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Huafeng Shen Date

EFFECTS OF CALCIUM AND VITAMIN D SUPPLEMENTATION ON CRYPT MORPHOLOGY IN NORMAL COLON MUCOSA

By

Huafeng Shen

Master of Public Health

Epidemiology

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Roberd M. Bostick
Committee Chair

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Committee Member

_____

Committee Member

_____

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Huafeng Shen

Bachelor of Medicine, Zhejiang University, 2005
Master of Medicine, Zhejiang University, 2007

Thesis Committee Chair: Roberd M. Bostick, MD, MPH

An abstract of
A thesis submitted to the Faculty of the
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Abstract

EFFECTS OF CALCIUM AND VITAMIN D SUPPLEMENTATION ON CRYPT MORPHOLOGY IN NORMAL COLON MUCOSA

By Huafeng Shen

To investigate the effects of calcium and vitamin D on crypt morphology (length, perimeter, and area of crypts) in the normal colorectal mucosa of sporadic colorectal adenoma patients, we conducted a pilot, randomized, double-blind, placebo-controlled 2×2 factorial chemoprevention clinical trial of supplemental calcium 2,000 mg/day and vitamin D_3 800 IU/day, alone and in combination, versus placebo over 6 months. Colorectal crypt length, perimeter, and area in the normal-appearing rectal mucosa were quantified by image analysis. The mean crypt length increased by 1% (p=0.92) in the calcium group, and decreased by 2% (P = 0.69) and 4% (P = 0.40) in the vitamin D and calcium plus vitamin D groups, respectively, relative to the placebo group. The mean crypt perimeter decreased by 2% (P = 0.70) and 4% (P = 0.40) in the vitamin D and calcium plus vitamin D groups, respectively, relative to the placebo group, but did not change appreciably in the calcium group. The mean crypt area decreased by 2% (P = 0.74), 5% (P = 0.41) and 7% (P = 0.30) in the calcium, vitamin D and calcium plus vitamin D groups, respectively, relative to the placebo group. These results indicate that calcium and/or vitamin D_3 supplementation does not change the crypt morphology (length, perimeter, and area of crypts) in the normal human colorectal epithelium of sporadic adenoma patients.

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CHAPTER I

Descriptive Epidemiology

Colorectal cancer (CRC) is the second leading cause of cancer deaths and the third most common cancer diagnosed among men and women, in the United States, with over 142,000 incident cases and 51,370 deaths expected in 2010 (1, 2). The age-adjusted colorectal cancer incidence rates in the U.S were 59.0 per 100,000 among males and 43.6 per 100,000 among females from 2002 to 2006 (1). There was an accelerated decrease in colorectal cancer incidence rates from 1998 to 2006, which largely reflects increases in screening that can detect and remove precancerous polyps (3). The colorectal cancer mortality rates among males were 30.8 per 100,000 in 1990 and decreased to 20.5 per 100,000 in 2006, while the mortality rates among females were 20.3 per 100,000 in 1990 and decreased to 14.5 per 100,000 in 2006 (1). However, despite advances in screening, treatment and prevention, the mortality due to colorectal cancer has declined only modestly over the past several decades (1, 2).

Incidence rates vary around the world, with the highest rates seen in the developed countries and the lowest in India (4). Migration studies have indicated increasing colorectal cancer rates among people moving from low incidence to high incidence areas (5-8). A population-based case-control study of colorectal cancer among Chinese men and women in western North America and the People's Republic of China showed that the colorectal cancer risk among migrants to North America increased with increasing years lived in North America (5). The incidence of colorectal cancer during 1973-1986 among Asian residents in the western United States in relation to country of birth was

examined (6). The results indicated that the incidence rate among US-born Japanese men was twice as high as that among foreign-born Japanese men and about 60% higher than those of US-born white men. Incidence among US-born Japanese women was about 40% higher than that among Japanese women born in Japan or US-born white women. Foreign-born Chinese men had about the same incidence of colorectal cancer as US-born white men, while US-born Chinese men experienced slightly reduced rates. Another study reported that the rates of colorectal cancer in Italian migrants decrease in the low risk area of Sao Paulo Brazil and increase in the high risk area of Argentina (8). The international differences and migrant data suggest a role for environmental factors, such as diet, in colorectal carcinogenesis.

Colorectal Carcinogenesis

Colorectal cancer typically develops from adenomatous polyps arising from the lining of the intestine. The size and number of adenomas, as well as their histologic type and the presence of epithelial dysplasia, are thought to affect the risk of colorectal cancer development (9, 10). Intervention studies with colorectal cancer as an endpoint are difficult to perform owing to the large number of patients and the long follow-up required. Therefore, studies using the appearance of colorectal adenomatous polyps as a surrogate endpoint are usually considered.

According to Potter's recent review, there are three main pathways depicting the progression from normal colonic cell to cancer cell (11). The first and most important pathway is the APC-β-catenin-Tcf-MYC pathway. Most colorectal cancers are initiated by a mutation of APC gene, which may result in the development of adenoma. If this mutation is inherited, it will result in the familial adenomatous polyposis syndrome

(FAP), which is characterized by the development of multiple colorectal adenomas. Mutation of the APC gene leads to increased concentrations of β-*catenin,* which after adhering to T-cell factor 4 (Tcf4), mediates transcription of certain genes, including the oncogene *c-myc* (12). Progression from adenoma to carcinoma is then dependent on accumulation of other genetic and epigenetic alterations, such as DNA hypomethylation and mutations of the *K-ras* and *p53* genes (13). The second carcinogenic pathway involves mutations in DNA mismatch repair genes, which is associated with microsatellite instability. Mutations in mismatch repair genes are found in approximately 90% of hereditary nonpolyposis colorectal cancer (HNPCC) and in up to 15% of sporadic colorectal cancers (14, 15). Several mismatch repair genes have been identified: *hMLH1*, *hMSH2, hPMS1, hPMS2* and *hMSH6*; mutations in the *hMLH1* and *hMSH2* genes are most commonly found in HNPCC (16). Mutations of the BAX gene and the TGF-β type II receptor gene have commonly been identified in colorectal cancers with microsatellite instability (11). The third carcinogenesis pathway has been suggested for persons with ulcerative colitis, which is associated with an approximately 20-fold increased risk of colorectal cancer. Chronic inflammation can result in genetic alterations, which can progress to dysplasia and subsequently to cancer (11, 17).

Environmental Factors in Colorectal Cancer

Western dietary pattern associated with a higher body mass index and a greater intake of total energy and dietary fat has been implicated by many studies as particularly high risk for CRC (18, 19). Recent data suggests that the beneficial effects of diets high in fruits, vegetables, and fiber may be less than had been previously believed (20-22). The association between high intakes of folate and risk of CRC has still been inconclusive

(23, 24). Evidence from the epidemiologic studies for protection against colorectal cancer by higher intakes of calcium is very strong, while the human evidence for a protective effect of vitamin D against colorectal cancer is not as strong as that for calcium (25). Physcial activity has been consistently inversely associated with CRC (26). The association between alcohol and CRC risk has been controversial, but the weight of evidence suggests that high intake of alcohol increases risks of CRC (27). The association of cigarette smoking and CRC risk appears to be dose-related. Long-term cigarette smoking is associated with increased risk of colorectal cancer (28, 29). Non-steroidal anti-inflammatory drugs (NSAIDs) lower both short-term and long-term risk of CRC (30).

