

## Effects of Calcium and Vitamin D on MLH1 and MSH2 Expression in Rectal Mucosa of Sporadic Colorectal Adenoma Patients

Eduard Sidelnikov<sup>1,2</sup>, Roberd M. Bostick<sup>1,2</sup>, W. Dana Flanders<sup>1,2</sup>, Qi Long<sup>2</sup>, Veronika Fedirko<sup>1,3</sup>, Aasma Shaukat<sup>5</sup>, Carrie R. Daniel<sup>6</sup>, and Robin E. Rutherford<sup>4</sup>

### Abstract

To further clarify and develop calcium and vitamin D as chemopreventive agents against colorectal cancer in humans and develop modifiable biomarkers of risk for colorectal cancer, we conducted a pilot, randomized, double-blind, placebo-controlled, 2 × 2 factorial clinical trial to test the effects of calcium and vitamin D<sub>3</sub>, alone and in combination, on key DNA mismatch repair proteins in the normal colorectal mucosa. Ninety-two men and women with at least one pathology-confirmed colorectal adenoma were treated with 2.0 g/d calcium or 800 IU/d vitamin D<sub>3</sub>, alone or in combination, versus placebo over 6 months. Colorectal crypt overall expression and distribution of MSH2 and MLH1 proteins in biopsies of normal-appearing rectal mucosa were detected by automated immunohistochemistry and quantified by image analysis. After 6 months of treatment, MSH2 expression along the full lengths of crypts increased by 61% ( $P = 0.11$ ) and 30% ( $P = 0.36$ ) in the vitamin D and calcium groups, respectively, relative to the placebo group. The estimated calcium and vitamin D treatment effects were more pronounced in the upper 40% of crypts (differentiation zone) in which MSH2 expression increased by 169% ( $P = 0.04$ ) and 107% ( $P = 0.13$ ) in the vitamin D and calcium groups, respectively. These findings suggest that higher calcium and vitamin D intakes may result in increased DNA MMR system activity in the normal colorectal mucosa of sporadic adenoma patients and that the strongest effects may be vitamin D related and in the differentiation zone of the colorectal crypt. *Cancer Epidemiol Biomarkers Prev*; 19(4); 1022–32. ©2010 AACR.

### Introduction

Colorectal cancer, the second leading cause of cancer deaths in the United States (1), is strongly associated with Western diets and life-styles (2, 3). Most “sporadic” colorectal cancer develops in the adenomatous polyp, a benign neoplastic intestinal tumor that is the only accepted biomarker of risk for the disease (4–6). There are no currently accepted preneoplastic biomarkers of risk for the disease that can be used for assessing whether someone has an at-risk colorectal mucosa molecular phenotype or whether dietary and other interventions may have preventive efficacy.

**Authors' Affiliations:** Department of <sup>1</sup>Epidemiology and <sup>2</sup>Biostatistics and Bioinformatics, Rollins School of Public Health, and <sup>3</sup>Winship Cancer Institute, Emory University; and <sup>4</sup>Emory University School of Medicine, Division of Digestive Diseases, Atlanta, Georgia; <sup>5</sup>Department of Medicine, GI Division, University of Minnesota, Minneapolis, Minnesota; and <sup>6</sup>Nutritional Epidemiology Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, NIH, Department of Health and Human Services, Bethesda, Maryland

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

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We recently reported that the protein expression of the DNA mismatch repair (MMR) genes *MSH2* and *MLH1* in the normal-appearing colorectal mucosa is lower in patients with incident, sporadic colorectal adenoma than in patients with no current or past adenoma (7, 8). We also reported that expression levels of these proteins were associated with modifiable risk factors for colorectal neoplasms, suggesting that a low *MSH2* and/or *MLH1* expression phenotype may respond to preventive interventions (7, 8). The DNA MMR pathway, which is responsible for ~15% of colorectal cancers, involves silencing the *MLH1* and/or *MSH2* genes (6). Silencing of either of these genes interrupts the normal review and repair of DNA errors after replication, which eventually leads to microsatellite instability (MSI) and cancer development. Levels of expression of *MLH1* and *MSH2* protein in colonic cells are likely to indicate the functional level of the MMR mechanism.

We also recently reported that calcium and/or vitamin D supplementation modulated the expression of various biomarkers of risk for colorectal neoplasms in a randomized, controlled trial (9, 10). Higher intakes of calcium and higher levels of circulating 25-OH-vitamin D have been consistently associated with reduced risk for colorectal cancer and adenomas, and calcium supplementation reduces adenoma recurrence (11–14). Proposed,

likely complementary, antineoplastic mechanisms of calcium include protection of the colorectal mucosa against bile and fatty acids (15, 16), direct effects on the cell cycle (17), and modulation of E-cadherin and  $\beta$ -catenin expression through the calcium-sensing receptor (17-19). Vitamin D, beyond its role in calcium metabolism and homeostasis, promotes bile acid degradation and xenobiotic metabolism; regulates cell proliferation, differentiation, and apoptosis; and influences DNA repair, angiogenesis, inflammation, and immune response (19-21).

To the authors' knowledge, there are no published studies that specifically investigated whether calcium or vitamin D may affect the expression of DNA MMR proteins in the normal colorectal mucosa. This article reports findings from a pilot clinical trial that assessed the individual and combined effects of calcium and vitamin D<sub>3</sub> supplementation on the expression of MLH1 and MSH2 proteins in the normal-appearing colorectal mucosa of colorectal adenoma patients.

## Materials and Methods

This study was approved by the Emory University Institutional Review Board. Written informed consent was obtained from each study participant.

### Participant population

Participants were recruited from the patient population attending the Digestive Diseases Clinic at Emory University. Eligibility for the study included 30 to 75 y of age; in general good health; capable of informed consent; a history of at least one pathology-confirmed adenomatous colonic or rectal polyp within the past 36 mo; no contraindications to calcium or vitamin D supplementation or rectal biopsy procedures; and no medical conditions, habits, or medication usage that would otherwise interfere with the study as described below. Detailed eligibility and exclusion criteria were previously reported (9, 10).

