

Research Article

A Randomized Clinical Trial of the Effects of Supplemental Calcium and Vitamin D₃ on the APC/ β -Catenin Pathway in the Normal Mucosa of Colorectal Adenoma PatientsThomas U. Ahearn^{1,4}, Aasma Shaukat⁵, W. Dana Flanders^{2,3,4}, Robin E. Rutherford⁶, and Roberd M. Bostick^{2,4}**Abstract**

APC/ β -catenin pathway perturbation is a common early event in colorectal carcinogenesis and is affected by calcium and vitamin D in basic science studies. To assess the effects of calcium and vitamin D on adenomatous polyposis coli (APC), β -catenin, and E-cadherin expression in the normal appearing colorectal mucosa of sporadic colorectal adenoma patients, we conducted a randomized, double-blinded, placebo-controlled 2 \times 2 factorial clinical trial. Pathology-confirmed colorectal adenoma cases were treated with 2 g/day elemental calcium and/or 800 IU/day vitamin D₃ versus placebo over 6 months ($N = 92$; 23/group). Overall APC, β -catenin, and E-cadherin expression and distributions in colon crypts in normal-appearing rectal mucosa biopsies were detected by standardized automated immunohistochemistry and quantified by image analysis. In the vitamin D₃-supplemented group relative to placebo, the proportion of APC in the upper 40% of crypts (Φ h APC) increased 21% ($P = 0.01$), β -catenin decreased 12% ($P = 0.18$), E-cadherin increased 72% ($P = 0.03$), and the Φ h APC/ β -catenin ratio (APC/ β -catenin score) increased 31% ($P = 0.02$). In the calcium-supplemented group Φ h APC increased 10% ($P = 0.12$), β -catenin decreased 15% ($P = 0.08$), and the APC/ β -catenin score increased 41% ($P = 0.01$). In the calcium/vitamin D₃-supplemented group, β -catenin decreased 11% ($P = 0.20$), E-cadherin increased 51% ($P = 0.08$), and the APC/ β -catenin score increased 16% ($P = 0.26$). These results support (i) that calcium and vitamin D modify APC, β -catenin, and E-cadherin expression in humans in directions hypothesized to reduce risk for colorectal neoplasms, (ii) calcium and vitamin D as potential chemopreventive agents against colorectal neoplasms, and (iii) the potential of APC, β -catenin, and E-cadherin expression as modifiable, preneoplastic risk biomarkers for colorectal neoplasms. *Cancer Prev Res*; 5(10); 1247–56. ©2012 AACR.

Introduction

Colorectal cancer (CRC), the second leading cause of cancer deaths in the United States (1), is responsible for approximately 8% of all cancer deaths worldwide (2, 3). The etiology of sporadic CRC is predominately rooted in dietary and lifestyle behaviors (2, 4), suggesting that it may be preventable. The molecular basis of colorectal carcinogenesis is becoming clearer (4); however, there are no validated,

treatable, preneoplastic biomarkers of risk for colorectal neoplasms.

Malfunction of the APC/ β -catenin signaling pathway is an early and common event in the pathogenesis of colorectal neoplasms. Impaired adenomatous polyposis coli (APC) function occurs in approximately 80% to 90% of sporadic CRCs (5), resulting in the increased potential of β -catenin to translocate to the nucleus and activate target genes responsible for promoting cell proliferation and inhibiting differentiation (5–7). E-cadherin may also antagonize β -catenin nuclear expression by sequestering β -catenin to its cytoplasmic tail, linking E-cadherin to actin filaments and promoting cell adhesion and differentiation (5, 7). We reported that APC expression [especially the proportion of APC in the upper 40% of colorectal crypts (Φ h APC)], β -catenin expression, and the Φ h APC/ β -catenin ratio (APC/ β -catenin score) in normal colorectal mucosa may be valid, potentially modifiable, preneoplastic biomarkers of risk for colorectal neoplasms (8).