Calcium, Vitamin D, and Colorectal Cancer Risk

There is strong biologic plausibility and animal experimental evidence for protection against colorectal cancer by calcium and vitamin D. Proposed mechanisms of calcium against colorectal cancer include protection of colonocytes against bile acids and fatty acids (31), direct effects on cell cycle regulation, modulation of the APC colon carcinogenesis pathway, and modulation of E-cadherin and β-catenin expression via the calcium-sensing receptor (32-34). Proposed mechanisms for vitamin D involve bile acid catabolism, direct effects on the cell cycle, growth factor signaling, cell adhesion, DNA repair and modulation of >200 genes (32, 35). There have been numerous observational studies of calcium and vitamin D and risk of CRC, but there have been only few clinical trials. Herein, the epidemiologic evidence for effects of calcium and/or vitamin D on risk of CRC is summarized.

Observational Studies of Calcium and Colorectal Cancer Risk

Data from the numerous observational studies, especially from the prospective cohort studies, are consistent with the hypothesis that higher intakes of calcium reduce risk of CRC. Of 42 analytic observational studies of calcium and CRC (22 case-control studies and 20 prospective cohort studies), inverse associations were found in 30 (71%) studies, of which 16 were statistically significant, three found null associations, and nine found increased risk with higher intake, none of which was statistically significant (25). A pooled analysis of 10 cohort studies from five countries reported a statistically significant 22% reduction in risk for incident CRC among those with the highest versus the lowest levels of calcium intake (36). The results of calcium and colorectal adenoma studies are relatively fewer. Of 11 observational studies (eight primary case-control studies, two case-control studies nested in cohort studies, and one prospective study in a clinical-trial cohort), nine (82%) found inverse associations, of which one was statistically significant, and two found statistically nonsignificant increased risk with higher intake (25).

Clinical trials of Calcium and Colorectal Cancer Risk

There are at least 17 trials of calcium and colorectal epithelial cell proliferation, most were pilot studies that primarily reported beneficial responses. Only two full-scale clinical trials have been reported. One found a statistically significant shift of proliferative zone to the lower 60% of the crypt (normalization), without reduction of the overall proliferation rate (37). The second one also found no effect on proliferation (38).

There have been five preliminary and two major clinical trials of calcium and adenoma recurrence, and one major trial of prevention of incident colorectal cancer. In a US multicenter, randomized, double-blind, placebo-controlled clinical trial (the Calcium

Polyp Prevention Study), a total of 930 subjects were randomly assigned into either 1,200 mg of elemental calcium daily or placebo to test the effect of calcium supplementation on the recurrence of colorectal adenomas (39). Calcium supplementation statistically significantly reduced colorectal adenoma recurrence by 15%, an effect that extended up to at least 5 years after cessation of active treatment (39, 40). A European multicenter randomized trial randomized 665 patients with a history of colorectal adenomas to three treatment groups: 2,000 mg of elemental calcium daily, fiber, or placebo. After 3 years of follow-up, adenoma recurrence was 34% lower in the calcium group, although the finding was not statistically significant (22). The summary risk ratio (RR) in a meta-analysis of all seven adenoma recurrence trials was 0.80 (95% CI: 0.68-0.93) (41). A recent trial involving 36,282 postmenopausal women tested the effect of calcium with vitamin D supplementation in the prevention of colorectal cancer (42). A total of 18,176 women received 1,000 mg of elemental calcium with 400 IU of vitamin D daily and 18,106 received a matching placebo for an average of 7 years. They concluded that daily supplementation of calcium with vitamin D for seven years had no effect on the incidence of colorectal cancer among postmenopausal women. However, these data are difficult to interpret due to the low doses administered and the relative short length of follow-up for colorectal cancer as an endpoint.

Observational Studies of Vitamin D and Colorectal Cancer Risk

Of 30 analytic observational studies of vitamin D and CRC (17 case-control studies and 13 prospective cohort studies), 20 (67%) found inverse associations, of which six were statistically significant, six found null associations, and four found statistically nonsignificant positive associations (25). A pooled analysis of five cohort studies

reported a statistically nonsignificant 7% reduction in risk for incident colorectal cancer among those consuming the highest levels of vitamin D versus those consuming the lowest levels (36).

Of 21 analytic observational studies of vitamin D and colorectal adenoma (12 primary case-control studies, four case-control studies nested in prospective cohort studies, and five prospective studies in clinical-trial cohorts), 12 (57%) found inverse associations of which three were statistically significant, seven found null associations, and two found statistically nonsignificant increased risk (25).

Those studies that investigated total vitamin D exposures based on diet alone without considering vitamin D exposure from sunlight and inconsistent vitamin D fortification of milk products may have serious error and misclassification and bias findings toward the null. The consistent results from the previous 25-(OH)-vitamin D blood level studies suggest the assessment of vitamin D exposure using 25-(OH)-vitamin D blood level in the future studies (25).

Studies of Calcium plus Vitamin D and Colorectal Cancer Risk

Of the numerous observational studies of calcium and vitamin D and colorectal cancer, only 13 have reported investigating the potential synergistic modification of risk for colorectal cancer. Only four of these presented complete data for assessing interactions (25). A large clinical trial of colorectal adenoma recurrence suggested that calcium supplementation was primarily effective among those with 25-(OH)-vitamin D levels greater than the cohort median (29.1 ng/mL) (RR = 0.71, 95% CI = 0.57-0.89, $P_{interaction}$ = 0.01) (43). These data indicate that assessment of vitamin D exposure, using 25-(OH)-

vitamin D blood level, may be particularly important for investigating calcium-vitamin D interactions.

Overall, evidence from the observational epidemiologic studies for protection against colorectal cancer by higher intakes of calcium is very strong, especially that from the prospective cohort studies. This observational evidence is supported by reduced adenoma recurrence with calcium supplementation in clinical trials. The human evidence for a protective effect of vitamin D against colorectal cancer is not as strong as that for calcium. Serum 25-(OH)-vitamin D is the best indicator of total vitamin D exposure. Human studies assessing calcium-vitamin D interactions have been inconclusive.

Surrogate End-Point Biomarkers (SEBs) in Colorectal Cancer

As with many other cancers, colon carcinogenesis is the result of a multistep process in which an increasing number of alterations occur as cells progress from normal to precancerous states of increasing size and dysplasia to cancer and finally to metastatic disease. Ideally, a comprehensive panel of biomarkers would show differential expression between the various phases of colon carcinogenesis from which to accurately categorize and quantify risk (44). Phenotypic biomarkers could “summarize” the result of complex interactions among genotype, gene-gene interactions, epigenetic phenomenon, environmental exposures, and gene-environment interactions. However, currently there are no generally accepted preneoplastic biomarkers of risk for colorectal cancer.

