Effects of calcium and vitamin D on MLH1 and MSH2 expression in rectal mucosa of sporadic colorectal adenoma patients

Eduard Sidelnikov, Emory University
Robert M Bostick, Emory University
W Dana Flanders, Emory University
Qi Long, Emory University
Veronika Fedirko, Emory University
Aasma Shaukat, University of Minnesota
Carrie R. Daniel, Department of Health and Human Services
Robin E Rutherford, Emory University

Journal Title: Cancer Epidemiology, Biomarkers and Prevention
Volume: Volume 19, Number 4
Publisher: American Association for Cancer Research | 2010-04, Pages 1022-1032
Type of Work: Article | Post-print: After Peer Review
Publisher DOI: 10.1158/1055-9965.EPI-09-0526
Permanent URL: http://pid.emory.edu/ark:/25593/fh92p

Final published version: http://cebp.aacrjournals.org/content/19/4/1022

Copyright information:
@ 2013 Society for Endocrinology

Accessed February 29, 2020 4:48 AM EST
Effects of calcium and vitamin D on MLH1 and MSH2 expression in rectal mucosa of sporadic colorectal adenoma patients

Eduard Sidelnikov1,2, Roberd M. Bostick1,2, W. Dana Flanders1,2,3, Qi Long2,3, Veronika Fedirko1,2, Aasma Shaukat4, Carrie R. Daniel5, and Robin E. Rutherford6
1Department of Epidemiology, Rollins School of Public Health, Emory University, Atlanta, GA 30322
2Winship Cancer Institute, Emory University, Atlanta, GA 30322
3Department of Biostatistics and Bioinformatics, Rollins School of Public Health, Emory University, Atlanta, GA 30322
4Department of Medicine, GI Division, University of Minnesota, Minneapolis, MN 55455
5Nutritional Epidemiology Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, Department of Health and Human Services, Bethesda, MD 20852
6Emory University School of Medicine, Division of Digestive Diseases, Atlanta, GA 30322

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 D3, 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 D3, alone or in combination, versus placebo over six 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 six 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) where 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.

Keywords
mutS-homolog 2 (MSH2); mutL-homolog 1 (MLH1); randomized controlled trial; DNA mismatch repair; normal colorectal mucosa

Reprints requests should be sent to: Roberd M. Bostick, MD, MPH, Department of Epidemiology, 1518 Clifton Road NE Atlanta, GA 30322, rmbosti@sph.emory.edu, Tel. (404)-727-2671, Fax (404)-727-8737.
Introduction

Colorectal cancer, the second leading cause of cancer deaths in the United States (1), is strongly associated with Western diets and lifestyles (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 pre-neoplastic 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.

We recently reported that the protein expression of the DNA mismatch repair 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 mismatch repair (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, anti-neoplastic 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 β-catenin expression via the calcium sensing receptor (CaSR) (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 mismatch repair proteins in the normal colorectal mucosa. This paper reports findings from a pilot clinical trial that assessed the individual and combined effects of calcium and vitamin D₃ supplementation on the expression of MLH1 and MSH2 proteins in the normal-appearing colorectal mucosa of colorectal adenoma patients.

Participants and Methods

This study was approved by the Emory University IRB. 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 30–75 years of age, in general good health, capable of informed consent, a history of at least one pathology-confirmed adenomatus colonic or rectal polyp within the past 36 months, 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 months 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 eight months, 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 socio-demographics, medical history and medication use, nutritional supplement use, lifestyle, family history, and others), and provided a blood sample. Diet was assessed with a semi-quantitative Willett food frequency questionnaire (22). Medical and pathology records were reviewed. Those still eligible and willing to participate then entered a 30-day 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 [NSAID] 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 D₃ supplementation group (400 IU twice daily), and a calcium plus vitamin D₃ supplementation group taking 2.0 g elemental calcium plus 800 IU of vitamin D₃ daily. Each group consisted of 23 participants.