### Clinical trial protocol

All age-eligible patients diagnosed with at least one pathology-confirmed adenomatous colonic or rectal polyp within the past 36 mo were identified as potential study participants. All patients passing initial chart screening for eligibility were sent an introductory letter, followed by a telephone interview. During the telephone interview, a few preliminary screening questions were asked and, if a person was willing, still appeared eligible, and could be available for the next 8 mo, an in-person eligibility visit was scheduled. Potential participants were asked to bring all medications and vitamins and minerals being taken to this appointment.

During the eligibility visit, potential participants were interviewed, signed a consent form, completed questionnaires (included questions on sociodemographics, medical history and medication use, nutritional supplement use, life-style, family history, and others), and provided a blood sample. Diet was assessed with a semiquantita-

tive Willett food frequency questionnaire (22). Medical and pathology records were reviewed. Those still eligible and willing to participate then entered a 30-d placebo run-in trial. Only participants without significant perceived side effects and who had taken at least 80% of their tablets were eligible for randomized assignment. Adherence for the run-in trial was assessed by questionnaire, interview, and pill count.

Eligible participants then had their vital signs taken, underwent a baseline rectal biopsy and, if still willing to participate, were randomly assigned (stratified by sex and nonsteroidal anti-inflammatory drug use) to one of four treatment groups. Of patients who passed initial chart eligibility, 42% were contacted and 20% were eligible and consented to participate.

Participants ( $n = 92$ ) were randomly assigned to the following four treatment groups: a placebo control group, a 2.0-g elemental calcium supplementation group (as calcium carbonate in equal doses twice daily), an 800-IU vitamin D<sub>3</sub> supplementation group (400 IU twice daily), and a calcium plus vitamin D<sub>3</sub> supplementation group taking 2.0 g elemental calcium plus 800 IU of vitamin D<sub>3</sub> daily. Each group consisted of 23 participants.

Study tablets were custom manufactured by Tishcon Corp. The corresponding supplement and placebo pills were identical in size, appearance, and taste. The placebo was free of calcium, magnesium, vitamin D, and chelating agents.

Calcium carbonate was chosen because it delivers more elemental calcium for a given tablet than other forms; therefore, fewer tablets are required, enhancing adherence. It was the form used in the Calcium and Polyp Prevention adenoma recurrence (23) and the Calcium and Colorectal Epithelial Cell Proliferation (24) trials, and in the majority of the larger studies using long-term calcium supplementation for other reasons; therefore, its safety record had been well established and it was the least expensive and most widely available calcium supplement form.

Vitamin D<sub>3</sub> was the chosen form of vitamin D for several reasons, the most important of which was to avoid the toxicity risks associated with 1,25(OH)<sub>2</sub>-vitamin D or 25(OH)-vitamin D. Multivitamins and calcium/vitamin D supplements typically provide 400 IU per day of vitamin D<sub>3</sub>, but numerous intervention studies show that this dose will not suppress parathyroid hormone (PTH) in the overwhelming majority of North American adults (25, 26). So we chose a more effective dose of 800 IU per day, which raises serum 25-(OH) vitamin D levels toward the desired range and leaves a substantial margin of safety, even after taking into consideration dietary intake.

The treatment period was 6 mo to replicate the treatment period of the Calcium and Colorectal Epithelial Cell Proliferation trial (24) and to ensure approximately 2 to 3 mo of 25-OH-vitamin D steady-state levels. Participants attended follow-up visits at 2 and 6 mo after randomization and were contacted by telephone at monthly intervals between the second and final follow-up visits.

At follow-up visits, pill-taking adherence was assessed by questionnaire, interview, and pill count. Participants were instructed to remain on their usual diet and not take any nutritional supplements not in use on entry into the study. At each of the follow-up visits participants were interviewed, filled out questionnaires, and had their vital signs taken. At the first and last visits, all participants had their blood drawn and underwent a rectal biopsy procedure. All participants were asked to abstain from aspirin use for 7 d before each biopsy visit. All visits for a given participant were scheduled at the same time of day to control for possible circadian variability in the outcome measures.

Factors hypothesized to be related to risk for colorectal neoplasms or to the expression of MMR proteins in normal colon mucosa (e.g., diet, medications, etc.) were assessed at baseline, several were reassessed at the first follow-up visit, and all were reassessed at the final follow-up visit. Participants did not have to be fasting for their visits and did not take a bowel cleansing preparation or enema.

Six sextant ~1 mm-thick biopsy specimens were taken from normal-appearing rectal mucosa 10 cm proximal to the external anal aperture through a rigid sigmoidoscope with a jumbo cup flexible endoscopic forceps mounted on a semiflexible rod. No biopsies were taken within 4.0 cm of a polypoid lesion. The biopsies were then immediately placed in PBS and examined and reoriented under a dissecting microscope to ensure that they were not twisted or curled on the bibulous paper. The biopsies were then immediately placed in 10% normal buffered formalin.

### Immunohistochemistry protocol

The biopsies in formalin were left undisturbed for at least 6 h, transferred to 70% ethanol 24 h after being placed in formalin, embedded in paraffin blocks (two blocks of three biopsies each) within 2 wk of the biopsy procedure, cut and stained within another 4 wk, and analyzed within another 4 wk. From one block, five slides with four section levels each taken 40  $\mu$ m apart were prepared for each antigen, yielding a total of 20 levels per antigen.