A clinical trial of 193 sporadic adenoma patients treated with placebo (n = 66), 1.0 g calcium (n = 64), or 2.0 g calcium (n = 63) daily for 6 months used colorectal epithelial cell proliferation as a biomarker (37). Calcium supplementation shifted the zone of

proliferation from the entire crypt to the lower 60% of the crypt without affecting the overall proliferation rate in the colorectal mucosa of sporadic adenoma patients. Some biomarker data from the current pilot, randomized, double-blind, placebo-controlled, 2×2 factorial chemoprevention clinical trial were published previously. The preliminary results suggested that calcium and vitamin D, individually or together, may enhance apoptosis (as indicated by the expression of Bcl-2, an apoptosis inhibitor, and Bax, an apoptosis promoter) (45); promote colorectal epithelial cell differentiation (as indicated by the expression of $p21^{waf1/cip1}$ a marker of differentiation; MIB-1, a marker of short-term proliferation; and hTERT, a marker of long-term proliferation) (46); increase DNA mismatch repair (MMR) activity (as indicated by expression of MSH2 and MLH1) (47); decrease oxidative DNA damage (as indicated by 8-hydroxy-2′-deoxyguanosine [8-OH-dG]) (48); and increase expression of the calcium receptor (CaR), the vitamin D receptor (VDR), and the P450 cytochromes, CYP27B1 and CYP24A1 (49), in the normal colorectal epithelium of sporadic adenoma patients.

Crypt Morphology and Colorectal Cancer

Almost all carcinomas of the colon and rectum develop from adenomatous polyps, which are thought to arise from the “susceptible” colorectal epithelium characterized by hyperproliferation, impaired apoptosis, and reduced differentiation. Crypt morphology (length, perimeter, and area crypts) are associated with colonic cell proliferation, apoptosis, and differentiation, and, therefore, may serve as modifiable biomarkers of risk for colorectal neoplasm. There are a few animal and human studies on the effects of calcium alone on crypt length in the normal colon mucosa. In a transmissible murine colonic hyperplasia (TMCH) model, diet with 1.0% calcium and 6% pectin inhibited

increases in crypt length compared with the standard diet with 0.5% calcium and 5% cellulose (50). In a small uncontrolled intervention study, no change in crypt length was observed after 12 weeks and 1 year of 1,500 mg calcium supplementation compared with the baseline data (51). In a randomized controlled trial, a total of 111 sporadic adenoma patients were randomized into one of three groups: the first group received two placebos, the second group received calcium 1,000 mg/day plus placebo, and the third group received placebo + resistant starch. After 2-months of follow-up, no difference in crypt length was observed among the three groups for the different colorectal areas. Crypt length in this study was obtained by counting the total number of cells in whole length cut colonic crypts and dividing by two (52).

Aberrant crypt foci (ACF) are tiny lesions at the earliest stage of colorectal carcinogenesis; they consist of large, thick crypts that can be identified by dense, methylene blue staining. ACF were first reported in a rodent model in 1987 (53). Numerous genetic and epigenetic changes, including frequent *K-ras* mutations (54) and infrequent APC mutations (55) or microsatellite instability (56), have been identified in ACF which are also present in colon cancers. It is thought that the progression of ACF to polyp and, subsequently, to cancer parallels the accumulation of several molecular alterations and mutations whereby a small fraction of ACF evolve to colorectal cancer (57, 58). Growing evidence in animal and human studies suggests that ACF could serve as surrogate biomarkers of colonic carcinogenesis for purposes of risk stratification or testing chemopreventive efficacy of new agents (59).

Although the preliminary results from the current pilot, randomized, double-blind, placebo-controlled, 2×2 factorial chemoprevention clinical trial from which the data for

the analysis reported herein were derived, found changes in the expression of biomarkers of apoptosis, differentiation and proliferation, DNA mismatch repair, oxidative DNA damage, CaR, VDR, CYP27B1, and CYP24A1 in the normal colorectal epithelium, the effects of calcium and vitamin D, individually or jointly, on crypt morphology in the normal human colorectal epithelium has not been previously investigated or reported by us or others. Herein, we report the first human trial results on the effects of calcium and/or vitamin D supplementation on colonic crypt morphology (length, perimeter, and area of crypts) as biomarkers of risk for colorectal neoplasms in the normal-appearing colorectal mucosa.

CHAPTER II

EFFECTS OF CALCIUM AND VITAMIN D SUPPLEMENTATION ON CRYPT MORPHOLOGY IN NORMAL COLON MUCOSA

Huafeng Shen

ABSTRACT

To investigate the effects of calcium and vitamin D on crypt morphology (length, perimeter, and area of crypts) in the normal colorectal mucosa of sporadic colorectal adenoma patients, we conducted a pilot, randomized, double-blind, placebo-controlled 2×2 factorial chemoprevention clinical trial of supplemental calcium 2,000 mg/day and vitamin D_3 800 IU/day, alone and in combination, versus placebo over 6 months. Colorectal crypt length, perimeter, and area in the normal-appearing rectal mucosa were quantified by image analysis. The mean crypt length increased by 1% (p=0.92) in the calcium group, and decreased by 2% (P = 0.69) and 4% (P = 0.40) in the vitamin D and calcium plus vitamin D groups, respectively, relative to the placebo group. The mean crypt perimeter decreased by 2% (P = 0.70) and 4% (P = 0.40) in the vitamin D and calcium plus vitamin D groups, respectively, relative to the placebo group, but did not change appreciably in the calcium group. The mean crypt area decreased by 2% (P = 0.74), 5% (P = 0.41) and 7% (P = 0.30) in the calcium, vitamin D and calcium plus vitamin D groups, respectively, relative to the placebo group. These results indicate that calcium and/or vitamin D_3 supplementation does not change the crypt morphology (length, perimeter, and area of crypts) in the normal human colorectal epithelium of sporadic adenoma patients.

INTRODUCTION

Colorectal cancer (CRC) is the second leading cause of cancer deaths and the third most common cancer diagnosed among men and women in the United States. Despite advances in screening, treatment and prevention, the mortality due to colorectal cancer has declined only modestly over the past several decades (1, 2). The international differences and migrant data suggest a role for environmental factors, such as diet, in colorectal carcinogenesis.

There is strong biologic plausibility and animal experimental evidence for protection against colorectal cancer by calcium and vitamin D. Proposed mechanisms of calcium against colorectal cancer include protection of colonocytes against bile acids and fatty acids (18), direct effects on cell cycle regulation, modulation of the APC colon carcinogenesis pathway, and modulation of E-cadherin and β-catenin expression via the calcium-sensing receptor (19-21). Proposed mechanisms for vitamin D involve bile acid catabolism, direct effects on the cell cycle, growth factor signaling, cell adhesion, DNA repair and modulation of >200 genes (19, 22).