Convincing evidence from experimental and observational studies and randomized, placebo-controlled clinical trials suggests that calcium and vitamin D have chemopreventive effects against colorectal neoplasms (9). The beneficial effects of calcium may partially be attributed to

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its binding of toxic secondary bile acids and ionized fatty acids, and/or by directly inhibiting proliferation and promoting differentiation and apoptosis (9). Vitamin D signaling may induce cell-cycle arrest and promote differentiation and apoptosis directly through vitamin D-mediated gene transcription and indirectly through modifying growth factors, and play roles in promoting oxidative DNA damage repair, inhibiting angiogenesis, and regulating immune cell function (9). Also, prospective cohort studies have consistently found higher total calcium intake to be associated with lower risk for colorectal neoplasms (9), calcium supplementation reduces colorectal adenoma recurrence (modified by vitamin D status; 10), and higher circulating 25(OH)-vitamin D (25(OH)D) is inversely associated with colorectal neoplasms (9, 11, 12).

Evidence from animal models (13, 14) and *in vitro* (15–17) studies suggest that the chemopreventive effects of calcium and vitamin D may, in part, include modification of the APC/ β -catenin signaling pathway. However, to our knowledge there are no reported human *in vivo* investigations on the effects of supplemental calcium and vitamin D₃ on the expression of APC, β -catenin, and E-cadherin in the normal colorectal mucosa. 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 APC, β -catenin, and E-cadherin expression 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 (18). 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 of 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%) attended the eligibility visit to be interviewed, sign a consent form, complete questionnaires, and provide blood samples (18). Diet was assessed using a semiquantitative Willett Food Frequency Questionnaire (19). 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 were randomly assigned, stratified on

sex and nonsteroidal anti-inflammatory drug use, 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 (18). Participants were instructed to maintain their usual diet and not take any new nutritional supplements 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 before 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 carried out by placing the slides in a preheated Pretreatment Module (Lab Vision Corp.) with 100× 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 APC (OP80; 1:70 dilution, Calbiochem), β -catenin (610154; 1:300 dilution; Transduction Laboratories), and E-cadherin (Zymed 33-4000; 1:50 dilution). No slides were counterstained. After processing, slides were coverslipped with a Leica CV5000 Coverslipper (Leica Microsystems, Inc.). 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 (18). Briefly, a scorable crypt was defined as an intact crypt extending from the muscularis mucosa to the colon lumen (20). Before "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. 1).

Reliability control was conducted by selecting samples of previously analyzed slides to be reanalyzed by the technician, who was blinded to the selection. Intrareader reliability was greater than 0.90 for APC, β -catenin, and E-cadherin.

Protocol for measuring serum 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 method as previously described (21, 22). 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

All statistical analyses were conducted using SAS 9.3 statistical software (SAS Institute Inc.). A *P* value ≤ 0.05

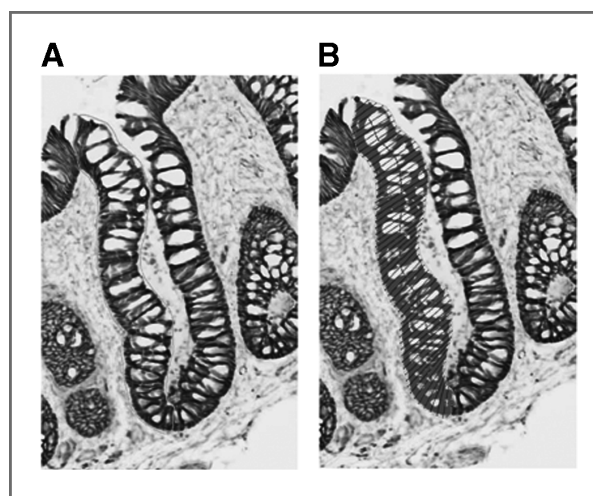


Figure 1. Quantitative image analysis. A, finding and tracing the hemicypt; B, automated sectioning and quantification of β -catenin labeling optical density (figure adapted from reference 8).

(2-sided) was considered statistically significant. Treatment groups were assessed for comparability of characteristics at baseline and at final follow-up by the Fisher exact test for categorical variables and ANOVA for continuous variables. Slide scoring reliability was analyzed using intraclass 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 (18). To evaluate distinct functional zones of crypts, measures of crypt biomarker distribution selected *a priori* were the upper 40% of the crypts (differentiation zone), the lower 60% of the crypts (proliferation zone), and the ratio of the upper 40% of crypts to the whole crypt (Φ_h). An APC/ β -catenin score was calculated by dividing an individual's Φ_h APC by their β -catenin expression in the whole crypt (Φ_h APC expression/ β -catenin expression). E-cadherin was not included in the APC/ β -catenin score because during carcinogenesis malfunctioning regulation of β -catenin by APC occurs most often earlier than E-cadherin downregulation (23). We hypothesize that a higher score is associated with reduced potential of β -catenin to promote proliferation.