Heat-mediated antigen retrieval was used to break the protein cross-links formed by formalin to uncover the epitope. To accomplish this, slides were placed in a preheated Pretreatment Module (Lab Vision Corp.) with 100  $\times$  Citrate Buffer (pH 6.0; DAKO S1699, DAKO Corp.; further called DAKO) and steamed for 40 min. After antigen retrieval, slides were placed in a DAKO Automated stainer (DAKO) and rinsed with warm Pretreatment Module Buffer. The Autostainer was programmed for each immunohistochemistry run and the reagents used were as follows: antibody (MLH1 antibody manufactured by BD Pharmingen, dilution 1:15; or MSH2 antibody manufactured by Calbiochem, dilution 1:500) diluted with Antibody Diluent (DAKO S0809 for MLH1 and S3022 for MSH2, DAKO); LSAB2 Detection System (DAKO K0675, DAKO) for MLH1; and Envision+ Detection System (DAKO K4007,

DAKO) for MSH2, diaminobenzidine (DAKO K3466 for MLH1 and K3438 for MSH2, DAKO), and TBS buffer (DAKO S1968, DAKO). The slides were not counterstained. After staining, the slides were coverslipped automatically with a Leica CV5000 Coverslipper (Leica Microsystems, Inc.) and placed in opaque slide folders. In each staining batch of slides, positive and negative control slides were included. A surgical specimen of normal colon tissue was used as a control tissue for both MMR biomarkers. The control tissue was processed in the same manner as the patient's tissue and the negative and the positive control slides were treated identically to the patient's slides except that antibody diluent was used rather than primary antibody on the negative control slide.

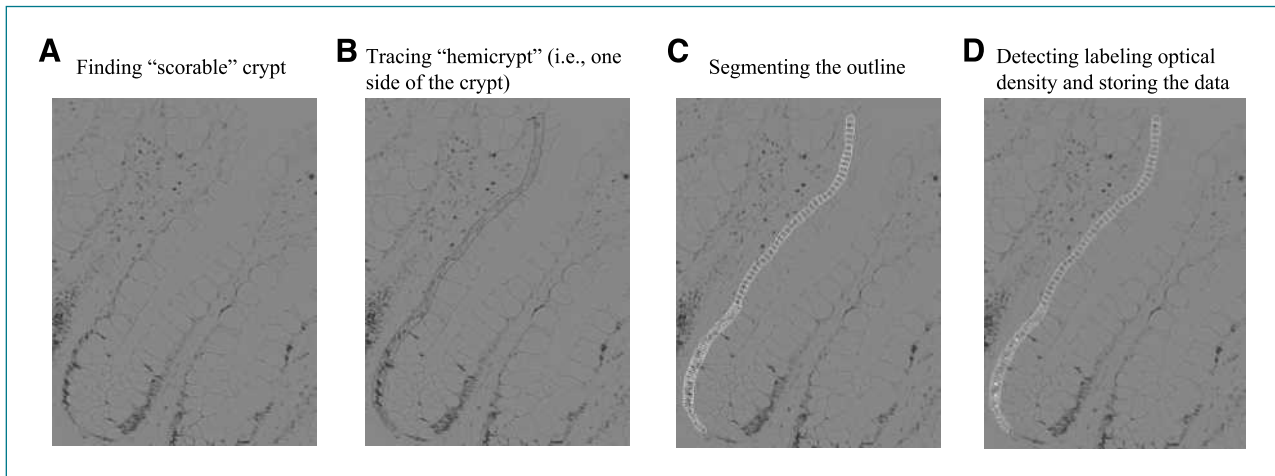
### Protocol for quantifying staining density of immunohistochemically detected biomarkers in normal colon crypts ("scoring")

The imaging and analysis unit was a "hemicypt," defined as one side of a colonic crypt bisected from base to colon lumen surface. Intact (at most two contiguous cells missing) hemicypts extending from the muscularis mucosae to the colon lumen were considered eligible for quantitative image analysis ("scorable"; Fig. 1). Before analysis, negative and positive control slides were checked for staining adequacy and the patient's slides were scanned to assess the adequacy of the biopsy specimen (that is, whether scorable crypts were present).

The major equipment and software for the image analysis procedures were as follows: personal computer, light microscope (Olympus BX40, Olympus Corp.) with appropriate filters and attached digital light microscope camera (Polaroid DMC Digital Light Microscope Camera, Polaroid Corp.), 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 Corp.).

The following preparations were done before starting the scoring program: (a) ensuring standardized settings on the microscope, digital camera, and imaging software; and (b) cleaning and visually scanning the slides. Then, participant ID number, scorer ID, visit number, and antigen, followed by the number of the first biopsy to be scored, whether it had scorable crypts, whether it was labeled, and if so, the section level number on the biopsy on which scoring was begun was recorded. Slides were oriented in a standardized fashion and the section levels on the slides were viewed in sequence using light microscopy. All images were taken at  $\times$ 200 magnification and stored as 16-bit grayscale 1,600  $\times$  1,200 pixel images.

For each patient, the two biopsies with the greatest number of scorable hemicypts were selected for quantitative image analysis (scoring). Intact hemicypts were "scored" in order from the first section of the first biopsy from left to right. The goal was to score at least 16 scorable hemicypts per biopsy (32 per patient). If the



**Figure 1.** Quantitative image analysis of MSH2 labeling optical density consists of several steps: (A) finding eligible crypts (see text for details), (B) manually tracing one side of the crypt (hemicypt), (C) automated division of the outline into segments of width of an average colonocyte, (D) automated background-corrected densitometry of overall and segment-specific labeling of the biomarker and entering the results into the database.

16th hemicypt was reached before the level was finished, the scorer continued scoring until either the level was finished or the 20th hemicypt was scored, whichever came first. No more than 20 hemicypts per biopsy were scored.

If the two best biopsies had <32 scorable biopsies, an attempt was made to cut more slides. If that did not solve the issue, scoring was completed if the two best biopsies had 16 or more scorable hemicypts between them. All three biopsies were scored only if there was less than a total of 16 scorable hemicypts between the two best biopsies.