Almost all carcinomas of the colon and rectum develop from adenomatous polyps, which are thought to arise from the “susceptible” colorectal epithelium characterized by hyperproliferation, impaired apoptosis, and reduced differentiation. Crypt morphology (length, perimeter, and area crypts) are associated with colonic cell proliferation, apoptosis, and differentiation, and, therefore, may serve as modifiable biomarkers of risk for colorectal neoplasm.

The effects of calcium and vitamin D, individually or jointly, on crypt morphology in the normal human colorectal epithelium have not been previously reported. 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 D_3, alone and in combination, versus placebo over 6 months, to test the effects of these agents on crypt morphologic characteristics (length, perimeter, and area of crypts) in the normal colorectal mucosa.

METHODS

Participant Population

Participants were recruited from the patient population attending the Digestive Diseases Clinic of Emory University. Eligibility included age 30 to 75 years, in general good health, capable of informed consent, and a history of at least one pathology-confirmed sporadic colon or rectal adenoma within the past 36 months. Exclusions included contraindications to calcium or vitamin D supplementation or rectal biopsy procedures, and medical conditions, habits, or medication usage that would otherwise interfere with the study. Specific exclusions were reported elsewhere (45). This study was approved by the Emory University Institutional Review Board. Written informed consent was obtained from each study participant.

Clinical Trial Protocol

Between April 2005 and January 2006, 522 age-eligible patients diagnosed with at least one pathology-confirmed adenomatous colonic or rectal polyp within the previous

36 months were identified as potential study participants. Medical charts were screened, and 224 (43%) potentially eligible patients were sent an introductory letter followed by a telephone interview to assess their willingness to participate and further eligibility. During the eligibility visit, potential participants were interviewed and signed a consent form. They also completed questionnaires (on sociodemographics, medical history, medication and nutrition supplement use, lifestyle, family history, and others) and provided a blood sample. Diet was assessed with a semiquantitative food frequency questionnaire (60). 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 took at least 80% of their tablets were randomized. Adherence for pill-taking was assessed by questionnaire, interview, and pill count. A total of 92 (88%) eligible participants then underwent a baseline rectal biopsy and were randomly assigned (stratified by sex and nonsteroidal anti-inflammatory drug use) to treatment group.

Participants (n = 92) were randomly assigned to the following four treatments: placebo (n = 23), 2.0 g elemental calcium supplementation (as calcium carbonate in equal doses twice daily; n = 23), 800 IU vitamin D_3 supplementation (400 IU twice daily; n = 23), and 2.0 g elemental calcium plus 800 IU vitamin D_3 (n = 23).

All 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 used for elemental calcium delivery in this trial. Vitamin D_3 was chosen as our form of

vitamin D. Additional details on the rationale for the doses and forms of calcium and vitamin D supplementation forms were described previously (45).

Over the 6-month treatment period, participants attended follow-up visits at 1 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 follow-up visit, participants were interviewed and completed questionnaires, and at the first and last visits, underwent venipuncture and a rectal biopsy procedure. Participants were asked to abstain from aspirin use for 7 days 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. Participants did not have to be fasting for their visits and did not take a bowel cleansing preparation or enema. The detailed protocol of this clinical trial was described previously (41).

Tissue Collection and Processing

Six 1-mm-thick biopsy specimens were taken from the 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, teased off the forceps with and onto a strip of bibulous paper, then immediately placed in PBS and oriented under a dissecting microscope to ensure that they were not twisted or curled on the bibulous paper, and then immediately placed in 10% normal buffered formalin. 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 within 2 weeks of the biopsy procedure, cut and

stained within another 4 weeks, and analyzed within another 4 weeks. The paraffin blocks were cut into 3.0-μm-thick sections. Five slides with four section levels each taken 40 μm apart were prepared, yielding a total of 20 levels per patient per visit.

Image Analysis of the Crypt Morphology (Length, Perimeter, and Area of Crypts) in Normal Colon Crypts

A quantitative image analysis method ("scoring") was used to evaluate the colonic crypt morphology (length, perimeter, and area of crypts). The unit of analysis was the "hemicrypt", defined as one half of a crypt bisected from crypt base to colon lumen. A "scorable" hemicrypt was defined as an intact hemicrypt that extended from the muscularis mucosa to the colon lumen. The major equipment and software for the image analysis procedures were a Scanscope CS digital scanner (Aperio Technologies), computer, digital drawing board, Matlab software (Math- Works), CellularEyes Image Analysis Suite (DivEyes), and MySQL (Sun Microsystems). First, slides were scanned with the Aperio Scanscope CS digital scanner, and electronic images were reviewed in the CellularEyes program to identify colon crypts acceptable for analysis. Before analysis, images of negative and positive control slides were checked for staining adequacy. Standardized settings were used on all equipment throughout the scoring procedures. Next, the hemicrypt was analyzed by precisely tracing the borders of the hemicrypt using a digital drawing board. The program then created a crypt length line midway along the hemicrypt axis, and then drew equally spaced perpendicular lines to the crypt length line at intervals to yield segments with the average widths of normal colonocytes. The created crypt length line provided the crypt length. The area inside the traced hemicrypt borders (the perimeter) was defined as crypt area. All these

measurements were automatically performed by the program. Then, the reader moved to the next hemicrypt on the same or next image, section level, biopsy, and/or slide and repeated the previously described analysis steps. The goal was to analyze a minimum of 16 hemicrypts on each of two biopsies, for a total of 32 hemicrypts (Fig. 1).

One slide reader analyzed all slides throughout the study. Blinded subsets of previously analyzed slides were resubmitted to the reader during the study to assess intrareader reliability, which was found to be 0.98 for crypt length, 0.98 for crypt perimeter, 0.95 for crypt area, and 0.94 for the number of crypts scored.

Statistical Analysis

Treatment groups were assessed for comparability of characteristics at baseline and final follow-up by the Fisher's exact test for categorical variables and ANOVA for continuous variables. Correlations among the crypt measures were assessed using the Pearson correlation coefficient. Slide scoring reliability was analyzed using intraclass correlation coefficients.

The mean length, perimeter, and area of colon crypts for each patient at baseline and 6-month follow-up were calculated by summing all the measurements from all analyzed crypts from the biopsy specimens and dividing by the number of crypts analyzed.

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 length, perimeter and area of crypts from baseline to the 6-month follow-up between participants in each active treatment group and those in the placebo group by a repeated-measures linear mixed-effects model. The model included

the intercept, follow-up visit effects (baseline and follow-up), and interactions between treatment groups and the follow-up visit effect (the absolute treatment effect). To provide perspective on the magnitude of the treatment effects, we also calculated relative effects, defined as [(treatment group follow-up mean) / (treatment group baseline mean)] / [(placebo follow-up mean) / (placebo baseline mean)]. 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 odds ratio (e.g., a relative effect of 3.0 would mean that the proportional change in the treatment group was three times as that in the placebo group). Since the treatment groups were balanced on risk factors at baseline, no adjustment was made for other covariates in the primary intent-to-treat analyses.