Study tablets were custom manufactured by Tishcon Corporation, NY, USA. 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 D₃ 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)₂-vitamin D or 25(OH)-vitamin D. Multivitamins and calcium/vitamin D supplements typically provide 400 IU per day of vitamin D₃, but numerous intervention studies show that this dose will not suppress 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 six months to replicate the treatment period of the Calcium and Colorectal Epithelial Cell Proliferation trial (24) and to ensure approximately 2 – 3 months of 25-OH-vitamin D steady state levels. Participants attended follow-up visits at 2 and 6 months 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 seven days prior to 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 approximately one millimeter-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 semi-flexible rod. No biopsies were taken within 4.0 cm of a polypoid lesion. The biopsies were then immediately placed in phosphate buffered saline 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 six hours, transferred to 70% ethanol 24 hours after being placed in formalin, embedded in paraffin blocks (two blocks of three biopsies each) within two weeks of the biopsy procedure, cut and stained within another four weeks, and analyzed within another four weeks. From one block, five slides with four section levels each taken 40 microns 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 (PT) Module (Lab Vision Corp., CA) with 100x Citrate Buffer pH 6.0 (DAKO S1699, DAKO Corp., Carpinteria, CA; further referred to as DAKO) and steamed for 40 minutes. After antigen retrieval, slides were placed in a DAKO Automated stainer (DAKO) and rinsed with warm PT Module Buffer. The Autostainer was programmed for each immunohistochemistry (IHC) run and the following reagents were used: antibody (MLH1 antibody manufactured by BD Pharmingen, catalog no. 554072, dilution 1:15; or MSH2 antibody manufactured by Calbiochem, catalog no. NA27, 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 MHS2, diaminobenzidine (DAB) (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., IL) 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 “hemicrypt”, defined as one side of a colonic crypt bisected from base to colon lumen surface. Intact (at most two contiguous cells missing) hemicrypts extending from the muscularis mucosae to the colon lumen were considered eligible for quantitative image analysis (“scorable”; Figure 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 (i.e., whether “scorable” crypts were present).

The major equipment and software for the image analysis procedures were: personal computer, light microscope (Olympus BX40, Olympus Corporation, Japan) with appropriate filters and attached digital light microscope camera (Polaroid DMC Digital Light Microscope Camera, Polaroid Corporation, USA), digital drawing board, ImagePro Plus image analysis software (Media Cybernetics, Inc., MD), our in-house developed plug-in software for colorectal crypt analysis, and Microsoft Access 2003 relational database software (Microsoft Corporation, WA).

The following preparations were performed before starting the scoring program: 1) ensuring standardized settings on the microscope, digital camera, and imaging software; and 2) 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 200x magnification and stored as 16-bit grayscale 1,600 × 1,200 pixel images.

For each patient the two biopsies with the greatest number of “scorable” hemicrypts were selected for quantitative image analysis (“scoring”). Intact hemicrypts 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” hemicrypts per biopsy (32 per patient). If the 16th hemicrypt was reached before the level was finished, the scorer continued scoring until either the level was finished or the 20th hemicrypt was scored, whichever came first. No more than 20 hemicrypts per biopsy were scored.

If the two best biopsies had less than 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” hemicrypts between them. All three biopsies were scored only if there was less than a total of 16 “scorable” hemicrypts 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. Hemicrypts were manually traced by the scorer (Figure 1). A traced hemicrypt was divided by the software into segments corresponding in width to that of an average normal crypt epithelial cell. Overall hemicrypt- 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 hemicrypt.

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 re-analyzed during the course of the trial to determine intra-reader reliability.

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

Laboratory assays for serum 25-OH-vitamin D and 1,25-(OH)2-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 assayed together, ordered randomly, and labeled to mask treatment group, follow-up visit, and quality control replicates. The average intra-assay coefficient of variation for serum 25-OH-vitamin D was 2.3%, and for 1,25-(OH)2-vitamin D, 6.2%.

**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 analysis of variance (ANOVA) for continuous variables. Slide scoring reliability was analyzed using intra-class 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 or DI) of the MMR markers (24, 29, 30).