To ensure adherence, a scorer was guided through the scoring protocol by the computer software. For each scored slide, background correction images were obtained and controlled for by the computer program. Hemicypts were manually traced by the scorer (Fig. 1). A traced hemicypt was divided by the software into segments corresponding in width to that of an average normal crypt epithelial cell. Overall hemicypt- and segment-specific optical signal densities were then calculated by the software and stored into a Microsoft Access database along with various dimensional parameters of the hemicypt.

One slide reader analyzed all of the MLH1- and MSH2-stained slides throughout the study. A reliability control sample previously analyzed by the reader was reanalyzed during the course of the trial to determine intrareader reliability.

#### **Protocol for measuring serum 25-OH-vitamin D and 1,25-(OH)<sub>2</sub>-vitamin D levels**

Laboratory assays for serum 25-OH-vitamin D and 1,25-(OH)<sub>2</sub>-vitamin D were done by Dr. Bruce W. Hollis at the Medical University of South Carolina using a RIA method as previously described (27, 28). Serum samples for baseline and follow-up visits for all subjects were as-

sayed together, ordered randomly, and labeled to mask treatment group, follow-up visit, and quality control replicates. The average intraassay coefficient of variation for serum 25-OH-vitamin D was 2.3% and 6.2% for 1,25-(OH)<sub>2</sub>-vitamin D.

#### **Statistical analysis**

Treatment groups were assessed for the 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 intraclass correlation coefficients.

Labeling optical densities for MLH1 and MSH2 for each study participant were adjusted for staining batch by dividing each person's measurement by the mean value for everyone included in the staining batch in which the participant's sample was run. We decided *a priori* to investigate overall (total) crypt expression, expression in the upper 40% (differentiation zone) and lower 60% (proliferation zone) of the crypts, and the ratio of expression in the upper 40% to the full length of the crypts as a measure of within-crypt distribution (distribution index) of the MMR markers (24, 29, 30).

Treatment effects were evaluated by assessing the differences in mean labeling optical densities from baseline to the 6-mo follow-up visit between patients in each active treatment group relative to the placebo group using linear mixed models to account for correlated data. Primary analyses were based on assigned treatment at the time of randomization, regardless of adherence status (intent-to-treat analysis). Two continuous outcomes—MLH1 and MSH2 labeling optical density measurements—were analyzed separately. To provide perspective on the magnitude of the absolute treatment effects [(follow-up – baseline in the active treatment group) – (follow-up – baseline in the placebo group)] of each outcome variable, we also calculated relative

effects, defined as (treatment group follow-up mean/treatment group baseline mean)/(placebo follow-up mean/placebo baseline mean). The interpretation of the relative effect is somewhat analogous to that of an odds ratio (e.g., a relative effect of 2.0 would mean that the relative proportional change in the treatment group was twice as great as that in the placebo group). No adjustment was made for other covariates in the primary intent-to-treat analyses.

Possible effects of calcium and/or vitamin D on the distribution of MLH1 and MSH2 in rectal crypts were also assessed graphically with the Loess procedure as implemented in the SAS version 9 statistical software (31). First, the number of cells within a hemicrypt was standardized to 50 segments (the average number of cells within a column of colonic crypt cells). Then, average Loess model predicted segment-specific levels of MSH2 at baseline and follow-up by treatment group were plotted in the graphs (Fig. 2) along with smoothing lines to make graphical evaluation easier.

In sensitivity analyses, we also analyzed data without standardization for batch, by including batch as a covariate, and using different transformations; the results from these analyses did not differ materially from those reported.

Statistical analyses were done using the SAS v.9.2 statistical software (Copyright 2002-2008 by SAS Institute,

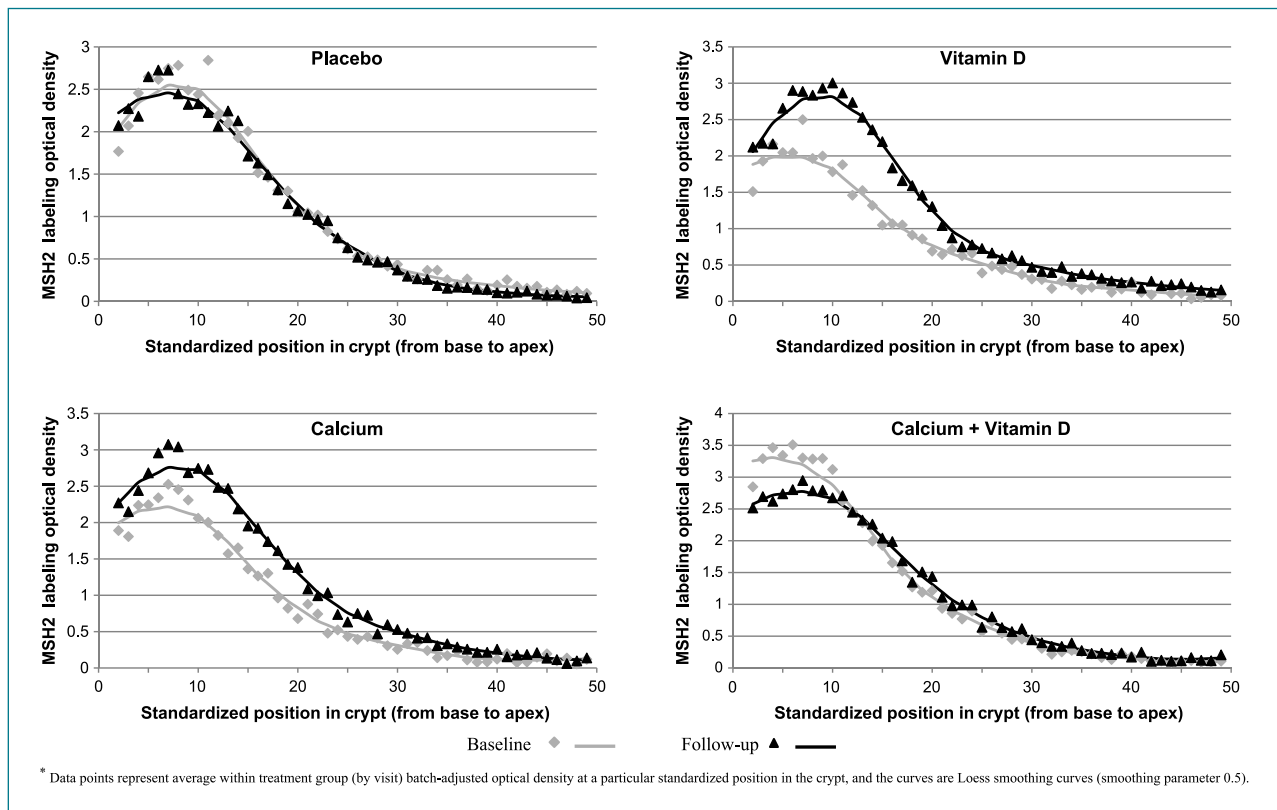
Inc.). A cutoff level of  $P \leq 0.05$  (two sided) was used for assessing statistical significance.