The distributions of batch standardized APC, β -catenin, and E-cadherin labeling optical densities along the full length of the crypts were graphically plotted and evaluated using the LOESS procedure. First, each hemicypt 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.

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), treatment groups, 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 2 times that in the placebo group).

Sensitivity analysis

To investigate the sensitivity of our analyses to missing data, we used multiple imputation to impute biomarker expression for study participants who did not have scorable crypts or were lost to follow-up. To create a monotone missing pattern, we used a Markov chain Monte Carlo method to impute a value for the few observations that were missing on biomarkers measured at baseline, based on an assumption of multivariate normality (SAS, V 9.3, Proc MI). Once a monotone missing pattern was created, we used a regression approach to impute the remaining values (24). Age and sex were included as covariates for imputation to create 6 completed data sets that we then analyzed as previously described.

Results

Characteristics of study participants

Baseline characteristics of study participants did not significantly differ by treatment group (Table 1). The mean

Table 1. Selected baseline characteristics of the study participants^a ($n = 92$)

Characteristics	Treatment group				P value ^b
	Placebo ($n = 23$)	Calcium ($n = 23$)	Vitamin D ($n = 23$)	Calcium + Vitamin D ($n = 23$)	
Demographics, medical history, habits, anthropometrics					
Age, years	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 CRC in first-degree relative (%)	17	30	17	13	0.60
Take NSAID ^c regularly ^d (%)	22	13	9	22	0.60
If woman ($n = 28$), 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 ^e					
Total energy intake, kcal/d	1,596 (528)	1,788 (691)	1,848 (821)	1,845 (752)	0.59
Total ^f calcium, mg/d	625	678	753	733	0.75
Total ^f 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

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

^bBy Fisher exact test for categorical variables, and ANOVA for continuous variables.

^cNonsteroidal anti-inflammatory drug.

^dAt least once a week.

^eAll nutrients energy adjusted using residual method.

^fDiet plus supplements.