Treatment effects were evaluated by assessing differences in mean labeling optical densities from baseline to the 6-month 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 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 for cases and controls by colon site were plotted in the graphs (Figure 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 SAS v.9.2 statistical software (Copyright 2002–2008 by SAS Institute Inc., Cary, NC, USA). A cutoff level of \( P \leq 0.05 \) (2-sided) was used for assessing statistical significance.

## Results

### Characteristics of Study Participants

Treatment groups did not differ significantly on characteristics measured at baseline (Table 1) or at the end of follow-up (data not shown). On average, participants were 61 years old, and 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 non-smokers. 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 6-months 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 persons from each of the other three groups. Intra-class 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)\(_2\)-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)\(_2\)-vitamin D did not change within any treatment group (data not shown).

### Effects of Calcium and/or Vitamin D\(_3\) 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; Figure 2). After 6-months of treatment, 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 2, A). Most of the absolute change in the calcium, vitamin D, and calcium plus vitamin D groups occurred in the lower 60% of crypts (Table 2, B, C; Figure 2); the relative treatment effect in this crypt zone was very similar to that for the entire crypt in each treatment group (Table 2, A, C). On the other hand, the greatest relative change occurred in the upper 40% of the crypt (the differentiation zone) where 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 2, B). 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 (DI) did not change appreciably in any of the treatment groups (data not shown).

Effects of Calcium and/or Vitamin D Supplementation on MLH1 Expression

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 (Figure 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 2, A). 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 2, B and C); this resulted in no change in the DI 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 D3. In this pilot trial we found large estimated increases in MLH1 and, especially, MSH2 in the calcium and, especially, the vitamin D3 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 D3 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 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 about 15% of colorectal cancers (6, 32). There are no known mechanisms of direct effects of calcium or vitamin D on MLH1 and MSH2 expression. Since 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 anti-neoplastic effect of vitamin D was stronger in animals given relative 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 more than 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 colorectal carcinomas with microsatellite instability (MSI). MSI develops due to impaired function of MLH1 and/or MSH2 and total absence of one of the proteins leads to high degree microsatellite instability (MSI-H) (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 1) in this same study (10) and in our previous trial (24) calcium supplementation did not affect the overall colorectal cell proliferation rate, and 2) 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 since 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 strength 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 mismatch repair markers in the normal rectal epithelium; there was high protocol adherence by study participants; immunostaining was automated; and, via 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 D3 individually may increase expression of MLH1 and MSH2 proteins in normal appearing rectal mucosa; b) the effect of vitamin D3 on both MLH1 and MSH2 expression may be stronger than that of calcium; c) combined treatment with calcium and vitamin D3 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 D3; 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 D3 as potential chemopreventive agents against colorectal neoplasms.

Acknowledgments

We thank Jill Joelle Woodard and Bonita Feinstein for managing the study, Dr. Bruce W. Hollis for conducting blood vitamin D assays, Dr. Mark M. Bouzyk for conducting vitamin D receptor genotyping analysis, Christopher Farino and Stuart Myerberg for development of the study database, Charles Reichley for development of the computer scoring software, the physicians of the Emory Clinic for work on biopsy procurement, and all study participants for their time and dedication to the study.

Grant support: National Cancer Institute, National Institutes of Health (R01 CA104637 to R.M.B.); Georgia Cancer Coalition Distinguished Scholar award (to R.M.B.); the Franklin Foundation. The National Cancer Institute, the Georgia Cancer Coalition, and the Franklin Foundation had no influence on the design of the study; the collection, analysis, and interpretation of the data; the decision to submit the manuscript for publication; or the writing of the manuscript.

References


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 ("hemicrypt"); 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.