## Results

### Characteristics of study participants

Treatment groups did not differ significantly on the characteristics measured at baseline (Table 1) or at the end of follow-up (data not shown). On average, participants were ages 61 years, 70% were male, 71% were white, and 19% had a history of colorectal cancer in a first-degree relative. Most of the participants were college graduates, overweight, and nonsmokers. Adequate biopsy specimens for image analysis for MSH2 and MLH1 were obtained from 87 and 78 participants at baseline and from 82 and 72 participants after a 6-month follow-up, respectively.

Adherence to visit attendance averaged 92% and did not differ significantly among the four treatment groups. On average, at least 80% of pills were taken by 93% of participants at the first follow-up visit and 84% at the final follow-up visit. There were no treatment or biopsy complications. Seven people (8%) were lost to follow-up due to perceived drug intolerance ( $n = 2$ ), unwillingness to continue participation ( $n = 3$ ), physician's advice ( $n = 1$ ), and death ( $n = 1$ ). Dropouts included one person from the vitamin D supplementation group and two



**Figure 2.** Expression of MSH2 protein at standardized positions within the crypts of normal-appearing rectal mucosa in four treatment groups. \*, the Calcium, Vitamin D versus Markers of Adenomatous Polyps Trial.

**Table 1.** Selected baseline characteristics of the study participants (*n* = 92)

Characteristics	Treatment group				<i>P</i> *
	Placebo ( <i>n</i> = 23)	Calcium ( <i>n</i> = 23)	Vitamin D ( <i>n</i> = 23)	Calcium + vitamin D ( <i>n</i> = 23)	
Demographics					
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	0.13
College graduate (%)	65	64	57	45	0.53
Medical history					
History of colorectal cancer in 1° relative (%)	17	30	17	13	0.60
Take NSAID <sup>†</sup> regularly <sup>‡</sup> (%)	22	13	9	17	0.77
Take aspirin regularly <sup>‡</sup> (%)	22	52	30	57	0.05
Habits					
Current smoker (%)	9	4	0	0	0.61
Take multivitamin (%)	30	30	26	39	0.86
Physical activity (MET/d)	14.5 (11.6)	17.9 (17.9)	20.7 (12.0)	20.9 (14.7)	0.43
Mean dietary intakes					
Total energy intake, kcal/d	1,596 (528)	1,788 (691)	1,848 (821)	1,845 (752)	0.59
Total calcium <sup>§</sup> , mg/d	618 (308)	746 (335)	843 (526)	824 (714)	0.41
Total vitamin <sup>§</sup> D, IU/d	277 (230)	336 (202)	360 (317)	415 (316)	0.40
Total fat, g/d	67 (32)	72 (35)	70 (32)	74 (28)	0.59
Dietary fiber, g/d	15 (7)	17 (9)	18 (9)	17 (11)	0.97
Alcohol, g/d	9 (14)	11 (15)	14 (18)	10 (20)	0.84
Anthropometrics					
Body mass index (BMI), kg/m <sup>2</sup>	30.6 (7.2)	29.4 (5.5)	28.9 (5.6)	31.6 (6.0)	0.44
Waist-to-hip ratio	0.9 (0.1)	0.9 (0.1)	0.9 (0.1)	1.0 (0.1)	0.17
Adenoma characteristics					
Multiple adenomas <sup>  </sup> (%)	17	22	39	26	0.45
Large adenoma ≥ 1 cm <sup>¶</sup> (%)	19	32	17	9	0.32
Villous/tubulovillous adenoma <sup>**</sup> (%)	4	9	9	4	1.00
Mild dysplasia <sup>††</sup> (%)	100	96	100	100	1.00
Baseline vitamin D serum levels					
25-OH-vitamin D (ng/mL)	20.44 (7.5)	25.67 (7.6)	21.04 (8.3)	20.93 (9.6)	0.12
1,25-(OH) <sub>2</sub> -vitamin D (pg/mL)	39.2 (12.2)	45.4 (35.3)	44.5 (22.6)	37.9 (12.5)	0.60

NOTE: Data are given as means (SD) unless otherwise specified.

\*By Fisher's exact test for categorical variables, and by ANOVA for continuous variables.

<sup>†</sup>Nonsteroidal anti-inflammatory drug.

<sup>‡</sup>At least weekly.

<sup>§</sup>Diet plus supplements.

<sup>||</sup>At least two adenomas.

<sup>¶</sup>At least one large adenoma.

<sup>\*\*</sup>At least one villous or tubulovillous adenoma.

<sup>††</sup>Mild dysplasia as highest degree of dysplasia in any adenoma.

persons from each of the other three groups. Intraclass correlation coefficients for biopsy scoring reliability were 0.95 and 0.98 for MLH1 and MSH2, respectively.

