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EFFECTS OF CALCIUM AND/OR VITAMIN D SUPPLEMENTATION ON GOBLET CELL MUCIN CONTENT IN NORMAL COLON MUCOSA OF SPORADIC COLORECTAL ADENOMA PATIENTS

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EFFECTS OF CALCIUM AND/OR VITAMIND SUPPLEMENTATION ON GOBLET CELL MUCIN CONTENT IN NORMAL COLON MUCOSA OF SPORADIC COLORECTAL ADENOMA PATIENTS

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Abstract

EFFECTS OF CALCIUM AND/OR VITAMIND SUPPLEMENTATION ON GOBLET CELL MUCIN CONTENT IN NORMAL COLON MUCOSA OF SPORADIC COLORECTAL ADENOMA PATIENTS

By Hsiao-Jung Tseng

Background: Calcium and vitamin D favorably modify molecular phenotypic profiles of colon crypts in the normal colorectal mucosa of colorectal adenoma patients, but their effects on goblet cell mucin content are unknown. **Methods:** We conducted a pilot, randomized, double-blind, placebo-controlled 2×2 factorial chemoprevention clinical trial of supplemental calcium 2,000 mg and/or vitamin D_3 800 IU daily versus placebo over six months in patients (n = 92) with a history of at least one pathology-confirmed colorectal adenoma. Biopsies of normal-appearing rectal mucosa obtained at baseline and at six-months follow-up were histologically sectioned, and goblet cell mucin area and distributions within full-length crypts were quantified by image analysis. The results were analyzed using mixed linear models. **Results:** Relative to the placebo group, the mean goblet cell area increased by 16% (p = 0.21), 6% (P = 0.53), and 3% (P = 0.96) in the vitamin D, calcium, and calcium plus vitamin D groups, respectively. There was little indication that any possible changes in goblet cell mucin area differed along the lengths of crypts except, possibly, in the lower 20% of crypts in the vitamin D group where there was an estimated 24% increase (p = 0.07). **Conclusion:** Calcium and/or vitamin D_3 supplementation do not appear to appreciably change goblet cell mucin content in the normal colorectal mucosa of sporadic adenoma patients. **Impact:** These results, taken together with previous findings, support the use of colon crypt molecular phenotypic markers over histological characteristics as modifiable pre-neoplastic biomarkers of risk for colorectal neoplasms in humans.

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CHAPTER I

BACKGROUND

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 141,210 incident cases and 49,380 deaths expected in 2011 (1). The age-adjusted colorectal cancer incidence rates in the U.S were 57.1 per 100,000 among males and 42.4 per 100,000 among females from 2003 to 2007 (1). Among men, cancers of the prostate, lung and bronchus, and colorectum account for nearly 52% of all newly diagnosed cancers; on the other hand, colorectal cancer is essentially the only cancer that occurs with similar frequency among women – about 53% of the estimated incidence for the most common causes of cancer death (2). Recent declines in colorectal cancer incidence largely reflect increases in screening, which can efficiently detect and remove precancerous polyps (3). The colorectal cancer mortality rate among males was 30.8 per 100,000 in 1990, decreasing to 20.1 per 100,000 in 2007, while the mortality rate among females was 20.3 per 100,000 in 1990, decreasing to 14.2 per 100,000 in 2007 (1). However, even with current advances in screening tests, treatment and prevention, there is limited improvement in decreasing colorectal cancer mortality rates (1, 2).

Colorectal cancer incidence rates vary across countries and are higher in economically transitioning countries in Eastern Europe, such as the Czech Republic, especially for males. In Asia, colorectal cancer incidence rates generally increased in the most recent period; however, CRC incidence in some countries, even those with the largest increases, such as India and Thailand, was still relatively low from 1983-1987 to 1998-2002 for both males and females (4).

Several migrant studies provide further evidence for a relationship of lifestyle and environment to colorectal carcinogenesis, essentially for diet, for persons who have moved from low risk countries to the United States (5-8). For example, the incidence rates for U.S.-born Japanese men were twice as high as those in Japan, which strongly implicates the role of environment, such as Western dietary patterns (5). Also, the results from the prospective Multiethnic Cohort study of over 35,000 Latinos of Mexican national origin also supported associations between colorectal cancer risk and certain dietary components, such as alcohol, non-starch polysaccharides, and vegetables, although some food traditions were retained (6). Among Asian residents in the western United States from 1973 to 1986, the association between incident colorectal cancer and the country of birth was also examined (7). Foreign-born Chinese men had about the same incidence of colorectal cancer as U.S.-born white men, while U.S.-born Chinese men experienced slightly lower rates. Chinese women had rates that were generally 30-40% lower than that of U.S.-born white women, regardless of place of birth. Also, 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 further found that risks for cancers of both the colon and the rectum increased with increasing years lived in North America, and were more strongly associated with dietary saturated fat and a sedentary lifestyle compared to the risk among the general population in China (8). Together, all the migrant data suggests that environmental factors, such as diet and lifestyle, may account for most of the international differences the incidence of the disease.

Colorectal Carcinogenesis

The majority of colorectal cancer develops from a long, multi-stage process that begins with a benign growth of cells in the lining of the intestine. The steps involved include the progression from normal epithelium to a hyper-proliferative epithelium; to early, intermediate, late adenoma; and subsequently to carcinoma and metastasis. Adenomas with a villous histology,

larger cells, and a greater degree of dysplasia are more likely to progress into cancer (9, 10). Also, persons with multiple adenomas are at higher risk for developing colorectal cancer. It is difficult to implement interventional studies with colorectal cancer as an endpoint due to the large sample size and long-term follow-up that are required. Using a surrogate endpoint, like the appearance of colorectal adenomatous polyps, is common.