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

RESULTS

Characteristics of Study Participants

Treatment groups did not differ significantly on characteristics measured at baseline (Table 1). The mean age of participants was 61 years, 70% were men, 70% were white, 15% reported taking NSAIDs at least once a week, and 21% had a family history of colorectal cancer in a first-degree relative. Most participants were nonsmokers and college graduates, and, on average, tended to be overweight. Baseline serum 25-OH-

vitamin D and 1,25-$(OH)_2$-vitamin D levels did not differ among the four treatment groups.

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. No adverse events were attributed to study procedures or treatments. Seven participants (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 other three groups.

Effects of Calcium and/or Vitamin D_3 Supplementation on Crypt Morphology (Length, Perimeter, and Area of Crypts) in Normal Colorectal Crypts

Crypt length, perimeter, and area were strongly correlated at baseline, with r = 1.00 ($P < 0.0001$) between crypt length and perimeter, r = 0.86 ($P < 0.0001$) between crypt length and area, and r = 0.88 ($P < 0.0001$) between crypt perimeter and area. The clinical trial crypt morphology end point results are shown in Table 2. At baseline, the four treatment groups did not differ significantly in crypt length, perimeter and area. After 6 months of treatment, the mean crypt length increased by 1% (P = 0.92) in the calcium group, and decreased by 2% (P = 0.69) and 4% (P = 0.40) in the vitamin D and calcium plus vitamin D groups, respectively, relative to the placebo group. The mean crypt perimeter decreased by 2% (P = 0.70) and 4% (P = 0.40) in the vitamin D and calcium plus vitamin D groups, respectively, relative to the placebo group, but did not change appreciably in the calcium group. The mean crypt area decreased by 2% (P = 0.74), 5% (P = 0.41) and

7% (P = 0.30) in the calcium, vitamin D and calcium plus vitamin D groups, respectively, relative to the placebo group.

Stratified Analyses

Potential treatment effect modification by age, sex, family history of colorectal cancer, smoking or NSAID use was investigated. However, the sample size was too small for most of these results to be reliable. In our preliminary findings, the effect of treatment on crypt length, perimeter and area did not vary by smoking status, or family history of colorectal cancer (data not shown). As summarized in Table 3, among women, the mean crypt length decreased by 10% (P = 0.28) in calcium plus vitamin D group, relative to the placebo group; among men, there were no appreciable changes of mean crypt length in any of the three active treatment groups, relative to the placebo group. Age was categorized into two groups according to median age (59 y). Among those age < 59, the mean crypt length decreased by 12% (P = 0.07) in the calcium plus vitamin D group, relative to the placebo group; while mean crypt length did not change appreciably in any of the three active treatment groups, relative to the placebo group, among those age ≥ 59. Among NSAID users, the mean crypt length decreased by 13% (P = 0.12) in the calcium plus vitamin D group, relative to the placebo group; among non-NSAID users, mean crypt length did not change appreciably in any of the three active treatment groups, relative to the placebo group.

DISCUSSION

The results of this pilot randomized, controlled clinical trial provide the first evidence on the effects of supplemental calcium and vitamin D_3, alone or jointly, on crypt

morphology (length, perimeter, and area of crypts) in the normal colorectal mucosa of sporadic adenoma patients. Although in the preliminary trial changes in the expression of biomarkers of apoptosis, differentiation and proliferation, DNA mismatch repair, oxidative DNA damage, CaR, VDR, CYP27B1 and CYP24A1 were found in the normal colorectal mucosa, in the current analysis we found no evidence to suggest that calcium and/or vitamin D supplementation substantially changes crypt length, perimeter, and area in normal colorectal mucosa. It also appeared that the effect of treatment on crypt length, perimeter and area did not vary by sex, age, or NSAID use. The findings from this study are consistent with the results of previous randomized controlled trials with 6-months follow-up, which indicated that calcium supplementation shifted the zone of proliferation to the lower 60% normal proliferative zone of the crypt (normalization), without affecting the overall proliferation rate in the colorectal mucosa of sporadic adenoma patients (37, 46). Another randomized controlled trial also provides evidence that rectal mucosal proliferation rate is not affected by calcium supplementation (38). Since the colorectal epithelial cell proliferation rate is not affected, crypt morphology (length, perimeter, and area of crypts) may not be changed.

There are several possible explanations for the null findings. The first is that calcium and/or vitamin D simply do not meaningfully affect crypt morphology. Second, is chance, especially considering the small sample size. A third possible explanation is that colon carcinogenesis is typically a slow, chronic process, which includes the progression from normal epithelium to hyperproliferative epithelium; to early, intermediate, and late adenoma; and then, subsequently to carcinoma and metastasis. It takes more than 10 years in most cases for an adenoma to develop into cancer, which involves multiple

genetic mutations of oncogenes and tumor suppressor genes (13, 61). So far no published studies have shown the progression time from normal colonic epithelium to change of crypt morphology. Since the follow-up period for this trial was only 6 months, it is possible that we were not able to capture the change of crypt morphology in such a short period of treatment.

A fourth possibility is that our vitamin D dose may have been too low. The optimal levels of serum 25-(OH)-vitamin D are suggested to be 33-100 ng/mL. To achieve these serum vitamin D levels in industrialized countries with increasing indoor lifestyles, total vitamin D exposures should be 1,000-4,000 IU/day (25). Evidence from a large clinical trial of colorectal adenoma recurrence suggested that calcium supplementation was primarily effective among those with 25-(OH)-vitamin D levels greater than the cohort median (29.1 ng/mL) (43). In our trial, only the vitamin D_3 supplementation group reached 25-(OH)-vitamin D levels above 29.1 ng/mL at 6-month follow-up (45). This suggests that vitamin D supplementation at 800 IU/day may not be adequate to reach sufficient serum vitamin D level, or, possibly, to affect crypt morphology in the normal colorectal mucosa. Higher doses of vitamin D supplementation may be needed in the future intervention trials.

There are only a few animal and human studies on the effects of calcium on crypt length in the normal colon mucosa. In a transmissible murine colonic hyperplasia (TMCH) model, diet with 1.0% calcium and 6% pectin inhibited increases in crypt length compared with the standard diet with 0.5% calcium and 5% cellulose (50). In a small uncontrolled intervention study, no change in crypt length was observed after 12 weeks and 1 year of 1,500 mg calcium supplementation (51). In a randomized controlled trial, a

total of 111 sporadic adenoma patients were randomized into one of three groups: the first group received two placebos, the second group received calcium 1,000 mg/day plus placebo, and the third group received placebo plus resistant starch. After 2-months of follow-up, no differences in crypt length were observed among the three treatment groups in four colorectal sites (cecum, transverse colon, sigmoid colon, and rectum). Crypt length in this study was measured as the total number of cells in whole length bisected colonic crypts divided by two (52).

