundation.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 10, 2010; revised October 7, 2010; accepted October 29, 2010; published OnlineFirst November 17, 2010.

References

- Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer Statistics 2009. *CA A Cancer Journal for Clinicians* 2009;59:225–49.
- Potter JD. Colorectal cancer: molecules and populations. *J Natl Cancer Inst* 1999;91:916–32.
- Peterlik M, Grant WB, Cross HS. Calcium, vitamin D and cancer. *Anticancer Res* 2009;29:3687–98.
- Kallay E, Bonner E, Wrba F, Thakker RV, Peterlik M, Cross HS. Molecular and functional characterization of the extracellular calcium-sensing receptor in human colon cancer cells. *Oncol Res* 2003;13:551–9.
- Kallay E, Bises G, Bajna E, Bieglmayer C, Gerdenitsch W, Steffan I, et al. Colon-specific regulation of vitamin D hydroxylases—a possible approach for tumor prevention. *Carcinogenesis* 2005;26:1581–9.
- McCullough ML, Giovannucci EL. Diet and cancer prevention. *Oncogene* 2004;23:6349–64.
- Jiménez-Lara AM. Colorectal cancer: potential therapeutic benefits of Vitamin D. *The Int J Biochem Cell Biol* 2007;39:672–7.
- 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;18:2933–41.
- Palmer HG, Gonzalez-Sancho JM, Espada J, Berciano MT, Puig I, Baulida J, et al. Vitamin D3 promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of β -catenin signaling. *J Cell Biol* 2001;154:369–88.
- Bostick RM, Goodman M, Sidelnikov E. *Genetics of Colorectal Cancer*. New York: Springer Science + Business Media, LLC; 2009.
- 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;95:1765–71.
- Gorham ED, Garland CF, Garland FC, Grant WB, Mohr SB, Lipkin M, et al. Optimal vitamin D status for colorectal cancer prevention: a quantitative meta analysis. *Am J Prev Med* 2007;32:210–6.
- Fedirko V, Bostick RM, Goodman M, Flanders WD, Gross MD. Blood 25-hydroxyvitamin D3 concentrations and incident sporadic colorectal adenoma risk: a pooled case-control study. *Am J Epidemiol* 2010;172:489–500.
- Whitfield JF. The calcium-sensing receptor—a driver of colon cell differentiation. *Curr Pharm Biotechnol* 2009;10:311–6.
- Cross HS, Kallay E. Regulation of the colonic vitamin D system for prevention of tumor progression: an update. *Future Oncol* 2009;5:493–507.
- Cross HS, Bareis P, Hofer H, Bischof MG, Bajna E, Kriwanek S, et al. 25-Hydroxyvitamin D3-1 α -hydroxylase and vitamin D receptor gene expression in human colonic mucosa is elevated during early carcinogenesis. *Steroids* 2001;66:287–92.
- Cross HS, Bises G, Lechner D, Manhardt T, Kállay E. The Vitamin D endocrine system of the gut—its possible role in colorectal cancer prevention. *J Steroid Biochem Mol Biol* 2005;97:121–8.
- Bises G, Kallay E, Weiland T, Wrba F, Wenzl E, Bonner E, et al. 25-Hydroxyvitamin D3-1 α -hydroxylase expression in normal and malignant human colon. *J Histochem Cytochem* 2004;52:985–9.
- Cross HS, Nittke T, Peterlik M. Modulation of vitamin D synthesis and catabolism in colorectal mucosa: a new target for cancer prevention. *Anticancer Res* 2009;29:3705–12.
- Fedirko V, Bostick RM, Flanders WD, Long Q, Shaikat A, Rutherford RE, et al. Effects of vitamin D and calcium supplementation on markers of apoptosis in normal colon mucosa: a randomized, double-blind, Placebo-controlled clinical trial. *Cancer Prev Res* 2009;2:213–23.
- Willett WC, Sampson L, Stampfer MJ, Rosner B, Bain C, Witschi J, et al. Reproducibility and validity of a semiquantitative food frequency questionnaire. *Am J Epidemiol* 1985;122:51–65.
- 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;6:931–42.
- Hollis BW. Quantitation of 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D by radioimmunoassay using radioiodinated tracers. *Meth-ods Enzymol* 1997;282:174–86.
- Hollis BW, Kamerud JQ, Kurkowski A, Beaulieu J, Napoli JL. Quantification of circulating 1,25-dihydroxyvitamin D by radioimmunoassay with 125I-labeled tracer. *Clin Chem* 1996;42:586–92.
- Tangrea J, Helzlsouer K, Pietinen P, Taylor P, Hollis B, Virtamo J, et al. Serum levels of vitamin D metabolites and the subsequent risk of colon and rectal cancer in Finnish men. *Cancer Causes Control* 1997;8:615–25.
- Wu K, Willett WC, Fuchs CS, Colditz GA, Giovannucci EL. Calcium intake and risk of colon cancer in women and men. *J Natl Cancer Inst* 2002;94:437–46.
- Whitfield JF. Calcium, calcium-sensing receptor and colon cancer. *Cancer Lett* 2009;275:9–16.
- Bhagavathula N, Hanosh AW, Nerusu KC, Appelman H, Chakrabarty S, Varani J. Regulation of E-cadherin and beta-catenin by Ca^{2+} in colon carcinoma is dependent on calcium-sensing receptor expression and function. *Int J Cancer* 2007;121:1455–62.
- 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 β -Catenin/TCF activation. *Cancer Res* 2003;63:67–71.
- 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 D3. *Cancer Res* 2005;65:493–8.
- Sheinin Y, Kallay E, Wrba F, Kriwanek S, Peterlik M, Cross HS. Immunocytochemical localization of the extracellular calcium-sensing receptor in normal and malignant human large intestinal mucosa. *J Histochem Cytochem* 2000;48:595–602.
- Kallay E, Bajna E, Wrba F, Kriwanek S, Peterlik M, Cross HS. Dietary calcium and growth modulation of human colon cancer cells: role of the extracellular calcium-sensing receptor. *Cancer Detect Prev* 2000;24:127–36.
- Cheng SX, Okuda M, Hall AE, Geibel JP, Hebert SC. Expression of calcium-sensing receptor in rat colonic epithelium: evidence for modulation of fluid secretion. *Am J Physiol Gastrointest Liver Physiol* 2002;283:G240–50.
- Brenner BM, Russell N, Albrecht S, Davies RJ. The effect of dietary vitamin D3 on the intracellular calcium gradient in mammalian colonic crypts. *Cancer Letters* 1998;127:43–53.
- Kállay E, Kifor O, Chattopadhyay N, Brown EM, Bischof MG, Peterlik M, et al. Calcium-dependent c-myc proto-oncogene expression and proliferation of CACO-2 cells: a role for a luminal extracellular calcium-sensing receptor. *Biochem Biophys Res Commun* 1997;232:80–3.
- Bhagavathula N, Kelley EA, Reddy M, Nerusu KC, Leonard C, Fay K, et al. Upregulation of calcium-sensing receptor and mitogen-activated protein kinase signalling in the regulation of growth and differentiation in colon carcinoma. *Br J Cancer* 2005;93:1364–71.
- Canaff L, Hendy GN. Human calcium-sensing receptor gene. *J Biol Chem* 2002;277:30337–50.
- Pence BC, Buddingh F. Inhibition of dietary fat-promoted colon carcinogenesis in rats by supplemental calcium or vitamin D3. *Carcinogenesis* 1988;9:187–90.
- Oh K, Willett WC, Wu K, Fuchs CS, Giovannucci EL. Calcium and vitamin D intakes in relation to risk of distal colorectal adenoma in women. *Am J Epidemiol* 2007;165:1178–86.
- Zheng W, Anderson KE, Kushi LH, Sellers TA, Greenstein J, Hong CP, et al. A prospective cohort study of intake of calcium, vitamin D, and other micronutrients in relation to incidence of rectal cancer among postmenopausal women. *Cancer Epidemiol Biomarkers Prev* 1998;7:221–5.
- Bareis P, Bises G, Bischof MG, Cross HS, Peterlik M. 25-hydroxyvitamin D metabolism in human colon cancer cells during tumor progression. *Biochem Biophys Res Commun* 2001;285:1012–7.
- Lechner D, Kállay E, Cross HS. 1 α ,25-Dihydroxyvitamin D3 down-regulates CYP27B1 and induces CYP24A1 in colon cells. *Mol Cellular Endocrinol* 2007;263:55–64.
- Cross HS, Peterlik M, Reddy GS, Schuster I. Vitamin D metabolism in human colon adenocarcinoma-derived Caco-2 cells: Expression of 25-hydroxyvitamin D3-1 α -hydroxylase activity and regulation of side-chain metabolism. *J Steroid Biochem Mol Biol* 1997;62:21–8.