In Giovannucci and Wu's review, there are three molecular pathways of colon carcinogenesis, characterized by several steps, each of which involve genetic mutations of oncogenes and tumor suppressor genes (11). The first molecular pathway is the APC-β-catenin-Tcf-MYC pathway proposed by Fearon and Vogelstein (12). Most colorectal cancers are initiated by a mutation of the *APC* gene (either somatic or inherited), 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. The *APC* gene encodes the APC protein, which is involved in the regulation of β-*catenin*. When the *APC* gene is mutated, the concentration of β-*catenin* will increase and thereafter adheres to the T-cell factor 4 (Tcf4), mediating transcription of certain genes including the oncogene *c-myc* (13). Progression from adenoma to carcinoma is then dependent on the accumulation of other genetic and epigenetic alterations, such as DNA hypomethylation and mutations of the *K-ras* and *p53* genes (12). 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 non-polyposis 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). Alterations of the genes encoding BAX and the TGF-β type II receptor have commonly been identified in colorectal cancers with microsatellite instability (17, 18). A third carcinogenesis pathway has been suggested for persons with

ulcerative colitis, an inflammatory bowel disease that is associated with an approximately 20-fold increased risk of colorectal cancer. In patients with ulcerative colitis, chronic inflammation can result in genetic alterations, which can progress to dysplasia and subsequently to cancer (19, 20).

Environmental Factors in Colorectal Cancers

Given that colorectal cancer is a multistep process involving multiple risk factors, many studies have examined the combined impact of multiple lifestyle factors on the primary prevention of colorectal cancer. Recent studies suggest that the importance of environmental factors, such as diet and lifestyle (21, 22), are closely associated with the development of CRC, especially diet (23, 24). A recent meta-analysis concluded that dietary fat might not be associated with increased risk of CRC (30), while previous studies proposed that a diet rich in meat might be associated with an increased risk of CRC (31). Some studies have also suggested that a diet rich in fruit, vegetables or fiber may be less associated with a decreased risk of CRC than had been previously believed (32-34). Physical activity has been found to be consistently inversely associated with risk of colon cancer (25). The association between alcohol and CRC risk has been controversial (26), but the evidence strongly suggests that high intake of alcohol increases risk of CRC (27). Cigarette smoking is directly associated with colorectal cancer incidence and mortality (28). Regular use of non-steroidal anti-inflammatory drugs (NSAIDs), including aspirin, reduced adenoma recurrence in randomized controlled trials and has been consistently, strongly inversely associated with risk of colorectal cancer (29).

Calcium, Vitamin D, and Colorectal Cancer Risk

Convincing evidence from observational and experimental studies and randomized, placebo-controlled clinical trials suggests that calcium and vitamin D have already emerged as promising chemopreventive agents against colorectal neoplasms (35), with the effects of (i) protecting colonocytes through binding of toxic secondary bile acids and ionized fatty acids (36);

(ii) directly inhibiting proliferation and promoting differentiation and apoptosis in cell cycle (37); (iii) modulating the APC colon carcinogenesis pathway; and (iv) modulating E-cadherin and β-catenin expression via the calcium-sensing receptor (38, 39).

Colonic luminal calcium binding to the calcium receptor (CaR, also referred to as the calcium-sensing receptor) may directly modulate the cell cycle of colonic cells, partly by (i) inhibiting the β-catenin/TCF transcription complex; (ii) promoting activation of E-cadherin (38); and (iii) reducing the concentration of 25-hydroxyvitamin D 24-hydroxylase (CYP24A1) (40). Luminal calcium is also hypothesized to bind to pro-inflammatory, secondary bile acids and ionized fatty acids (38, 41). On the other hand, vitamin D, binding to the vitamin D receptor (VDR), plays a role in cell-cycle regulation, partly by: (i) competitively binding β-catenin (42); (ii) up-regulating p21 (43) and E-cadherin expression (44); and (iii) regulating growth factors (35). The VDR also promotes bile acid degradation (35). In addition, prospective cohort studies have consistently found that: higher total calcium intake is associated with reduced risk for colorectal neoplasms (35); calcium supplementation reduces colorectal adenoma recurrence (modified by vitamin D status) (45); and higher circulating concentrations of 25(OH)D are inversely associated with colorectal neoplasms (35, 46, 47). Although there is abundant evidence from observational studies of the effects of calcium and vitamin D, there have been only few clinical trials focused on the risk of colorectal cancers. The evidence from epidemiological studies to date on the effects of calcium and/or vitamin D in relation to the risk of colorectal cancers is summarized below.

Human 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 the 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 studies (71%). Of these, 16 were statistically significant, three found null associations, nine found increased risk with higher intake, and none was statistically significant (35). A pooled analysis of 10 cohort studies from five countries reported a statistically significant (22%) lower risk for incident CRC among those with the highest versus the lowest levels of calcium intake (48). Of 11 observational studies of calcium and colorectal adenoma (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 a statistically non-significant increased risk with higher intake (35).

Among all clinical trials of calcium and CRC risk, there are at least 17 trials of calcium and colorectal epithelial cell proliferation, and 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 (49). The second trial found no proliferative effects (50). There have been five preliminary and two major clinical trials of calcium and adenoma recurrence, and one major trial of incident colorectal cancer prevention. In a U.S. multicenter, randomized, double-blind, placebo-controlled clinical trial (the Calcium Polyp Prevention Study), a total of 930 subjects were randomly assigned into either the 1,200 mg of elemental calcium daily or the placebo group to test the effect of calcium supplementation on the recurrence of colorectal adenomas (51). Calcium supplementation significantly reduced any meta-chronous colorectal adenoma recurrence by 15% (52), an effect that extended up to at least five years after cessation of active treatment (53). A smaller European multicenter randomized trial placed 665 randomized patients who had a history of colorectal adenomas into three treatment groups: 2,000 mg of elemental calcium daily, fiber, or placebo. After three years of follow up, adenoma recurrence was 34% lower in the calcium group, although the finding was not

statistically significant (34). The summary risk ratio (RR) in a meta-analysis of all seven adenoma recurrence trials was 0.80 (95% CI: 0.68-0.93) (54).

Recently, the Women's Health Initiative randomized, double-blinded, placebo-controlled clinical trials involving 36,282 postmenopausal women tested the effect of calcium with vitamin D supplementation in the prevention of colorectal cancer (55). 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 seven 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 high rates of treatment drop in and drop out, the low doses administered, and the relatively short length of follow up for colorectal cancer as an endpoint.