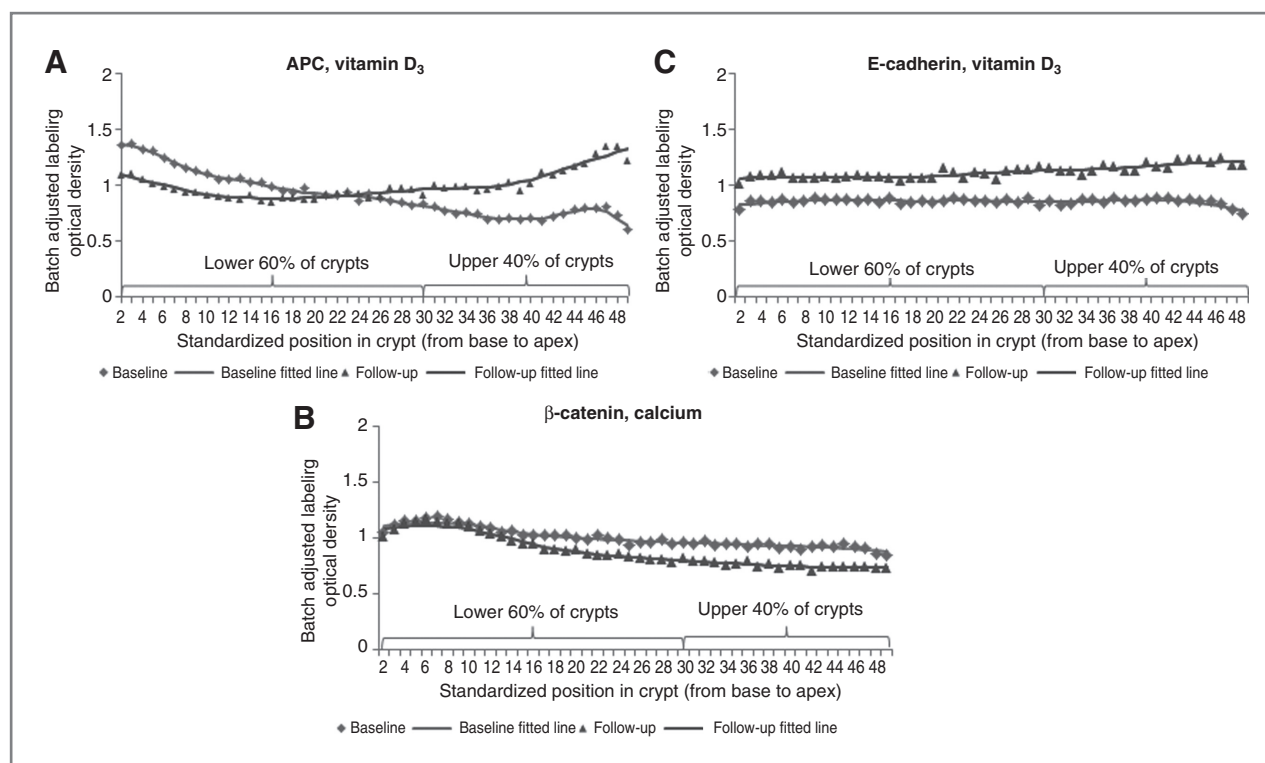


Figure 2. Representative examples of labeling expression distribution of (A) APC, (B) β -catenin, and (C) E-cadherin along normal colorectal crypts by treatment group at baseline and 6-month follow-up.

age of study participants was 61 years, 70% were men, 71% were white, and 20% had a family history of CRC 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 (18).

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 (18). There was no evidence of treatment effect modification by obesity status [body mass index (BMI) ≥ 30].

APC

A graphical comparison of APC crypt expression distribution at baseline and 6-month follow-up indicated that APC expression decreased in approximately the

lower 40% of the crypt and increased in the upper 60% of the crypt (Fig. 2A). As shown in Table 2, following 6 months of treatment, APC expression increased in the vitamin D₃ treatment group 25% ($P = 0.14$) in the full length of crypts, 48% ($P = 0.03$) in the upper 40% of crypts, 11% in the lower 60% of crypts ($P = 0.47$), and 21% ($P = 0.01$) in the Φ h of crypts, relative to the placebo group. In the calcium group APC expression decreased 2% ($P = 0.91$) in the full length of crypts, increased 7% ($P = 0.66$) in the upper 40% of crypts, decreased 10% ($P = 0.51$) in the lower 60% of crypts, and increased 10% ($P = 0.12$) in the Φ h of crypts, relative to the placebo group. APC expression tended to increase in the calcium/vitamin D₃ less than in the vitamin D₃ group, and these findings were not statistically significant (Table 2).

β -catenin

A graphical evaluation of β -catenin crypt expression distribution at baseline and 6-month follow-up indicated that β -catenin expression did not change in approximately the lower 20% of the crypt, but steadily decreased toward the crypt apex (Fig. 2B). As shown in Table 2, following 6 months of treatment, β -catenin expression decreased along the full length of crypts by 15% ($P = 0.08$), 12% ($P = 0.18$), and 11% ($P = 0.20$) in the calcium, vitamin D₃, and calcium/vitamin D₃ groups, respectively, relative to the placebo group. The findings in the upper 40% and lower

Table 2. Expression of APC, β -catenin, E-cadherin, and the APC/ β -catenin score^a in the normal-appearing colorectal mucosa