This study had several strengths and limitations. The most obvious limitation was the small sample size, which may have increased the probability of chance findings in detecting or not detecting a treatment effect. The small size also limited our ability to conduct additional stratified analyses. Although animal and human studies suggest that ACF can serve as surrogate biomarkers of colonic carcinogenesis for purposes of testing chemopreventive efficacy (59), crypt morphology (length, perimeter and area) are not proven biomarkers of risk for colon cancer. Another limitation was that treatment effects could not be examined in parts of the colon other than the rectum. The effects of vitamin D alone or in combination with calcium on crypt morphology in different parts of the colon (other than the rectum) are not clear, as there have been no such studies in humans. On the other hand, this study is the first randomized, double-blind, placebo-controlled trial to have assessed the independent and combined effects of supplemental calcium and vitamin D on crypt morphology in the normal colorectal epithelium; protocol adherence by study participants was high; novel quantitative image analysis procedures was used; and biopsy analysis reliability was high.

Overall, the results from this pilot clinical trial suggest that supplemental calcium and vitamin D_3, individually or together, does not appear to change the crypt morphology (length, perimeter, and area of crypts) in the normal human colorectal epithelium of sporadic adenoma patients. Intervention trials with larger sample sizes, longer follow-up, and sufficient supplementation with vitamin D_3 would be needed to definitively determine whether these agents alone or in combination meaningfully affect the morphology of crypts in the normal colorectal mucosa in humans.

CHAPTER III

This study is the first pilot, randomized, double-blind, placebo-controlled, 2×2 factorial chemoprevention clinical trial to test the effects of calcium and vitamin D, individually or jointly, on crypt morphology in the normal human colorectal epithelium. Our preliminary findings do not provide evidence that calcium and/or vitamin D_3 supplementation change the crypt morphology (length, perimeter, and area of crypts) in the normal human colorectal epithelium of sporadic adenoma patients.

Our findings suggest that crypt morphology (length, perimeter, and area of crypts) may not be a plausible biomarker of risk for colorectal cancer. In the future, we should focus on developing a panel of plausible, reliable biomarkers that describe molecular phenotypes from which to accurately categorize and quantify risk for colorectal cancer. Since previous studies indicate a significant reduction or loss of goblet in clinical samples of colon adenocarcinomas, investigation of goblet cells in colonic crypts as potential biomarkers of risk for colorectal cancer may be a fruitful area of research. Our study only tested the effects of calcium and/or vitamin D on markers of crypt morphology in the normal human colorectal epithelium of sporadic adenoma patients. Since some studies provided evidence that the efficacy of calcium supplementation in adenoma patients is different from that in the normal population (62), it may be more convincing to investigate the effects of calcium and/or vitamin D in both sporadic adenoma patients and the normal population.

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TABLES AND FIGURES

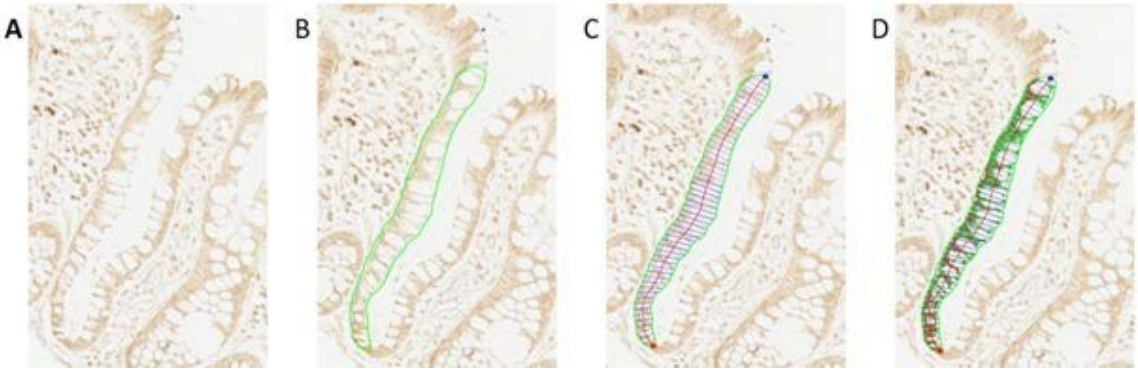

Figure 1. Quantitative image analysis using Aperio Scanscope and CellularEyes software to measure crypt length, perimeter and area in normal-appearing colorectal mucosa. A, choosing scorable crypts; B, tracing borders of hemicrypt; C, dividing hemicrypt into sections; D, automated quantification of crypt length, perimeter and area.

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

Characteristics	Treatment Group: Placebo (n=23)	Calcium (n=23)	Vitamin D (n=23)	Calcium + Vit. D (n=23)	*P*-value**
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 colorectal cancer in 1° relative (%)	17	30	17	13	0.60
Take NSAID¥ regularly§ (%)	22	13	9	22	0.60
Take aspirin regularly§ (%)	22	52	30	56	0.05
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
Body mass index (BMI), kg/m^2	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
Mean dietary intakes* **					
Total energy intake, kcal/d	1,596 (528)	1,788 (691)	1,848 (821)	1,845 (752)	0.59
Total§§ calcium, mg/d	625	678	753	733	0.75
Total§§ 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

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

** By Fisher's exact $\chi 2$ test for categorical variables, and ANOVA for continuous variables.

¥ Nonsteroidal anti-inflammatory drug.

§ At least once a week.

*** All nutrients energy adjusted using residual method.

§§ Diet plus supplements.

Table 2. Crypt length, perimeter and area in the normal-appearing colorectal mucosa at baseline and 6-month follow-up during the clinical trial

	Baseline				6-month follow-up				Absolute Rx effect			
Treatment Group	*n*	Mean	Std Err	*P*	*n*	Mean	Std Err	*P*	Rx effect*	Std Err	*P***	Relative Effect¥
Crypt length (µm)												
Placebo	20	359.1	10.1		19	382.0	10.3		.	.	.	1.00
Calcium	22	348.9	9.7	0.47	20	373.5	10.1	0.56	1.7	16.7	0.92	1.01
Vitamin D	23	350.8	9.5	0.55	19	367.0	10.3	0.30	-6.7	16.8	0.69	0.98
Ca + Vit D	22	371.0	9.7	0.40	20	379.7	10.1	0.87	-14.2	16.8	0.40	0.96
Crypt perimeter (µm)												
Placebo	20	708.5	18.7		19	751.0	19.1		.	.	.	1.00
Calcium	22	690.8	17.9	0.50	20	734.8	18.7	0.55	1.5	30.9	0.96	1.00
Vitamin D	23	693.2	17.5	0.55	19	723.5	19.1	0.31	-12.2	31.1	0.70	0.98
Ca + Vit D	22	732.3	17.9	0.36	20	748.4	18.6	0.92	-26.4	31.0	0.40	0.96
Crypt area (μm^2)												
Placebo	20	10,771	467.5		19	12,044	477.4		.	.	.	1.00
Calcium	22	10,658	450.0	0.86	20	11,692	467.5	0.60	-239.0	708.0	0.74	0.98
Vitamin D	23	10,378	440.1	0.54	19	11,055	475.4	0.15	-596.4	712.7	0.41	0.95
Ca + Vit D	22	11,718	447.9	0.15	20	12,241	465.5	0.77	-750.0	711.8	0.30	0.93

* Rx effect = [(treatment group follow-up) − (treatment group baseline)] − [(placebo group follow-up) − (placebo group baseline)].