Studies of Vitamin D and Colorectal Cancer Risk

Of 30 analytic observational studies of vitamin D and CRC (seventeen case-control studies and thirteen prospective cohort studies), twenty (67%) found inverse associations, of which six were statistically significant, six found null associations, and four found statistically non-significant positive associations (35). A pooled analysis of five cohort studies reported a statistically non-significant 7% reduction in risk for incident colorectal cancer among those consuming the highest levels of vitamin D versus those consuming the lowest levels (48).

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 non-significant increased risk (35). 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 had serious exposure misclassification that biased findings toward the null. The consistent results now emerging from studies that measured 25-(OH)-vitamin D blood level suggest that future studies should assess vitamin D exposures in this way (35).

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 (35). 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) (45).

Overall, the observational epidemiologic studies, especially the prospective cohort studies, provide strong evidence in support of higher intakes of calcium providing protection against colorectal cancer. Calcium also has conclusively been shown to reduce adenoma recurrence in clinical trials. The human evidence for a protective effect of vitamin D against colorectal cancer from studies that have assessed vitamin D exposure by measuring serum 25-(OH)-vitamin D, the best indicator of total vitamin D exposure, is growing and consistent, but the number of such studies remains small. Human studies that have assessed calcium-vitamin D interactions, all observational, have been relatively few and inconclusive.

Surrogate End-Point Biomarkers (SEBs) in Colorectal Cancer

As with a number of other cancers, colon carcinogenesis is the result of a multistep process in which an increasing number of alterations, including specific gene mutations, occur as cells progress from normal to precancerous states of increasing size and dysplasia to cancer and finally to metastatic disease. To shorten a long follow-up and reduce large sample sizes, surrogate

end-point biomarkers (SEBs) have become widely used in short-term cancer prevention trials in place of cancer end-points (56). An ideal SEB could both (i) show different expressions as modulated by chemopreventive agents between the various phases of colon carcinogenesis, and (ii) be used to accurately categorize and determine an individual's risk of developing colon cancer. Phenotypic biomarkers could "summarize" the complex interaction results from cumulative changes among genotypes, gene-gene interactions, epigenetic phenomena, environmental exposures, and gene-gene interactions. However, this field is still in the developmental stage and in general, no single SEB is currently accepted as a pre-neoplastic biomarker of risk for colorectal cancer.

To date, the most studied candidate biomarker has been colorectal epithelial cell proliferation. In a clinical trial of 193 patients with sporadic adenoma who were assigned treatment with placebo (n=66), 1.0 g calcium (n=64), or 2.0 g calcium (n=63) daily for six months, colorectal epithelial cell proliferation as a biomarker was measured (49). The results showed that calcium supplementation could shift expanded proliferative zones back into their normal locations in the lower 60% of the crypt without affecting the overall proliferation rate in the colorectal mucosa of sporadic adenoma patients. In a recently published pilot, randomized, double-blind, placebo-controlled, 2x2 factorial chemoprevention clinical trial, the results showed that calcium and vitamin D could: (i) enhance cell apoptosis through affecting the expression of Bcl-2 (an inhibitor of cell apoptosis) and Bax (a promoter of cell apoptosis) (57); (ii) promote colorectal cell differentiation as indicated by increased expression of $p21^{waf1/cip1}$; (iii) decrease proliferation as indicated by decreased expression of MIB-1 (a marker of short-term proliferation) and hTERT (a marker of long-term proliferation) (58); (iv) increase DNA mismatch repair (MMR) gene activity (as indicated by increased expression of MSH2 and MLH1) (59); and (v) increase expression of the calcium receptor (CaR), the vitamin D receptor (VDR), and the P450 cytochrome enzymes CYP27B1 and CYP24A1 (60) in the normal colorectal epithelium of sporadic adenoma patients.

Crypt Histology, Goblet Cells, and Colorectal Cancer

The normal human colonic epithelium maintains a dynamic equilibrium among proliferation, differentiation, and apoptosis. Cells at the base of colonic crypt divide rapidly before they begin to differentiate and move upwards in the crypt. Areas of dysplasia develop in the colonic mucosa and eventually some of the dysplastic cells give rise to raised tubular polyps (adenomas). Some of these adenomatous polyps may exist for years as premalignant cells before progressing to colorectal carcinomas; as these types of polyps evolve, they undergo a progression from an abnormal colorectal epithelium characterized by hyper-proliferation, impaired apoptosis, and reduced differentiation (61). Later-stage crypts contain larger cells with atypical characteristics, such as abnormal nuclear and/or cellular shapes (i.e., aberrant crypt foci, as indicated by ACF) that efface normal-appearing crypts, often in the context of *k-ras* mutations, which can be visualized histologically (62). ACF are also thought to be the precursor lesions for most adenoma polyps. Cumulative molecular defects within phenotypically normal mucosa or those expressed as higher-level pathologies (e.g., ACF and adenomas) represent an even greater risk for CRC (63).

Our present understanding of colonic epithelial cell physiology and pathophysiology mostly comes from experimental animal models (64) and *in vitro* human colon-cancer-derived cell lines (65). In a recent human colonic organ culture study, scientists maintained the metabolic activity of human colon tissue during an incubation period; these tissues included pre-malignant adenomas and invasive colon cancer, as well as normal colonic mucosa (66, 67). The histological characteristics were observed over a 2-day period. In normal tissue, elongated crypts with small densely packed cells were found at the crypt base and mucin-containing goblet cells were found in the upper portion of the crypt. Proliferating cells were confined to the lower third of the crypt, while CaR expression was seen in the upper third of the crypt, and cell membrane E-cadherin and

β-catenin were expressed throughout the crypt. In neoplastic tissue, the same cells were disorganized with observed abnormal glandular structures after the incubation period. In addition, the majority of cells in these structures were mucin-poor, but occasional goblet cells were seen and mucin staining was present. The proliferative cells were also seen throughout the abnormal epithelium but CaR expression was weak and variable. Finally, intense cytoplasmic β-catenin staining was observed in cultured tumor tissue.