At baseline, there were no significant differences in serum levels of 25-OH-vitamin D or 1,25-(OH)<sub>2</sub>-vitamin D among the four study groups. As previously reported (9), at the end of follow up, 25-OH-vitamin D serum levels had statistically significantly increased in the vitamin D and calcium plus vitamin D groups, and decreased

minimally in the placebo and calcium groups; the decrease, however, was not statistically significant. As expected, serum levels of 1,25-(OH)<sub>2</sub>-vitamin D did not change within any treatment group (data not shown).

### Effects of calcium and/or vitamin D<sub>3</sub> supplementation on MSH2 expression

At baseline, the four treatment groups did not differ significantly in their expression of MSH2 or MLH1 in

the rectal mucosa. The graphical assessment of MSH2 distribution showed that after the treatment period, the MSH2 protein retained its normal within-rectal crypt distribution in all four treatment groups with most MSH2 expression concentrated in the lower 60% of the crypt (the proliferation zone; Fig. 2). After 6 months of treat-

ment, MSH2 expression along the full lengths of crypts increased by 30% ( $P = 0.36$ ) and 61% ( $P = 0.11$ ) in the calcium and vitamin D groups, respectively, relative to the placebo group, but did not change appreciably in the calcium plus vitamin D treatment group (Table 2A). Most of the *absolute* change in the calcium, vitamin D, and

**Table 2.** MLH1 and MSH2 expression in colorectal crypts at baseline and 6-mo follow-up shown as staining batch–standardized optical density of staining of the immunohistochemically detected biomarkers

	Baseline				6-mo follow-up				Absolute treatment effect*				Relative effect†
	n	Mean‡	SEM	P§	n	Mean	SEM	P	n	Mean	SEM	P	
<b>A. Entire crypts</b>													
<b>MSH2</b>													
Placebo	20	1.01	0.14		20	1.01	0.14		17				1.00
Calcium	23	0.86	0.13	0.41	21	1.11	0.14	0.60	21	0.26	0.28	0.36	1.30
Vitamin D	22	0.75	0.13	0.17	20	1.20	0.14	0.34	19	0.46	0.28	0.11	1.61
Calcium + vitamin D	22	1.13	0.13	0.57	21	1.10	0.14	0.66	20	-0.03	0.28	0.93	0.98
<b>MLH1</b>													
Placebo	17	1.04	0.07		18	1.05	0.07		13				1.00
Calcium	18	0.98	0.07	0.59	19	1.11	0.07	0.58	17	0.11	0.15	0.44	1.11
Vitamin D	21	0.93	0.07	0.27	18	1.11	0.07	0.59	18	0.17	0.14	0.24	1.18
Calcium + vitamin D	22	1.05	0.07	0.93	17	1.11	0.07	0.56	16	0.05	0.14	0.71	1.05
<b>B. Upper 40% of crypts</b>													
<b>MSH2</b>													
Placebo	20	0.10	0.02		20	0.06	0.02		17				1.00
Calcium	23	0.07	0.02	0.27	21	0.09	0.02	0.31	21	0.06	0.04	0.13	2.07
Vitamin D	22	0.06	0.02	0.18	20	0.11	0.02	0.12	19	0.08	0.04	0.04	2.69
Calcium + vitamin D	22	0.07	0.02	0.30	21	0.09	0.02	0.41	20	0.05	0.04	0.18	1.90
<b>MLH1</b>													
Placebo	17	0.33	0.03		18	0.31	0.03		13				1.00
Calcium	18	0.31	0.03	0.64	19	0.33	0.03	0.57	17	0.04	0.06	0.47	1.14
Vitamin D	21	0.30	0.03	0.44	18	0.35	0.03	0.26	18	0.07	0.05	0.18	1.26
Calcium + vitamin D	22	0.35	0.03	0.58	17	0.36	0.03	0.20	16	0.03	0.06	0.58	1.10
<b>C. Lower 60% of crypts</b>													
<b>MSH2</b>													
Placebo	20	0.91	0.13		20	0.94	0.13		17				1.00
Calcium	23	0.79	0.12	0.48	21	1.02	0.13	0.68	21	0.20	0.26	0.44	1.25
Vitamin D	22	0.68	0.12	0.20	20	1.09	0.13	0.43	19	0.38	0.26	0.16	1.54
Calcium + vitamin D	22	1.05	0.12	0.44	21	1.01	0.13	0.73	20	-0.08	0.26	0.77	0.92
<b>MLH1</b>													
Placebo	17	0.72	0.05		18	0.75	0.05		13				1.00
Calcium	18	0.68	0.05	0.58	19	0.78	0.05	0.62	17	0.07	0.10	0.45	1.11
Vitamin D	21	0.63	0.05	0.22	18	0.76	0.05	0.86	18	0.10	0.10	0.32	1.15
Calcium + vitamin D	22	0.70	0.04	0.85	17	0.76	0.05	0.89	16	0.02	0.10	0.81	1.03

\*Absolute treatment effect = (treatment group follow-up – treatment group baseline) – (placebo group follow-up – placebo group baseline).

†Relative effect = [(treatment group follow-up/treatment group baseline)/(placebo follow-up/placebo baseline)]; interpretation as for odds ratio (e.g., a relative effect of 1.6 indicates a proportional increase of 60% in the treatment group relative to that in the placebo group).

‡Standardization for staining batch done by dividing each individual's labeling optical density measurement by the mean measurement of their staining batch. Batch-specific means were calculated among all subjects for the baseline visit and among the placebo group for the follow-up visit.

§Evaluates the difference between each treatment group and the placebo group.

calcium plus vitamin D groups occurred in the lower 60% of crypts (Table 2B and C; Fig. 2); the relative treatment effect in this crypt zone was very similar to that for the entire crypt in each treatment group (Table 2A and C). On the other hand, the greatest *relative* change occurred in the upper 40% of the crypt (the differentiation zone) in which MSH2 expression increased by 107% ( $P = 0.13$ ), 169% ( $P = 0.04$ ), and 90% ( $P = 0.18$ ) in the calcium, vitamin D, and calcium plus vitamin D groups, respectively, relative to the placebo group (Table 2B). Because of the overall low expression of MSH2 in the differentiation zone, the absolute differences here were much lower than in the lower portion of the crypt. The proportion of MSH2 in the upper 40% of the crypt (distribution index) did not change appreciably in any of the treatment groups (data not shown).