Figure 1.
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 vs. Markers of Adenomatous Polyps Trial

Data points represent average within treatment group (by visit) batch-adjusted optical density at a particular standardized position in the crypt, and the curves are Loess smoothing curves (smoothing parameter 0.5).
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
* Data points represent average within treatment group (by visit) batch-adjusted optical density at a particular standardized position in the crypt, and the curves are Loess smoothing curves (smoothing parameter 0.5).
### Table 1

Selected Baseline Characteristics of the Study Participants * (n=92)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Placebo (n=23)</th>
<th>Calcium (n=23)</th>
<th>Vitamin D (n=23)</th>
<th>Calcium + vitamin D (n=23)</th>
<th>P-value ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demographics</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>58.5 (8.2)</td>
<td>61.9 (8.2)</td>
<td>60.2 (8.1)</td>
<td>62.1 (7.5)</td>
<td>0.39</td>
</tr>
<tr>
<td>Men (%)</td>
<td>70</td>
<td>70</td>
<td>70</td>
<td>70</td>
<td>1.00</td>
</tr>
<tr>
<td>White (%)</td>
<td>74</td>
<td>83</td>
<td>65</td>
<td>61</td>
<td>0.13</td>
</tr>
<tr>
<td>College graduate (%)</td>
<td>65</td>
<td>64</td>
<td>57</td>
<td>45</td>
<td>0.53</td>
</tr>
<tr>
<td>Medical history</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>History of colorectal cancer in 1° relative (%)</td>
<td>17</td>
<td>30</td>
<td>17</td>
<td>13</td>
<td>0.60</td>
</tr>
<tr>
<td>Take NSAID ‡ regularly ‡ (%)</td>
<td>22</td>
<td>13</td>
<td>9</td>
<td>17</td>
<td>0.77</td>
</tr>
<tr>
<td>Take aspirin regularly ‡ (%)</td>
<td>22</td>
<td>52</td>
<td>30</td>
<td>57</td>
<td>0.05</td>
</tr>
<tr>
<td>Habits</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current smoker (%)</td>
<td>9</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0.61</td>
</tr>
<tr>
<td>Take multivitamin (%)</td>
<td>30</td>
<td>30</td>
<td>26</td>
<td>39</td>
<td>0.86</td>
</tr>
<tr>
<td>Physical activity (METs/day)</td>
<td>14.5 (11.6)</td>
<td>17.9 (17.9)</td>
<td>20.7 (12.0)</td>
<td>20.9 (14.7)</td>
<td>0.43</td>
</tr>
<tr>
<td>Mean dietary intakes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total energy intake, kcal/d</td>
<td>1,596 (528)</td>