There has been little study of the histologic characteristics of colon crypts in the normal appearing colorectal mucosa *in vivo* as potential biomarkers of risk for colorectal neoplasms or whether they can be modulated by dietary or other interventions. Crypt length, perimeter, area, and area occupied by goblet cell mucin may be related to colonic cell proliferation, apoptosis, and differentiation, and, therefore, may serve as more simply measured, modifiable biomarkers of risk for colorectal neoplasms. Crypt length in the normal colon mucosa was inhibited by calcium in an animal study but not in a small, uncontrolled trial (n=17) or a larger randomized controlled trial (n=111) (68-70). Also, in our present study, calcium and/or vitamin D_3 supplementation did not appear to appreciably change crypt length, perimeter, or area in the normal colorectal mucosa of sporadic adenoma patients (unpublished data).

Review of Studies in Calcium, Vitamin D, and Colon Crypt Goblet Cells or Mucin

To our knowledge there are no published human clinical studies on the effects of calcium and/or vitamin D treatment on goblet cell mucin contents in the normal colon tissue. For my thesis, we report the first human trial results on the effects of calcium and/or vitamin D supplementation on colonic crypt goblet cell mucin content in the normal-appearing colorectal mucosa of sporadic colorectal adenoma patients.

CHAPTER II

MANUSCRIPT FOR SUBMISSION FOR PUBLICATION IN A PEER-REVIEWED JOURNAL TO REPORT THE THESIS FINDINGS

Effects of Calcium and/or Vitamin D Supplementation on Goblet Cell Mucin Content in Normal Colon Mucosa of Sporadic Colorectal Adenoma Patients: A Randomized Clinical Trial

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Abstract

Background: Calcium and vitamin D favorably modify molecular phenotypic profiles of colon crypts in the normal colorectal mucosa of colorectal adenoma patients, but their effects on goblet cell mucin content are unknown.

Methods: We conducted a pilot, randomized, double-blind, placebo-controlled 2×2 factorial chemoprevention clinical trial of supplemental calcium 2,000 mg and/or vitamin D_3 800 IU daily versus placebo over six months in patients (n = 92) with a history of at least one pathology-confirmed colorectal adenoma. Biopsies of normal-appearing rectal mucosa obtained at baseline and at six-months follow-up were histologically sectioned, and goblet cell mucin area and distributions within full-length crypts were quantified by image analysis. The results were analyzed using mixed linear models.

Results: Relative to the placebo group, the mean goblet cell area increased by 16% (p = 0.21), 6% (P = 0.53), and 3% (P = 0.96) in the vitamin D, calcium, and calcium plus vitamin D groups, respectively. There was little indication that any possible changes in goblet cell mucin area differed along the lengths of crypts except, possibly, in the lower 20% of crypts in the vitamin D group where there was an estimated 24% increase (p = 0.07).

Conclusion: Calcium and/or vitamin D_3 supplementation do not appear to appreciably change goblet cell mucin content in the normal colorectal mucosa of sporadic adenoma patients.

Impact: These results, taken together with previous findings, support the use of colon crypt molecular phenotypic markers over histological characteristics as modifiable pre-neoplastic biomarkers of risk for colorectal neoplasms in humans.

Introduction

Colorectal cancer (CRC) remains the second leading cause of cancer-related deaths in the United States, despite advances in screening and treatment (1). Valid treatable pre-neoplastic biomarkers of risk for colorectal neoplasms that can be used as endpoints for screening for the potential efficacy of preventive interventions, such as supplemental vitamin D and calcium, are needed.

The normal human colonic epithelium maintains a dynamic equilibrium among proliferation, differentiation, and cell apoptosis. Colon crypts are epithelial invaginations into the large intestinal mucosa and have vigorous proliferation potential. Acidic mucins, mainly secreted from crypt goblet cells, are indicators of colonic epithelial cell secretory function and form a mucus barrier between the epithelial surface and the luminal contents. Without this protective barrier, colon cells would be more susceptible to various kinds of stress (71). Colorectal cancer typically develops from adenomatous polyps that arise from the colorectal epithelium. Evidence from *in vitro* and animal studies indicates that loss of the mucus layer results in spontaneous colitis and carcinogenesis (72-74). In addition, a recent study in human colon tissue using organ cultures indicated that, in neoplastic tissue, most crypt cells were mucin-poor, with occasional goblet cells, disorganized epithelium, and weak calcium-sensing receptor expression. This suggests the possibility that calcium, perhaps acting via the calcium-sensing receptor, may be involved in regulating cell growth and differentiation as well as impacting goblet cell number and mucin production (66). Therefore, colon crypt goblet cell area may serve as a modifiable, pre-neoplastic biomarker of risk for colorectal neoplasms that would be much easier to measure than are molecular markers.

Numerous epidemiologic studies indicate that calcium and vitamin D may reduce the risk of developing colorectal adenomas and cancer (48, 55, 75). From the same preliminary trial

reported herein, we previously reported changes in the expression of biomarkers of proliferation, apoptosis, differentiation, DNA mismatch repair, the APC/β-catenin pathway, oxidative DNA damage, and calcium and vitamin D metabolism in the normal colorectal mucosa in response to calcium or vitamin D_3 supplementation (57, 60, 76, 77). To date, there are no published animal or human studies on the effects of calcium and/or vitamin D supplementation on goblet cell mucin content in the normal-appearing colorectal mucosa of sporadic colorectal adenoma patients. We hypothesized that supplemental calcium and/or vitamin D would increase goblet cell mucin content (as indicated by goblet cell mucin area) in the normal-appearing colorectal mucosa of sporadic colorectal adenoma patients.

Patients and Methods

Participant Population

The detailed study protocol, including recruitment procedures and specific exclusions, was published previously (57). The Emory University Institutional Review Board approved this study. 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, a history of at least one pathology-confirmed sporadic colon or rectal adenoma 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 interpretation of the study results.