### Effects of calcium and/or vitamin D supplementation on MLH1 expression

The graphical assessment of MLH1 expression within the crypt indicated that the most baseline to follow-up change occurred in the vitamin D group, in which MLH1 expression appeared to increase uniformly along the entire length of the crypt. In the other three groups, the expression curves for the baseline and the follow-up visits were virtually identical (Fig. 3).

Changes in MLH1 expression in the calcium and/or vitamin D supplementation groups relative to the placebo group were similar but less pronounced than those for MSH2 (Table 2). At the end of the treatment period, MLH1 expression in the entire crypt increased by 11% ( $P = 0.44$ ), 18% ( $P = 0.24$ ), and 5% ( $P = 0.71$ ) in calcium, vitamin D, and calcium plus vitamin D groups, respectively, relative to the placebo group (Table 2A). The increase in MLH1 expression occurred uniformly along the crypt length and was of approximately the same magnitude in the proliferation and differentiation zones of the crypt (Table 2B and C); this resulted in no change in the distribution index in any of the treatment groups (data not shown).

## Discussion

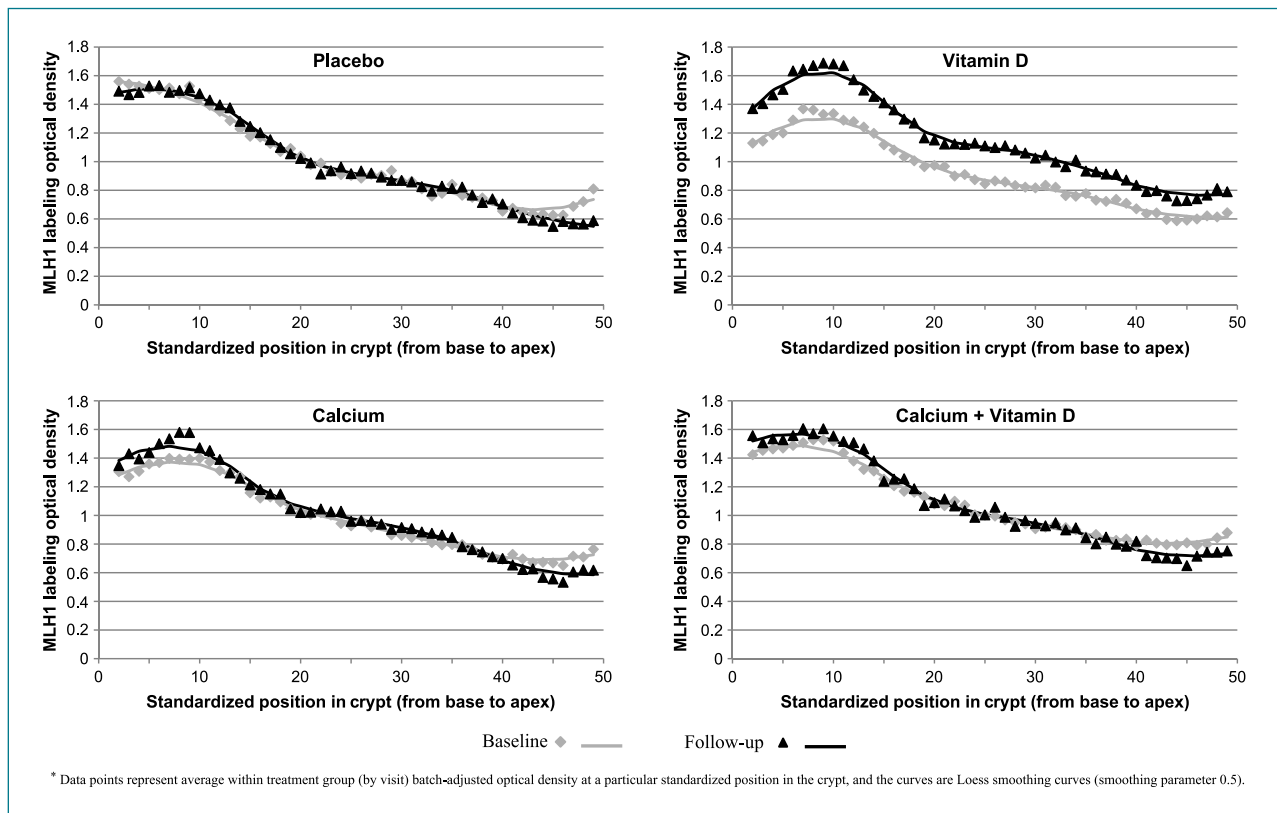
This clinical trial had intertwined missions of developing modifiable biomarkers of risk for colorectal cancer and assessing whether the molecular phenotype of the normal-appearing colorectal mucosa defined by these biomarkers of risk may be modifiable by calcium and/or vitamin D<sub>3</sub>. In this pilot trial, we found large estimated increases in MLH1 and, especially, MSH2 in the calcium and, especially, the vitamin D<sub>3</sub> groups relative to the placebo group. Despite the small sample size, the result for the change in MSH2 expression in the differentiation zone of the crypts in the vitamin D<sub>3</sub> group was statistically significant. To our knowledge, the study reported here is the first study to investigate individual and combined effects of calcium and/or vitamin D supplementation on the expression of MLH1 and MSH2 in the normal-appearing rectal mucosa in sporadic adenoma patients.