Treatment group	Baseline				6-Month follow-up				Absolute Rx effect			Relative effect ^d
	n	Mean	SE	P	n	Mean	SE	P	Rx effect ^b	SE	P ^c	
APC												
Whole crypts												
Placebo	22	1.09	0.08		21	0.97	0.08		0.00	.	.	1.00
Calcium	23	1.07	0.08	0.90	21	0.94	0.08	0.80	-0.02	0.15	0.91	0.98
Vitamin D	23	0.90	0.08	0.10	22	1.00	0.08	0.80	0.22	0.14	0.14	1.25
Ca + vitamin D	23	0.97	0.08	0.29	21	0.91	0.08	0.61	0.06	0.15	0.68	1.06
Upper 40% of crypts												
Placebo	22	0.39	0.04		21	0.41	0.04		0.00	.	.	1.00
Calcium	23	0.40	0.04	0.91	21	0.44	0.04	0.51	0.03	0.06	0.66	1.07
Vitamin D	23	0.30	0.04	0.07	22	0.46	0.04	0.31	0.15	0.06	0.03	1.48
Ca + vitamin D	23	0.36	0.04	0.47	21	0.41	0.04	0.39	0.04	0.06	0.58	1.10
Lower 60% of crypts												
Placebo	22	0.68	0.05		21	0.57	0.05		0.00	.	.	1.00
Calcium	23	0.70	0.05	0.81	21	0.53	0.05	0.53	-0.06	0.09	0.51	0.90
Vitamin D	23	0.60	0.05	0.24	22	0.56	0.05	0.84	0.07	0.09	0.47	1.11
Ca + vitamin D	23	0.60	0.05	0.24	21	0.52	0.05	0.49	0.03	0.09	0.73	1.04
Φh^e												
Placebo	22	0.35	0.01		21	0.42	0.01		0.00	.	.	1.00
Calcium	23	0.34	0.01	0.06	21	0.46	0.01	0.06	0.04	0.03	0.12	1.10
Vitamin D	23	0.31	0.01	0.10	22	0.46	0.01	0.05	0.07	0.03	0.01	1.21
Ca + vitamin D	23	0.35	0.01	0.86	21	0.44	0.01	0.30	0.01	0.03	0.64	1.03
β -Catenin												
Whole crypts												
Placebo	21	0.99	0.04		18	0.98	0.04		0.00	.	.	1.00
Calcium	22	1.01	0.04	0.69	19	0.85	0.05	0.04	-0.15	0.08	0.08	0.85
Vitamin D	21	0.94	0.04	0.45	21	0.82	0.04	0.03	-0.11	0.08	0.18	0.88
Ca + vitamin D	22	1.04	0.04	0.38	17	0.92	0.05	0.37	-0.11	0.08	0.20	0.89
Upper 40% of crypts												
Placebo	21	0.38	0.02		18	0.37	0.02		0.00	.	.	1.00
Calcium	22	0.40	0.02	0.52	19	0.33	0.02	0.07	-0.06	0.03	0.06	0.85
Vitamin D	21	0.36	0.02	0.36	21	0.33	0.02	0.12	-0.02	0.03	0.61	0.95
Ca + vitamin D	22	0.39	0.02	0.69	17	0.36	0.02	0.61	-0.02	0.03	0.50	0.94
Lower 60% of crypts												
Placebo	21	0.61	0.02		18	0.62	0.03		0.00	.	.	1.00
Calcium	22	0.63	0.02	0.44	19	0.56	0.02	0.11	-0.08	0.05	0.09	0.87
Vitamin D	21	0.58	0.02	0.38	21	0.54	0.02	0.02	-0.05	0.05	0.27	0.91
Ca + vitamin D	22	0.63	0.02	0.58	17	0.59	0.03	0.46	-0.05	0.05	0.36	0.93
Φh^e												
Placebo	21	0.39	0.01		18	0.38	0.01		0.00	.	.	1.00
Calcium	22	0.38	0.01	0.87	19	0.36	0.01	0.16	-0.01	0.01	0.31	0.97
Vitamin D	21	0.39	0.01	0.94	21	0.38	0.01	0.81	0.00	0.01	0.80	1.01
Ca + vitamin D	22	0.38	0.01	0.88	17	0.37	0.01	0.67	0.00	0.01	0.81	0.99
E-cadherin												
Whole crypts												
Placebo	21	1.13	0.12		19	1.04	0.13		0.00	.	.	1.00
Calcium	21	1.23	0.12	0.69	21	1.09	0.12	0.78	-0.05	0.25	0.85	0.96
Vitamin D	21	0.82	0.12	0.05	18	1.29	0.13	0.17	0.56	0.25	0.03	1.72
Ca + vitamin D	22	0.91	0.12	0.14	19	1.26	0.13	0.23	0.46	0.25	0.08	1.51

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Table 2. Expression of APC, β -catenin, E-cadherin, and the APC/ β -catenin score^a in the normal-appearing colorectal mucosa (Cont'd)