Clinical Trial Protocol

Between April 2005 and January 2006, 522 potentially eligible patients were identified after initial chart screening, and 224 (43%) were randomly selected and sent an introductory letter followed by a telephone interview. A total of 105 (47%) potential participants attended an eligibility visit during which they were interviewed, signed a consent form, completed

questionnaires, and provided a blood sample. Diet was assessed with a semi-quantitative food frequency questionnaire (78). Medical and pathology records were reviewed. After a 30-day placebo run-in trial, 92 (88%) eligible participants who had no significant perceived side effects and who took at least 80% of their assigned tablets, if still willing to participate, underwent a baseline rectal biopsy and were randomly assigned to the following four treatment groups (n = 23/treatment group): placebo; 2.0 g elemental calcium supplementation (as calcium carbonate in equal doses twice daily); 800 IU vitamin D_3 supplementation (400 IU twice daily); and 2.0 g elemental calcium plus 800 IU vitamin D_3 supplementation. Additional details on the rationale for the doses and forms of the calcium and vitamin D supplements were described previously (57). Over the 6-month treatment period, participants attended follow-up visits at 1 and 6 months after randomization during which they were interviewed and completed questionnaires about adherence and adverse events; at the final follow-up visit, they also underwent venipuncture and a rectal biopsy procedure.

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 jumbo cup flexible endoscopic forceps mounted on a semi-flexible rod. The biopsies were placed onto a strip of bibulous paper and immediately placed in phosphate buffered saline (PBS), oriented under a dissecting microscope, placed in 10% normal buffered formalin, and then transferred to 70% ethanol 24 h after initial placement in formalin. Then, within a week, the biopsies were processed and embedded in paraffin blocks with three biopsies per block. 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. For the analysis reported herein, we used whole slide digital images previously acquired and stored for our previously reported analysis of

immunohistochemically detected 8-OH-dG (76). Since the immunostaining is irrelevant to the present study, the details for these methods are not included here.

Image Analysis of the Normal Colon Crypts

A quantitative image analysis method ("scoring") was used to evaluate colon crypt length, perimeter, area, tissue area with immunohistochemically-detected biomarkers, tissue area without detected biomarkers, and mucin areas of goblet cells as depicted in **Figure 1**. The major equipment and software for the image analysis procedures were: a ScanScope CS digital scanner (Aperio Technologies, Inc., CA); a computer; a digital drawing board; MatLab software (MathWorks, Inc., MA); CellularEyes Image Analysis Suite (DivEyes LLC, GA); and MySQL (Sun Microsystems Inc., CA). First, slides were scanned with the Aperio ScanScope CS digital scanner. Electronic images were then reviewed in the CellularEyes program to identify colon crypts acceptable for analysis. A "scorable" crypt was defined as an intact crypt extending from the muscularis mucosa to the colon lumen (79, 80). 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. The technician reviewed slides in the CellularEyes program and selected two of three biopsies with 16 to 20 "scorable" hemicrypts (one half of the crypt) per biopsy. Using the digital drawing board, the borders of each selected hemicrypt were traced. The program then divided the outline into the equally spaced segments corresponding to the average widths of normal colonocytes. Finally, the program measured the background corrected optical densities of the biomarker labeled and non-labeled epithelium as well as the goblet cell mucin areas across the entire hemicrypt and within each segment. Then, the technician moved to the next identified hemicrypt and repeated all the previously described analysis steps. A reliability control sample previously analyzed by the reader was re-analyzed during the course of the trial to determine intra-reader "scoring" reliability by intra-class correlation coefficient, which was 0.94.

Statistical Analysis

Primary analyses were based on assigned treatment at the time of randomization, regardless of adherence status (intent-to-treat analysis). The baseline characteristics of the participants in the four treatment groups were compared using the Fisher's exact test for categorical variables and ANOVA for continuous variables. Treatment effects were evaluated by assessing the differences in goblet cell mucin areas in the full lengths of the colon crypts as well as in specific zones of the crypts, from the baseline to the 6-month follow-up visits between participants in each active treatment group and those in the placebo group using 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 average proportional change in the treatment group relative to that in the placebo group. The interpretation of the relative effect is somewhat analogous to that of an odds ratio (e.g., a relative effect of 2.0 means that the relative proportional change in the treatment group was twice as great as that in the placebo group). Stratified analyses were conducted to investigate potential differential treatment effects by sex, family history of colorectal cancer in a first degree relative, and baseline age, nonsteroidal anti-inflammatory drug (NSAID) use, body mass index (BMI), and serum 25-OH-vitamin D levels.

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

The treatment groups did not differ significantly on characteristics measured at baseline (**Table 1**). The mean age of participants was 61 years, 70% were men, 71% were white, 13% reported taking NSAIDs at least once a week, and 20% 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, as previously reported (57).

Adherence to visit attendance averaged 92% and did not differ significantly among the four treatment groups. On average, 93% of participants at the first follow-up visit and 84% at the final follow-up visit had taken at least 80% of their pills. 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.

Graphical Assessment of Goblet Cell Mucin Areas in Normal Colorectal Crypts

Goblet cell mucin area distributions along the colorectal crypts at the baseline and 6 months of follow-up visits, by treatment group, are shown in **Figure 2**. In each treatment group, goblet cell mucin area appeared to be greatest in the middle portions of the crypts (as oriented from the base to the luminal surface of the crypts, between the 20^{th} to 90^{th} percentiles), with a peak at 60^{th} percentile of the crypts), and smallest in the very lower and upper portions of the crypts. There were very little indications of changes in crypt goblet cell mucin area along the lengths of crypts, although there was a possible suggestion in the vitamin D group of a very

modest, relatively uniform increase throughout the crypt length, perhaps especially in the lower 90% of the crypts.

Effects of Calcium and/or Vitamin D Supplementation on Goblet Cell Mucin Area in Normal Colorectal Crypts

There were no changes in overall crypt area in any of the active treatment groups relative to placebo (**Appendix Table 4**). At baseline, there were no differences in goblet cell area along the full lengths of crypts among the four treatment groups (**Table 2**). After six months, the mean goblet cell mucin area increased by 6% (P = 0.53), 16% (P = 0.21), and 3% (P = 0.96) in the calcium, vitamin D, and calcium plus vitamin D groups, respectively, relative to the placebo group. The findings in functional zones of the crypt, the upper 40% (differentiation zone) and the lower 60% (proliferation zone) of the crypts, were similar to those for the full lengths of the crypts. The only finding that was nearly statistically significant was that in the vitamin D relative to the placebo group, there was a 24% (P = 0.07) increase in goblet cell mucin area in the lower 20% of the crypts. In the analyses stratified by age, sex, family history of colorectal cancer or polyps in a first-degree relative, BMI, and serum 25-OH-vitamin D level, the estimated changes in the active treatment groups relative to the placebo group, overall and in functional zones of the crypts were similar to those in the primary analyses (**Appendix Table 5 and Table 6**).