MLH1 and MSH2 were chosen as potential modifiable biomarkers of risk for colorectal cancer because of their crucial role in the human DNA MMR mechanism and of our previous findings of lower expression of these proteins in the normal-appearing mucosa of incident, sporadic colorectal adenoma patients relative to adenoma-free controls and the associations of their expression with modifiable risk factors for colorectal neoplasms (7, 8). Loss or insufficient function of either of these proteins is the main cause of MMR mechanism impairment and is responsible for ~15% of colorectal cancers (6, 32). There are no known mechanisms of direct effects of calcium or vitamin D on MLH1 and MSH2 expression. Because in sporadic colorectal carcinomas in which the *MLH1* and/or *MSH2* gene is silenced, the silencing is primarily through epigenetic phenomena (33), the effects of calcium and vitamin D may be through epigenetic modification of the *MLH1* and *MSH2* genes. Alternatively, the multiple potential mechanisms through which calcium and vitamin D may modify the at-risk molecular phenotype of the normal-appearing colorectal mucosa may indirectly lead to changes in MLH1 and MSH2 expression. Given the strong, intriguing preliminary results from this study, basic science studies directed at elucidating the mechanism(s) are needed.

Although we hypothesized that the combined effect of calcium plus vitamin D on the MMR proteins would be greater than from either agent alone, we found that it was the smallest among all active treatment groups. At least one experiment in rodents found that calcium and vitamin D individually suppressed cancer development, but their combination was ineffective (34). The Women's Health Initiative randomized clinical trial also found no overall treatment effect from the combination of calcium plus vitamin D on colorectal cancer incidence; however, this trial used lower daily doses of calcium (1,000 mg) and vitamin D (400 IU) and had substantial treatment drop in and drop out (35). On the other hand, many animal studies that investigated the combination of calcium and vitamin D reported that the antineoplastic effect of vitamin D was stronger in animals given relatively high-calcium diets (36, 37) and at least two large cohort studies (38, 39) found clear indications of a positive interaction between the two nutrients. In a randomized clinical trial of recurrent colorectal adenoma, there was strong evidence that vitamin D may enhance the chemopreventive effect of calcium; the investigators found that calcium supplementation reduced colorectal adenoma recurrence only in people with blood levels of 25-OH-vitamin D of >29.1 ng/mL (40). In our trial, all treatment groups had mean baseline levels of 25-OH-vitamin D below 29.1 ng/mL and only the vitamin D supplementation group exceeded that level at the end of follow-up, which may be another explanation of why we did not see any appreciable effect in the calcium plus vitamin D group.

Previous human studies of calcium and/or vitamin D and MLH1 and MSH2 have been limited to investigations of associations of calcium and/or vitamin D with





**Figure 3.** Expression of MLH1 protein at standardized positions within the crypts of normal-appearing rectal mucosa in four treatment groups\*, the Calcium, Vitamin D, and Markers of Adenomatous Polyps Trial.

colorectal carcinomas with MSI. MSI develops due to impaired function of MLH1 and/or MSH2 and total absence of one of the proteins leads to high-degree MSI (41-43). Two American case-control studies reported inverse associations between increased calcium intake and colorectal carcinomas with MSI (44, 45). We did not investigate MSI neoplasms, but our findings suggest that calcium may decrease risk of MSI by directly or indirectly increasing the abundance of MLH1 and MSH2 proteins. On the other hand, a Dutch study reported that increased calcium intake was associated with increased risk of MSI colorectal carcinomas, but their results were not statistically significant (46).

The increase in MLH1 and MSH2 expression in the calcium and vitamin D groups that we observed in our study suggests that calcium and vitamin D may have increased the activity of the DNA MMR mechanism. Such an increase in activity may be due to an increased capacity of a previously impaired MMR mechanism or it may be a response of the MMR mechanism to an increase in the number of DNA mismatches caused by increased cell proliferation. The latter is unlikely because (a) in this same study (10) and in our previous trial (24), calcium supplementation did not affect the overall colorectal cell proliferation rate; and (b) in both studies, there was a downward shift of the proliferative zone (10, 24), whereas there was no evidence for

a crypt zone shift for either MSH2 or MLH1 expression in the current study.

In our study, we observed stronger effects of calcium and vitamin D on MSH2 expression along the length of colorectal crypts than on MLH1 expression. A biological mechanism for this finding is unclear. One possible explanation is that because in the steady-state there is substantially more MSH2 than MLH1 protein in the cell (47), an increase in MMR function would also require a greater increase in MSH2 concentration.

Our study has several strengths and limitations. It is the only randomized, double-blind, placebo-controlled trial to have assessed the independent and combined effects of supplemental calcium and vitamin D on DNA MMR markers in the normal rectal epithelium; there was high protocol adherence by study participants; immunostaining was automated; and through the use of novel quantitative image analysis procedures, biopsy analysis reliability was high. On the other hand, MLH1 and MSH2 are not proven biomarkers of risk for colorectal cancer, but substantial basic science and epidemiologic literature support their role in colorectal carcinogenesis (4-6, 32). This study cannot prove that calcium and/or vitamin D increase the capacity of DNA MMR system, but its results suggest that calcium and vitamin D could have at least an indirect effect on MLH1 and MSH2 expression and thus the entire MMR mechanism.

Overall, the results of this pilot clinical trial suggest that (a) calcium and vitamin D<sub>3</sub> individually may increase expression of MLH1 and MSH2 proteins in normal-appearing rectal mucosa; (b) the effect of vitamin D<sub>3</sub> on both MLH1 and MSH2 expression may be stronger than that of calcium; (c) combined treatment with calcium and vitamin D<sub>3</sub> may have an appreciable effect on MSH2 and MLH1 expression only in the differentiation zone of the crypt, but this effect may be weaker than the separate effects of calcium or vitamin D<sub>3</sub>; and (d) MLH1 and MSH2 proteins may be potential modifiable biomarkers of risk for colorectal cancer, but further investigation in a full-scale study is required to obtain definitive results. Our trial adds to the body of knowledge supporting calcium and vitamin D<sub>3</sub> as potential chemopreventive agents against colorectal neoplasms.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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