<td>1,788 (691)</td>
<td>1,848 (821)</td>
<td>1,845 (752)</td>
<td>0.59</td>
</tr>
<tr>
<td>Total calcium ‡, mg/d</td>
<td>618 (308)</td>
<td>746 (335)</td>
<td>843 (526)</td>
<td>824 (714)</td>
<td>0.41</td>
</tr>
<tr>
<td>Total vitamin D ‡, IU/d</td>
<td>277 (230)</td>
<td>336 (202)</td>
<td>360 (317)</td>
<td>415 (316)</td>
<td>0.40</td>
</tr>
<tr>
<td>Total fat, gm/d</td>
<td>67 (32)</td>
<td>72 (35)</td>
<td>70 (32)</td>
<td>74 (28)</td>
<td>0.59</td>
</tr>
<tr>
<td>Dietary fiber, gm/d</td>
<td>15 (7)</td>
<td>17 (9)</td>
<td>18 (9)</td>
<td>17 (11)</td>
<td>0.97</td>
</tr>
<tr>
<td>Alcohol, gm/d</td>
<td>9 (14)</td>
<td>11 (15)</td>
<td>14 (18)</td>
<td>10 (20)</td>
<td>0.84</td>
</tr>
<tr>
<td>Anthropometrics</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body mass index (BMI), kg/m²</td>
<td>30.6 (7.2)</td>
<td>29.4 (5.5)</td>
<td>28.9 (5.6)</td>
<td>31.6 (6.0)</td>
<td>0.44</td>
</tr>
<tr>
<td>Waist-to-hip ratio</td>
<td>0.9 (0.1)</td>
<td>0.9 (0.1)</td>
<td>0.9 (0.1)</td>
<td>1.0 (0.1)</td>
<td>0.17</td>
</tr>
<tr>
<td>Adenoma characteristics</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multiple adenomas ‡ (%)</td>
<td>17</td>
<td>22</td>
<td>39</td>
<td>26</td>
<td>0.45</td>
</tr>
<tr>
<td>Characteristics</td>
<td>Treatment Group</td>
<td>P-value $^f$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------------</td>
<td>-----------------</td>
<td>-------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Placebo (n=23)</td>
<td>Calcium (n=23)</td>
<td>Vitamin D (n=23)</td>
<td>Calcium + vitamin D (n=23)</td>
<td></td>
</tr>
<tr>
<td>Large adenoma ≥ 1 cm $^{††}$ (%)</td>
<td>19</td>
<td>32</td>
<td>17</td>
<td>9</td>
<td>0.32</td>
</tr>
<tr>
<td>Villous/tubulovillous adenoma $^{‡‡}$ (%)</td>
<td>4</td>
<td>9</td>
<td>9</td>
<td>4</td>
<td>1.00</td>
</tr>
<tr>
<td>Mild dysplasia $^{§§}$ (%)</td>
<td>100</td>
<td>96</td>
<td>100</td>
<td>100</td>
<td>1.00</td>
</tr>
<tr>
<td><strong>Baseline vitamin D serum levels</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25-OH-vitamin D (ng/ml)</td>
<td>20.44 (7.5)</td>
<td>25.67 (7.6)</td>
<td>21.04 (8.3)</td>
<td>20.93 (9.6)</td>
<td>0.12</td>
</tr>
<tr>
<td>1,25-(OH)$_2$-vitamin D (pg/ml)</td>
<td>39.2 (12.2)</td>
<td>45.4 (35.3)</td>
<td>44.5 (22.6)</td>
<td>37.9 (12.5)</td>
<td>0.60</td>
</tr>
</tbody>
</table>