Discussion

The results of this pilot, randomized, controlled clinical trial provide the first data on whether supplemental calcium and vitamin D, alone or jointly, may affect goblet cell mucin area in the normal-appearing colorectal epithelium of patients with sporadic adenoma. Although in the same preliminary trial changes in the expression of biomarkers of proliferation, apoptosis, differentiation, DNA mismatch repair, the APC/β-catenin pathway, oxidative DNA damage, and calcium and vitamin D metabolism were found in the normal colorectal mucosa (8-13), in the

current analysis we found little evidence to suggest that calcium and/or vitamin D supplementation substantially changes goblet cell mucin area in the normal colorectal mucosa. Given the small sample size, a modest effect of vitamin D on goblet cell mucin area cannot be ruled out.

There are several possible explanations for the essentially null findings. First, calcium and/or vitamin D may simply not meaningfully affect goblet cell mucin content in the human normal rectal mucosa. In our study we could not rule out the possibility that our intervention agents may affect goblet cell mucin content higher in the colon. The second explanation is chance, especially considering the small sample size. A third 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 of 1,000 - 4,000 IU/day are required (77). 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) (45). In our trial, the vitamin D_3 supplementation groups reached 25-(OH)-vitamin D levels of only approximately 29 ng/mL at 6-month follow-up (57). This suggests that vitamin D supplementation at 800 IU/day may not yield a sufficient serum vitamin D level to substantially affect crypt goblet cell mucin content in the normal colorectal mucosa. It remains possible that a higher dose of supplemental vitamin D may increase goblet cell mucin. Fourth, we cannot rule out the possibility that it may require more than six months of treatment with calcium and/or vitamin D to affect changes in goblet cell mucin content.

Most basic and animal studies of colon carcinogenesis have focused on genetic and molecular modifications in the mechanisms of cell differentiation and proliferation; only a few investigated histological features in colonic epithelial cells, and these are only marginally relevant to our study. An early comparative study in 1984 found that vitamin D deficiency and a surplus of calcium in the diet significantly increased the amount of goblet cells in the small intestines of

chicks (81). On the other hand, in a transgenic mouse model with progastrin overexpression, a pharmacologic antagonist (AG1024) that modifies autocrine and paracrine pathways, reduced the proportion of crypt goblet and enteroendocrine cells as well as hyperplasia and proliferation in the colonic epithelium (82). Moreover, in a recent human colonic organ culture study there were few goblet cells in sessile adenoma tissue relative to normal tissue (66).

Our study had several strengths and limitations. The most obvious limitation of this pilot clinical trial was its small sample size and thus limited statistical power for detecting modest treatment effects. Another limitation was that possible treatment effects could not be examined in parts of the colon other than the rectum. There have been no other reported studies in humans or animals of the effects of vitamin D alone or in combination with calcium on crypt goblet cell mucin in colon sites other than the rectum. 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 goblet cell mucin content in the normal colorectal mucosa in humans. Also, protocol adherence by the study participants was high, novel quantitative image analysis procedure were used, and biopsy analysis reliability was high.

In conclusion, although in the same preliminary trial, changes in the expression of biomarkers of proliferation, apoptosis, differentiation, DNA mismatch repair, oxidative DNA damage, the APC/β-catenin pathway, and calcium and vitamin D metabolism were found in the normal rectal mucosa, overall, the current analysis suggests that supplemental calcium and vitamin D_3, alone or combined, may not substantially change crypt goblet cell mucin content in the normal human rectal epithelium of sporadic adenoma patients. The possibilities that vitamin D supplementation at the 800 I.U./day dose used in this trial or in higher doses may modestly increase goblet cell mucin content in the rectum or higher in the colon cannot be ruled out, but would require a trial with larger sample size than used in the present study to definitively test the hypothesis .

TABLES and FIGURES

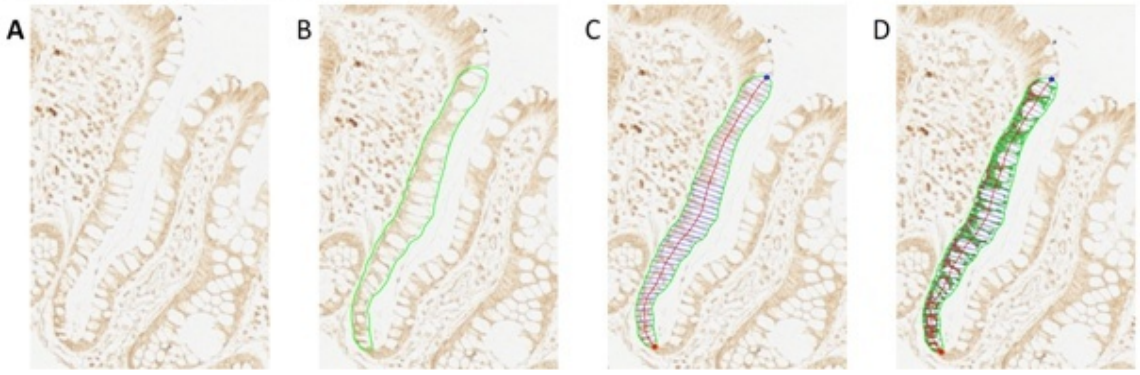

Figure 1. Quantitative image analysis using Aperio ScanScope and CellularEyes software to measure crypt morphologic characteristics in normal-appearing colorectal mucosa. A, choosing scorable crypts; B, tracing borders of hemicrypt; C, dividing hemicrypt into sections; D, automated quantification of crypt length, perimeter, area, and biomarker positive tissue, biomarker negative tissue, and goblet cell mucin areas.