Treatment group	Baseline				6-Month follow-up				Absolute Rx effect			Relative effect ^d
	n	Mean	SE	P	n	Mean	SE	P	Rx effect ^b	SE	P ^c	
Upper 40% of crypts												
Placebo	21	0.44	0.05		19	0.42	0.05		0.00	.	.	1.00
Calcium	21	0.47	0.05	0.70	21	0.45	0.05	0.70	0.00	0.10	0.99	1.01
Vitamin D	21	0.33	0.05	0.09	18	0.56	0.05	0.06	0.25	0.10	0.02	1.78
Ca + vitamin D	22	0.37	0.05	0.26	19	0.55	0.05	0.09	0.20	0.10	0.05	1.56
Lower 60% of crypts												
Placebo	21	0.64	0.07		19	0.60	0.08		0.00	.	.	1.00
Calcium	21	0.69	0.07	0.68	21	0.63	0.07	0.78	-0.01	0.15	0.93	0.98
Vitamin D	21	0.48	0.07	0.12	18	0.75	0.08	0.19	0.31	0.16	0.05	1.68
Ca + vitamin D	22	0.55	0.07	0.38	19	0.70	0.08	0.39	0.19	0.15	0.23	1.35
Φh^e												
Placebo	21	0.41	0.02		19	0.40	0.02		0.00	.	.	1.00
Calcium	21	0.41	0.02	0.92	21	0.41	0.02	0.66	0.01	0.03	0.71	1.03
Vitamin D	21	0.40	0.02	0.61	18	0.45	0.02	0.06	0.05	0.03	0.10	1.14
Ca + vitamin D	22	0.40	0.02	0.60	19	0.46	0.02	0.01	0.07	0.03	0.03	1.18
APC/ β -catenin score												
Placebo	21	0.37	0.03		18	0.44	0.03		0.00	.	.	1.00
Calcium	22	0.35	0.03	0.49	18	0.58	0.03	0.003	0.16	0.06	0.01	1.41
Vitamin D	21	0.36	0.03	0.83	20	0.57	0.03	0.004	0.13	0.06	0.02	1.31
Ca + vitamin D	22	0.37	0.03	0.84	17	0.50	0.03	0.18	0.07	0.06	0.26	1.16

^aAPC/ β -catenin score = Φh APC/ β -catenin expression in the whole crypt, where Φh APC = ratio of APC expression in the upper 40% of the crypts to the whole crypts.

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

^cP value for difference between each active treatment group and placebo group from repeated-measures MIXED model.

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

^e Φh = proportion of expression in the distribution zone (i.e., ratio of expression in upper 40% to expression in whole crypt).

60% of crypts did not appreciably differ from those observed in the full length of crypts. There were no apparent treatment effects on β -catenin expression in the Φh of crypts.

E-cadherin

A graphical evaluation of E-cadherin crypt expression distribution at baseline and 6-month follow-up indicated that E-cadherin expression uniformly increased along the full length of the crypt (Fig. 2C). As shown in Table 2, following 6 months of treatment, E-cadherin expression increased in the vitamin D₃ group 72% ($P = 0.03$) in the full length of crypts, 78% ($P = 0.02$) in the upper 40% of crypts, 68% ($P = 0.05$) in the lower 60% of crypts, and 14% ($P = 0.10$) in the Φh of crypts. E-cadherin expression also increased in the calcium/vitamin D₃ group, but less so than in the vitamin D₃ group, except in the Φh of crypts where E-cadherin expression increased 18% ($P = 0.03$). In the calcium group E-cadherin did not appreciably change relative to the placebo group (Table 2).

APC/ β -catenin score

The APC/ β -catenin score increased 41% ($P = 0.01$), 31% ($P = 0.02$), and 16% ($P = 0.26$) in the calcium, vitamin D₃, and calcium/vitamin D₃ groups, respectively, relative to the placebo group (Table 2).

Sensitivity analysis

There were no apparent differences in findings following imputation of missing observations (Supplementary Table S1).

Discussion

The results of this pilot, randomized, placebo-controlled clinical trial provide the first human *in vivo* evidence that supplemental calcium and vitamin D₃, alone or in combination, may increase APC and E-cadherin expression and the APC/ β -catenin score and decrease β -catenin expression in the normal colorectal mucosa of sporadic adenoma patients. These findings support the hypothesis that the anticarcinogenic effects calcium and vitamin D, alone or

in combination, may in part operate by modifying the APC/ β -catenin signaling pathway. These findings are relevant because, in light of our previous report of differences in APC and β -catenin expression between persons with incident sporadic adenomas and persons with no past or current adenomas, they (i) provide further support that APC and β -catenin expression and the APC/ β -catenin score in the normal colorectal mucosa may be modifiable, preneoplastic biomarkers of risk for colorectal adenomas, and (ii) provide human *in vivo* mechanistic evidence of the possible protective effects of calcium and vitamin D₃ against colorectal neoplasms.