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

¥ Relative effect = [(treatment group follow-up) / (treatment group baseline)] / [(placebo follow-up) / (placebo baseline)]; interpretation similar to that for an odds ratio (e.g., a relative effect of 1.5 indicates a 50% proportional increase in the treatment group relative to that in the placebo group).

Table 3. Crypt length in the normal-appearing colorectal mucosa at baseline and 6-month follow-up stratified by sex, age, and NSAID use

	Baseline				6-month follow-up				Absolute Rx effect			
Treatment Group	*n*	Mean (µm)	Std Err	*P*	*n*	Mean (µm)	Std Err	*P*	Rx effect* (µm)	Std Err	*P***	Relative Effect¥
Male												
Placebo	15	357.5	10.6		13	370.3	11.3		.	.	.	1.00
Calcium	15	359.9	10.6	0.87	13	380.7	11.3	0.51	8.1	18.1	0.66	1.02
Vitamin D	16	369.1	10.2	0.44	15	376.4	10.5	0.69	-5.4	17.5	0.76	0.98
Ca + Vit D	15	369.7	10.5	0.42	13	380.3	11.2	0.53	-2.2	18.2	0.91	0.99
Female												
Placebo	5	361.8	22.6		6	409.3	20.6		.	.	.	1.00
Calcium	7	325.3	19.1	0.23	7	358.9	19.1	0.09	-14.0	37.8	0.72	0.98
Vitamin D	7	308.9	19.1	0.09	4	342.1	25.2	0.05	-14.3	41.2	0.73	0.98
Ca + Vit D	7	373.9	19.1	0.69	7	379.0	19.1	0.29	-42.5	37.8	0.28	0.90
Age < 59												
Placebo	12	360.7	12.5		11	389.3	13.0		.	.	.	1.00
Calcium	10	342.2	14.9	0.35	10	372.6	14.9	0.40	1.7	23.3	0.94	1.01
Vitamin D	11	364.9	14.2	0.83	9	376.6	15.5	0.53	-16.9	23.6	0.48	0.96
Ca + Vit D	9	387.9	15.6	0.18	9	370.3	15.6	0.35	-46.3	24.3	0.07	0.88
Age ≥ 59												
Placebo	8	355.0	17.7		8	366.1	17.7		.	.	.	1.00
Calcium	12	354.5	12.7	0.98	10	374.6	13.7	0.71	9.0	25.2	0.72	1.02
Vitamin D	12	337.8	12.7	0.44	10	358.7	13.7	0.75	9.8	25.2	0.70	1.03
Ca + Vit D	13	359.5	12.2	0.84	11	387.1	13.1	0.35	16.6	24.8	0.51	1.04

(Continued)

	Baseline				6-month follow-up				Absolute Rx effect			
Treatment Group	***n***	**Mean (µm)**	**Std Err**	***P***	***n***	**Mean (µm)**	**Std Err**	***P***	**Rx effect[*] (µm)**	**Std Err**	***P*[**]**	**Relative Effect[¥]**
NSAID users												
Placebo	4	343.2	19.6		4	413.1	19.6		.	.	.	1.00
Calcium	3	347.3	23.2	0.89	3	399.9	23.2	0.67	-17.4	31.4	0.60	0.96
Vitamin D	2	327.1	28.4	0.65	2	370.3	28.4	0.25	-26.8	35.3	0.47	0.94
Ca + Vit D	4	375.9	20.0	0.28	4	395.2	20.0	0.54	-50.7	29.2	0.12	0.87
Non-NSAID users												
Placebo	16	363.3	11.5		15	373.5	11.9		.	.	.	1.00
Calcium	19	349.2	10.6	0.37	17	368.7	11.2	0.77	9.3	18.8	0.62	1.03
Vitamin D	21	353.0	10.1	0.51	17	366.4	11.1	0.67	3.2	18.7	0.87	1.01
Ca + Vit D	18	370.0	10.9	0.67	16	376.0	11.5	0.88	-4.3	19.2	0.82	0.99

[*] Rx effect = [(treatment group follow-up) − (treatment group baseline)] − [(placebo group follow-up) − (placebo group baseline)].

[**] P value for difference between each active treatment group and placebo group from repeated-measures MIXED model.

[¥] Relative effect = [(treatment group follow-up) / (treatment group baseline)] / [(placebo follow-up) / (placebo baseline)]; interpretation similar to that for an odds ratio (e.g., a relative effect of 1.5 indicates a 50% proportional increase in the treatment group relative to that in the placebo group).

APPENDIX

Table 4. Crypt perimeter in the normal-appearing colorectal mucosa at baseline and 6-month follow-up stratified by sex, age, and NSAID use

	Baseline				6-month follow-up				Absolute Rx effect			
Treatment Group	***n***	**Mean (µm)**	**Std Err**	***P***	***n***	**Mean (µm)**	**Std Err**	***P***	**Rx effect[*] (µm)**	**Std Err**	***P*[**]**	**Relative Effect[¥]**
Male												
Placebo	15	706.0	19.4		13	728.9	20.7		.	.	.	1.00
Calcium	15	711.2	19.4	0.85	13	748.3	20.7	0.51	14.3	33.3	0.67	1.02
Vitamin D	16	726.6	18.8	0.45	15	740.5	19.3	0.68	-8.9	32.3	0.78	0.99
Ca + Vit D	15	728.4	19.3	0.42	13	748.4	20.6	0.51	-2.8	33.5	0.93	1.00
Female												
Placebo	5	711.5	42.3		6	801.5	38.7		.	.	.	1.00
Calcium	7	647.0	35.9	0.26	7	707.5	35.9	0.09	-29.5	70.3	0.68	0.97
Vitamin D	7	616.8	35.9	0.11	4	678.9	47.2	0.06	-27.8	76.6	0.72	0.98
Ca + Vit D	7	740.5	35.9	0.61	7	748.9	35.9	0.33	-81.5	70.3	0.26	0.90
Age < 59												
Placebo	12	710.9	23.1		11	764.7	23.9		.	.	.	1.00
Calcium	10	679.1	27.4	0.38	10	733.0	27.4	0.39	0.1	43.6	1.00	1.00
Vitamin D	11	716.7	26.1	0.87	9	741.4	28.7	0.54	-29.1	44.1	0.51	0.96
Ca + Vit D	9	762.2	28.8	0.17	9	732.6	28.8	0.40	-83.4	45.4	0.07	0.89
Age ≥ 59												
Placebo	8	701.3	33.0		8	720.9	33.0		.	.	.	1.00
Calcium	12	700.5	23.6	0.99	10	737.3	25.5	0.70	17.2	46.3	0.71	1.02
Vitamin D	12	671.6	23.6	0.47	10	708.6	25.5	0.77	17.4	46.3	0.71	1.03
Ca + Vit D	13	711.6	22.7	0.80	11	760.3	24.4	0.34	29.2	45.6	0.53	1.04