Table 1. Summary of baseline characteristics of the study participants (N = 92)

	Treatment group				*P-Value**
	Placebo (N=23)	**Calcium** (N=23)	**Vitamin D** (N=23)	**Calcium + vitamin D** (N=23)	
Demographics, medical history, habits, and anthropometrics					
Age, yrs.	58.5 (8.2)	61.9 (8.2)	60.2 (8.2)	62.1 (7.5)	0.39
Men (%)	70	70	70	70	1.00
White (%)	74	83	65	61	0.40
College graduate (%)	65	64	57	45	0.53
History of colorectal cancer in first-degree relative (%)	17	30	17	13	0.60
Take NSAID regularly+(%)	22	13	4	13	0.43
Take aspirin regularly+ (%)	22	52	30	57	0.05
If women (n=28), taking estrogens (%)	4	4	4	9	1.00
Current smoker (%)	9	4	0	0	0.61
Take multivitamin (%)	30	30	26	41	0.86
Body mass index, kg/m^2	30.6 (7.2)	29.4 (5.5)	28.9 (5.6)	31.6 (6.0)	0.44
Mean dietary intakes					
Total energy intake, kcal/d	1,596 (5278)	1,788 (691)	1,848 (821)	1,845 (752)	0.59
Physical activity (METs/d)[§]	14.5 (11.6)	17.3 (17.9)	20.7 (12.0)	20.9 (14.7)	0.43
Red and processed meats (servings/wk.)	8.1 (5.9)	6.7 (4.9)	7.2 (4.7)	9.0 (6.5)	0.51
Total fruit & vegetables intake (servings/wk.)	4.7 (3.1)	4.7 (2.6)	4.8 (3.3)	3.9 (2.1)	0.73
Total[¶] calcium, mg/d	618.6 (307.9)	745.8 (334.9)	843.1 (525.8)	823.6 (713.9)	0.40
Total vitamin, IU/d	277.4 (229.9)	335.8 (202.2)	360.5 (317.1)	414.9 (315.5)	0.41
Total fat, g/d	66.8 (32.2)	72.3 (34.9)	69.8 (31.9)	73.8 (27.7)	0.89
Dietary fiber, g/d	14.8 (7.2)	17.4 (8.8)	17.5 (9.1)	17.1 (10.6)	0.70
Alcohol, g/d	8.6 (14.3)	10.9 (15.1)	13.8 (18.4)	10.2 (19.6)	0.76
Serum vitamin D					
Serum 25-(OH)-vitamin D (ng/mL)	21.5 (7.0)	25.2 (9.8)	21.5 (8.3)	23.3 (13.4)	0.57

NOTES: 1) Data are given as mean (SD) unless otherwise specified. 2) Abbreviation: NSAID, non-steroid anti-inflammatory drug.

*By Fisher's exact test for categorical variables and ANOVA for continuous variables

[+]At least once a week

[§]METs: metabolic equivalents of moderate plus vigorous exercise

¶Diet plus supplements

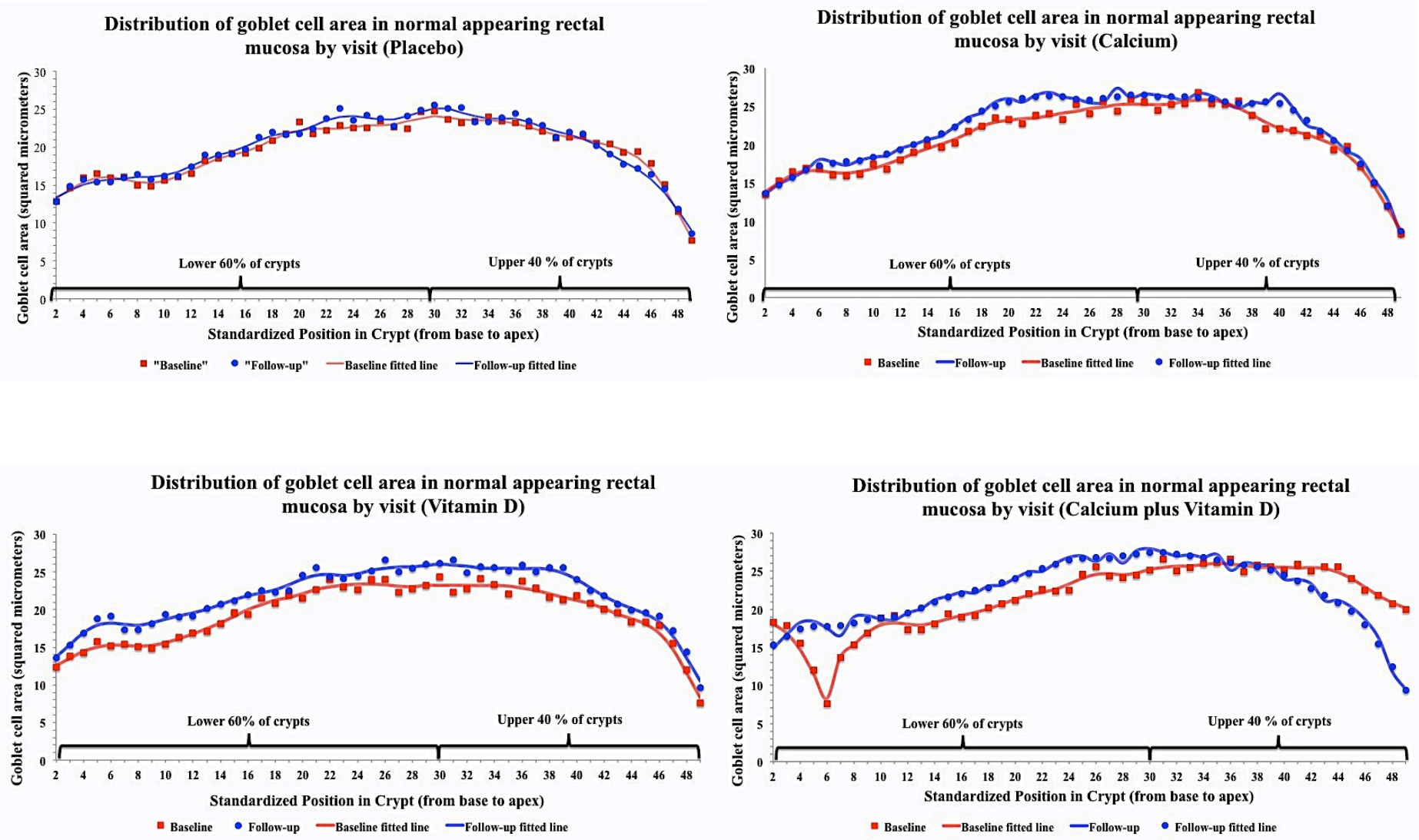

Figure 2. Distributions of goblet cell mucin areas along normal colorectal crypts at baseline and 6-month follow-up, by treatment group.