APC and β -catenin are appealing candidates for being preneoplastic biomarkers of risk because malfunctioning of the APC/ β -catenin signaling pathway is a common and early event in the colorectal neoplastic transition (5). In normal colorectal mucosa APC, axin, glycogen synthase kinase 3, and casein kinase negatively regulate Wnt signaling by forming the " β -catenin destruction" complex, and, in the absence of Wnt signaling, phosphorylate and promote the degradation of free β -catenin (5). Normal functioning of the APC gene is inhibited in approximately 80% to 90% of sporadic CRC, resulting in increased potential for β -catenin to translocate to the nucleus and activate Wnt target genes (5). In normal colorectal mucosa APC, β -catenin, and E-cadherin are all strongly expressed—APC primarily in the cytoplasm, and E-cadherin and β -catenin primarily at the cell membrane. During the adenoma–carcinoma sequence, APC and E-cadherin expression markedly decrease (although the decrease in E-cadherin tends to occur in later stages; 25–27), and β -catenin expression appears to steadily increase and translocate from the membrane to the cytoplasm and eventually into the nucleus (25, 28). We previously proposed that the APC/ β -catenin score may represent the potential of β -catenin to translocate to the nucleus and promote proliferative signaling (8). We found the APC/ β -catenin score in the normal colorectal mucosa of sporadic colorectal adenoma patients to be statistically significantly lower than in the normal colorectal mucosa of healthy controls, and that Φ h APC and β -catenin expression and the APC/ β -catenin score may be modifiable as suggested by their being associated with lifestyle and dietary risk factors for colorectal neoplasms (8).

The etiology of CRC is heavily influenced by modifiable dietary and lifestyle behaviors. Dietary-induced epigenetic modifications to the APC/ β -catenin signaling pathway may initiate or be a "second hit" in the adenoma–carcinoma pathway. Calcium and vitamin D are 2 promising chemopreventive agents that may act against colorectal neoplasms; however, the mechanisms by which they operate are not entirely clear (9). CRC cell line studies suggest that calcium and 1,25(OH)₂D upregulate E-cadherin expression and promote the translocation of β -catenin from the nucleus and cytoplasm to the plasma membrane (15–17, 29). Mice fed a diet comparable to a typical "Western" diet had increased *β -catenin* and *Tcf* gene expression and decreased APC gene expression; however, supplementation of the "Western" diet with increased dietary calcium and vitamin

D decreased *β -catenin* and *Tcf* gene expression, but had no apparent effect on APC gene expression (13). In a transmissible murine colonic hyperplasia model high dietary calcium modestly reduced total β -catenin expression (14). A diet supplemented with the vitamin D analog 1 α (OH)D₅ inhibited β -catenin nuclear expression in azoxymethane-treated mice (30), and endometrial E-cadherin expression increased in mice fed a diet high in vitamin D₃ (31). Investigations of the *in vivo* effects of calcium and/or vitamin D on APC expression are limited (13), but dietary modification of APC expression is supported by reports that diets moderately deficient in B-vitamin methyl donors reduced APC expression and increased β -catenin/Tcf signaling in rodents (32, 33).

Our results are consistent with the hypothesis that calcium and vitamin D reduce cell proliferation and promote differentiation in the colorectal mucosa. To our knowledge this is the first human *in vivo* study to suggest that calcium and/or vitamin D₃ may affect APC expression in the normal colorectal mucosa; however, the mechanism by which calcium and/or vitamin D₃ may modify APC expression remains unclear. We did not evaluate β -catenin localization, but consistent with the observed increase in APC expression, particularly Φ h APC, we observed decreased β -catenin expression and an increased APC/ β -catenin score in all 3 active treatment groups, suggesting that calcium and vitamin D₃ treatment may decrease the potential of β -catenin to promote proliferative signaling. These results are in line with our reports that suggested that calcium and/or vitamin D₃ treatment reduced human telomerase reverse transcriptase expression (a potential cofactor in the β -catenin transcriptional complex; ref. 34) and increased p21 expression (which is negatively regulated by β -catenin/TCF signaling refs. 35, 36). These results also corroborate our report that the APC/ β -catenin score may be a valid, potentially modifiable, preneoplastic biomarker of risk for colorectal neoplasms, providing *in vivo* evidence that supplemental calcium and/or vitamin D₃ may promote a greater APC/ β -catenin score in the normal rectal mucosa.