$^*$ Data are given as means (SD) unless otherwise specified.

$^†$ By Fisher’s exact test for categorical variables, and by ANOVA for continuous variables.

$^‡$ Nonsteroidal anti-inflammatory drug.

$^‡‡$ At least once a week.

$§§$ Diet plus supplements.

$**$ At least two adenomas.

$††$ At least two adenomas.

$‡‡$ At least one large adenoma.

$§§$ At least one villous or tubulovillous adenoma.

$^{§§}$ Mild dysplasia as highest degree of dysplasia in any adenoma.
Table 2
MLH1 and MSH2 Expression in Colorectal Crypts at Baseline and 6-months Follow-Up Shown as Staining Batch Standardized Optical Density of Staining of the Immunohistochemically-detected Biomarkers

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>6-Month Follow-up</th>
<th>Absolute Treatment Effect</th>
<th>Relative Effect $\dagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N Mean SE p</td>
<td>N Mean SE p</td>
<td>N Mean SE p</td>
<td>N Mean SE p</td>
</tr>
<tr>
<td><strong>A. Entire crypts</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSH2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>20 1.01 0.14</td>
<td>20 1.01 0.14</td>
<td>17 1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Calcium</td>
<td>23 0.86 0.13 0.41</td>
<td>21 1.11 0.14 0.60</td>
<td>21 0.26 0.28 0.36</td>
<td>1.30</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>22 0.75 0.13 0.17</td>
<td>20 1.20 0.14 0.34</td>
<td>19 0.46 0.28 0.11</td>
<td>1.61</td>
</tr>
<tr>
<td>Calcium + vitamin D</td>
<td>22 1.13 0.13 0.57</td>
<td>21 1.10 0.14 0.66</td>
<td>20 −0.03 0.28 0.93</td>
<td>0.98</td>
</tr>
<tr>
<td>MLH1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>17 1.04 0.07</td>
<td>18 1.05 0.07</td>
<td>13</td>
<td>1.00</td>
</tr>
<tr>
<td>Calcium</td>
<td>18 0.98 0.07 0.59</td>
<td>19 1.11 0.07 0.58</td>
<td>17 0.11 0.15 0.44</td>
<td>1.11</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>21 0.93 0.07 0.27</td>
<td>18 1.11 0.07 0.59</td>
<td>18 0.17 0.14 0.24</td>
<td>1.18</td>
</tr>
<tr>
<td>Calcium + vitamin D</td>
<td>22 1.05 0.07 0.93</td>
<td>17 1.11 0.07 0.56</td>
<td>16 0.05 0.14 0.71</td>
<td>1.05</td>
</tr>
<tr>
<td><strong>B. Upper 40% of crypts</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSH2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>20 0.10 0.02</td>
<td>20 0.06 0.02</td>
<td>17</td>
<td>1.00</td>
</tr>
<tr>
<td>Calcium</td>
<td>23 0.07 0.02 0.27</td>
<td>21 0.09 0.02 0.31</td>
<td>21 0.06 0.04 0.13</td>
<td>2.07</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>22 0.06 0.02 0.18</td>
<td>20 0.11 0.02 0.12</td>
<td>19 0.08 0.04 0.04</td>
<td>2.69</td>
</tr>
<tr>
<td>Calcium + vitamin D</td>
<td>22 0.07 0.02 0.30</td>
<td>21 0.09 0.02 0.41</td>
<td>20 0.05 0.04 0.18</td>
<td>1.90</td>
</tr>
<tr>
<td>MLH1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>17 0.33 0.03</td>
<td>18 0.31 0.03</td>
<td>13</td>
<td>1.00</td>
</tr>
<tr>
<td>Calcium</td>
<td>18 0.31 0.03 0.64</td>
<td>19 0.33 0.03 0.57</td>
<td>17 0.04 0.06 0.47</td>
<td>1.14</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>21 0.30 0.03 0.44</td>
<td>18 0.35 0.03 0.26</td>
<td>18 0.07 0.05 0.18</td>
<td>1.26</td>
</tr>
<tr>
<td>Calcium + vitamin D</td>
<td>22 0.35 0.03 0.58</td>
<td>17 0.36 0.03 0.20</td>
<td>16 0.03 0.06 0.58</td>
<td>1.10</td>
</tr>
<tr>
<td><strong>C. Lower 60% of crypts</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSH2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>20 0.91 0.13</td>
<td>20 0.94 0.13</td>
<td>17</td>
<td>1.00</td>
</tr>
<tr>
<td>Calcium</td>
<td>23 0.79 0.12 0.48</td>
<td>21 1.02 0.13 0.68</td>
<td>21 0.20 0.26 0.44</td>
<td>1.25</td>
</tr>
<tr>
<td></td>
<td>Baseline</td>
<td>6-Month Follow-up</td>
<td>Absolute Treatment Effect</td>
<td>Relative Effect</td>
</tr>
<tr>
<td>----------</td>
<td>----------</td>
<td>-------------------</td>
<td>---------------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>Mean*</td>
<td>SE†</td>
<td>p‡</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>22</td>
<td>0.68</td>
<td>0.12</td>
<td>0.20</td>
</tr>
<tr>
<td>Calcium + vitamin D</td>
<td>22</td>
<td>1.05</td>
<td>0.12</td>
<td>0.44</td>
</tr>
<tr>
<td>MLH1 Placebo</td>
<td>17</td>
<td>0.72</td>
<td>0.05</td>
<td>18</td>
</tr>
<tr>
<td>MLH1 Calcium</td>
<td>18</td>
<td>0.68</td>
<td>0.05</td>
<td>0.58</td>
</tr>
<tr>
<td>MLH1 Vitamin D</td>
<td>21</td>
<td>0.63</td>
<td>0.05</td>
<td>0.22</td>
</tr>
<tr>
<td>MLH1 Calcium + vitamin D</td>
<td>22</td>
<td>0.70</td>
<td>0.04</td>
<td>0.85</td>
</tr>
</tbody>
</table>

* 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.
† SE – standard error
‡ Evaluates the difference between each treatment group and the placebo group.
§ 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).