(Continued)

Treatment Group	Baseline				6-month follow-up				Absolute Rx effect			Relative Effect[¥]
	n	Mean (µm)	Std Err	*P*	*n*	Mean (µm)	Std Err	*P*	Rx effect[*] (µm)	Std Err	*P*[**]	
NSAID users												
Placebo	4	681.2	36.5		4	809.7	36.5		.	.	.	1.00
Calcium	3	685.2	43.3	0.95	3	783.1	43.3	0.65	-30.6	57.3	0.61	0.96
Vitamin D	2	654.5	53.0	0.69	2	730.4	53.0	0.25	-52.7	64.5	0.44	0.94
Ca + Vit D	4	744.6	37.5	0.26	4	780.7	37.5	0.59	-92.4	53.3	0.12	0.88
Non-NSAID users												
Placebo	16	715.7	21.3		15	734.8	21.9		.	.	.	1.00
Calcium	19	691.7	19.6	0.41	17	726.0	20.6	0.77	15.4	34.9	0.66	1.02
Vitamin D	21	696.9	18.6	0.51	17	722.6	20.5	0.69	6.7	34.7	0.85	1.01
Ca + Vit D	18	729.6	20.1	0.64	16	740.6	21.2	0.85	-8.1	35.6	0.82	0.99

[*] Rx effect = [(treatment group follow-up) − (treatment group baseline)] − [(placebo group follow-up) − (placebo group baseline)].

[**] P value for difference between each active treatment group and placebo group from repeated-measures MIXED model.

[¥] Relative effect = [(treatment group follow-up) / (treatment group baseline)] / [(placebo follow-up) / (placebo baseline)]; interpretation similar to that for an odds ratio (e.g., a relative effect of 1.5 indicates a 50% proportional increase in the treatment group relative to that in the placebo group).

Table 5. Crypt area in the normal-appearing colorectal mucosa at baseline and 6-month follow-up stratified by sex, age, and NSAID use

	Baseline				6-month follow-up				Absolute Rx effect			
Treatment Group	***n***	**Mean (μm^2)**	**Std Err**	***P***	***n***	**Mean (μm^2)**	**Std Err**	***P***	**Rx effect* (μm^2)**	**Std Err**	***P*****	**Relative Effect¥**
Male												
Placebo	15	10,804	476.8		13	11,390	506.5		.	.	.	1.00
Calcium	15	10,961	476.8	0.82	13	11,979	506.5	0.41	433.0	774.2	0.58	1.04
Vitamin D	16	11,082	461.7	0.68	15	11,532	474.3	0.84	-135.1	751.7	0.86	0.99
Ca + Vit D	15	11,152	474.4	0.61	13	11,879	504.3	0.50	141.1	780.1	0.86	1.01
Female												
Placebo	5	10,423	1058.9		6	13,469	979.7		.	.	.	1.00
Calcium	7	10,010	919.0	0.77	7	11,103	919.0	0.33	-1,953.3	1,527.5	0.22	0.86
Vitamin D	7	8,768.5	919.0	0.25	4	9,714.8	1164.8	0.89	-2,099.7	1,686.8	0.23	0.86
Ca + Vit D	7	12,932	919.0	0.09	7	13,052	919.0	0.45	-2,925.8	1,527.5	0.07	0.78
Age < 59												
Placebo	12	10,800	561.2		11	12,240	581.0		.	.	.	1.00
Calcium	10	10,413	665.5	0.66	10	11,767	665.5	0.60	-85.9	1,072.6	0.94	1.00
Vitamin D	11	10,545	634.5	0.77	9	11,334	697.1	0.33	-650.7	1,083.7	0.55	0.95
Ca + Vit D	9	12,321	699.1	0.10	9	12,041	699.1	0.83	-1,719.9	1,115.7	0.13	0.86
Age ≥ 59												
Placebo	8	10,529	866.3		8	11,542	866.3		.	.	.	1.00
Calcium	12	10,862	631.8	0.76	10	11,650	667.1	0.92	-225.2	971.2	0.82	0.98
Vitamin D	12	10,224	631.8	0.78	10	10,931	667.1	0.58	-306.2	971.2	0.75	0.98
Ca + Vit D	13	11,263	607.1	0.49	11	12,314	637.9	0.48	37.7	956.3	0.97	1.00

(Continued)

Treatment Group	Baseline				6-month follow-up				Absolute Rx effect			Relative Effect[¥]
	n	Mean (μm^2)	Std Err	*P*	*n*	Mean (μm^2)	Std Err	*P*	Rx effect[*] (μm^2)	Std Err	*P*[**]	
NSAID users												
Placebo	4	10,617	1,115.7		4	13,422	1,115.7		.	.	.	1.00
Calcium	3	10,205	1,360.4	0.82	3	12,762	1,360.4	0.72	-248.2	1,386.1	0.86	0.99
Vitamin D	2	10,120	1,666.1	0.81	2	11,203	1,666.1	0.30	-1,722.3	1,556.4	0.30	0.88
Ca + Vit D	4	12,656	1,178.1	0.24	4	13,667	1,178.1	0.88	-1,794.8	1,292.6	0.20	0.85
Non-NSAID users												
Placebo	16	10,830	515.3		15	11,653	529.7		.	.	.	1.00
Calcium	19	10,730	475.0	0.89	17	11,490	498.1	0.82	-63.8	802.5	0.94	1.00
Vitamin D	21	10,420	451.8	0.54	17	11,052	494.7	0.41	-174.1	797.6	0.83	0.99
Ca + Vit D	18	11,507	486.1	0.34	16	11,922	511.3	0.72	-409.4	818.3	0.62	0.96

[*] Rx effect = [(treatment group follow-up) − (treatment group baseline)] − [(placebo group follow-up) − (placebo group baseline)].

[**] P value for difference between each active treatment group and placebo group from repeated-measures MIXED model.

[¥] Relative effect = [(treatment group follow-up) / (treatment group baseline)] / [(placebo follow-up) / (placebo baseline)]; interpretation similar to that for an odds ratio (e.g., a relative effect of 1.5 indicates a 50% proportional increase in the treatment group relative to that in the placebo group).