Conflicting with previous *in vitro* studies (15, 16), we did not observe increased E-cadherin expression in the calcium group. The explanation for this lack of consistency in findings is not clear and may be a chance finding; however, the difference may be attributed to our investigation evaluating the effects of calcium on E-cadherin expression in the normal mucosa of free living humans rather than *in vitro*, or that the ability of calcium to increase E-cadherin expression may be limited to neoplastic tissue.

Contrary to our hypothesis and what has been reported in some studies (10, 37), the estimated treatment effect of calcium plus vitamin D₃ was not greater than that of either the calcium or vitamin D₃ alone in increasing Φ h APC, E-cadherin, or the APC/ β -catenin score, or in decreasing β -catenin. We previously reported that calcium combined with vitamin D₃ may mitigate treatment effects of calcium and vitamin D₃ alone on colorectal mucosa markers of apoptosis and differentiation (18, 36). There are several

plausible explanations for why this was observed, the first being that these could have been chance findings given the small sample size of our study. At least one study reported that calcium and vitamin D individually suppressed tumorigenesis in rodents, but the combination of the 2 was ineffective (38). We previously observed that calcium combined with vitamin D₃ increased CYP24A1 expression (39), which may reduce the effects of 1,25(OH)₂D in the colorectal mucosa. A large clinical trial of colorectal adenoma recurrence suggested that calcium supplementation was primarily effective among people with 25(OH)D concentrations greater than the median in the study population (29.1 ng/mL; 10). In our study population only participants in the vitamin D₃ group reached 25(OH)D concentrations greater than 29.1 ng/mL (18), suggesting the possibility of a threshold effect.

We previously reported that calcium and vitamin D₃ supplementation in this same trial favorably modified the expression of markers of calcium and vitamin D metabolism (39), proliferation (36), differentiation (36), apoptosis (18), mismatch repair (40), and oxidative DNA damage (41) in the normal human colorectal mucosa. Our current results, taken together with our previous findings, support the hypothesized effects of calcium and vitamin D on favorably modulating the molecular phenotype of the normal colorectal mucosa and reducing risk for colorectal neoplasms.

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 conduct stratified analyses. We were unable to evaluate β -catenin subcellular localization; however, our previous findings (8) suggested that sporadic colorectal adenoma cases relative to normal controls may have greater total β -catenin expression in the normal colorectal mucosa. We propose that the APC/ β -catenin score may represent the potential of β -catenin to promote proliferative signaling, and needs to be investigated in basic science studies. 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; however, evidence from our pilot case-control study suggests that APC, β -catenin expression, and the APC/ β -catenin score may be preneoplastic biomarkers of risk.

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 APC/ β -catenin signaling pathway 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 APC, β -catenin, and E-cadherin, resulting in high biomarker measurement reliability.

In summary, the results of this pilot, randomized, placebo-controlled clinical trial provide human *in vivo* evidence that supplemental calcium and vitamin D₃, alone and in combination, may increase APC and E-cadherin expression and the APC/ β -catenin score and decrease β -catenin expression in the normal colorectal mucosa of sporadic colorectal adenoma cases. These results suggest that the anticarcinogenic effects of supplemental calcium and vitamin D₃ may, in part, depend on the ability of these agents to favorably modulate the expression of APC, β -catenin, and E-cadherin and thus, possibly, inhibit proliferative β -catenin signaling. Taken together with our previous findings, APC (especially ϕ h APC) and β -catenin expression, the APC/ β -catenin score, and E-cadherin may be modifiable, preneoplastic 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

No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T.U. Ahearn, A. Shaukat, W.D. Flanders, R.M. Bostick

Writing, review, and/or revision of the manuscript: T.U. Ahearn, A. Shaukat, W.D. Flanders, R.E. Rutherford, R.M. Bostick

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T.U. Ahearn, A. Shaukat

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Cancer Prevention Research

A Randomized Clinical Trial of the Effects of Supplemental Calcium and Vitamin D₃ on the APC/ β -Catenin Pathway in the Normal Mucosa of Colorectal Adenoma Patients

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