44. Bareis P, Kállay E, Bischof MG, Bises G, Hofer H, Pötzi C, et al. Clonal differences in expression of 25-hydroxyvitamin D3-1 α -hydroxylase, of 25-hydroxyvitamin D3-24-hydroxylase, and of the vitamin D receptor in human colon carcinoma cells: effects of epidermal growth factor and 1 α ,25-dihydroxyvitamin D3. *Exp Cell Res* 2002;276:320-7.
45. Murillo G, Matusiak D, Benya RV, Mehta RG. Chemopreventive efficacy of 25-hydroxyvitamin D3 in colon cancer. *J Steroid Biochem Mol Biol* 2007;103:763-7.
46. Nittke T, Selig S, Kallay E, Cross HS. Nutritional calcium modulates colonic expression of vitamin D receptor and pregnane X receptor target genes. *Mol Nutr Food Res* 2008;52: S45-51.
47. Nittke T, Kallay E, Manhardt T, Cross HS. Parallel elevation of colonic 1,25-dihydroxyvitamin D3 levels and apoptosis in female mice on a calcium-deficient diet. *Anticancer Res* 2009;29:3727-32.
48. Fedirko V, Bostick RM, Long Q, Flanders WD, McCullough ML, Sidelnikov E, et al. Effects of supplemental vitamin D and calcium on oxidative DNA damage marker in normal colorectal mucosa: a randomized clinical trial. *Cancer Epidemiol Biomarkers Prev* 2010;19:280-91.
49. Liu G, Hu X, Chakrabarty S. Vitamin D mediates its action in human colon carcinoma cells in a calcium-sensing receptor-dependent manner: Downregulates malignant cell behavior and the expression of thymidylate synthase and survivin and promotes cellular sensitivity to 5-FU. *Int J Cancer* 2010;126:631-9.