Table 2. Goblet cell mucin area in the normal-appearing colorectal mucosa at baseline and 6-month follow-up during the clinical trial

	Baseline				6-month follow-up				Absolute treatment effect*				Relative effect
	N	Mean	(SE)	*P-value*	N	Mean	(SE)	*P-value*	N	Mean	(SE)	*P-value*	
(A) Whole crypts													
Placebo	23	912.3	58.1	--	21	959.2	54.9	--	21	--	--	--	1.00
Calcium	23	976.7	55.3	0.43	21	1086.7	58.4	0.15	21	52.7	84.5	0.53	1.06
Vitamin D_3	23	855.5	61.3	0.49	22	1041.5	55.1	0.31	22	105.0	83.7	0.21	1.16
Calcium + Vitamin D_3	23	971.0	56.2	0.47	21	1047.7	70.8	0.31	21	-4.5	84.5	0.96	1.03
(B) Upper 40% of crypts													
Placebo	23	390.8	28.5	--	21	408.8	24.3	--	21	--	--	--	1.00
Calcium	23	413.6	27.4	0.56	21	459.3	27.8	0.26	21	21.9	39.5	0.58	1.06
Vitamin D_3	23	364.2	27.5	0.49	22	446.3	26.1	0.33	22	48.3	39.1	0.22	1.17
Calcium + Vitamin D_3	23	423.2	25.7	0.40	21	449.5	32.6	0.33	21	-8.0	39.5	0.84	1.02
(C) Lower 60% of crypts													
Placebo	23	521.5	31.3	--	21	550.4	31.6	--	21	--	--	--	1.00
Calcium	23	563.1	29.0	0.36	21	627.4	33.0	0.11	21	31.1	48.1	0.52	1.06
Vitamin D_3	23	491.4	34.9	0.50	22	595.2	30.5	0.32	22	57.6	47.7	0.23	1.15
Calcium + Vitamin D_3	23	547.9	31.7	0.56	21	598.2	39.7	0.32	21	4.3	48.1	0.93	1.03
(D) Upper 20% of crypts													
Placebo	23	148.5	12.6	--	21	150.2	10.0	--	21	--	--	--	1.00
Calcium	23	152.7	12.9	0.81	21	169.7	11.4	0.31	21	-1.7	17.7	0.92	1.10
Vitamin D_3	23	138.1	11.5	0.54	22	169.7	12.8	0.28	22	23.6	17.5	0.18	1.22
Calcium + Vitamin D_3	23	159.4	10.3	0.52	21	166.0	13.7	0.39	21	12.5	17.7	0.48	1.03
(E) Lower 20% of crypts													
Placebo	23	139.7	10.2	--	21	142.9	8.8	--	21	--	--	--	1.00
Calcium	23	154.0	8.7	0.31	21	161.2	10.1	0.22	21	3.9	16.0	0.81	1.02
Vitamin D_3	23	129.2	10.0	0.45	22	164.4	9.9	0.12	22	29.0	15.9	0.07	1.24
Calcium + Vitamin D_3	23	140.7	10.2	0.94	21	161.4	9.8	0.18	21	14.2	16.0	0.38	1.12
(F) Upper 40%/whole crypt													
Placebo	23	0.4	0.0	--	21	0.4	0.0	--	21	--	--	--	1.00
Calcium	23	0.4	0.0	0.62	21	0.4	0.0	0.403	21	0.0	0.0	0.95	1.00
Vitamin D_3	23	0.4	0.0	0.91	22	0.4	0.0	0.964	22	0.0	0.0	0.95	1.00
Calcium + Vitamin D_3	23	0.4	0.0	0.34	21	0.4	0.0	0.690	21	0.0	0.0	0.64	0.99

* Treatment 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).

Chapter III

STUDY IMPLICATIONS AND FUTURE RESEARCH DIRECTIONS

This study is the first pilot, randomized, double-blind, placebo-controlled, 2x2 factorial chemoprevention clinical trial to examine the effects of calcium and vitamin D, separately or combined, on crypt histological features, such as goblet cell area, in the normal human colorectal epithelium. Our main finding was that we found evidence that calcium and/or vitamin D_3 supplementation substantially change the crypt goblet cell mucin content in the normal human rectal epithelium among patients with history sporadic colorectal adenomas. Although there was a possible suggestion that vitamin D_3 supplementation may modestly increase goblet cell mucin content throughout the crypt length, perhaps especially in the lower 90% of the crypts, our results indicated that there was very little change in crypt goblet cell mucin area along the lengths of crypts in the other active treatment groups. Overall, our findings suggest that colon crypt goblet cell mucin content may not be a strongly modifiable biomarker of risk for colorectal cancer.

In the future, it may be more productive to focus on developing molecular phenotypic biomarkers of risk that can be used to accurately categorize and quantify risk for colorectal cancer and to screen for the potential efficacy of preventive interventions. Further basic science and animal studies are needed to assess the reasons why there are reductions or loss of goblet cells in colon adenocarcinomas. If in the future the modest estimated changes in goblet cell mucin content found in this study come to be considered clinically important, then trials with larger sample sizes and downstream endpoints, such as colorectal adenomas, would be needed. Also, higher doses of vitamin D, for longer periods of time should be considered. Other avenues of investigation may include investigating other populations, such as persons who have had no previous colorectal neoplasms (83); or other potential preventive interventions; and effects in more proximal areas of the colon.

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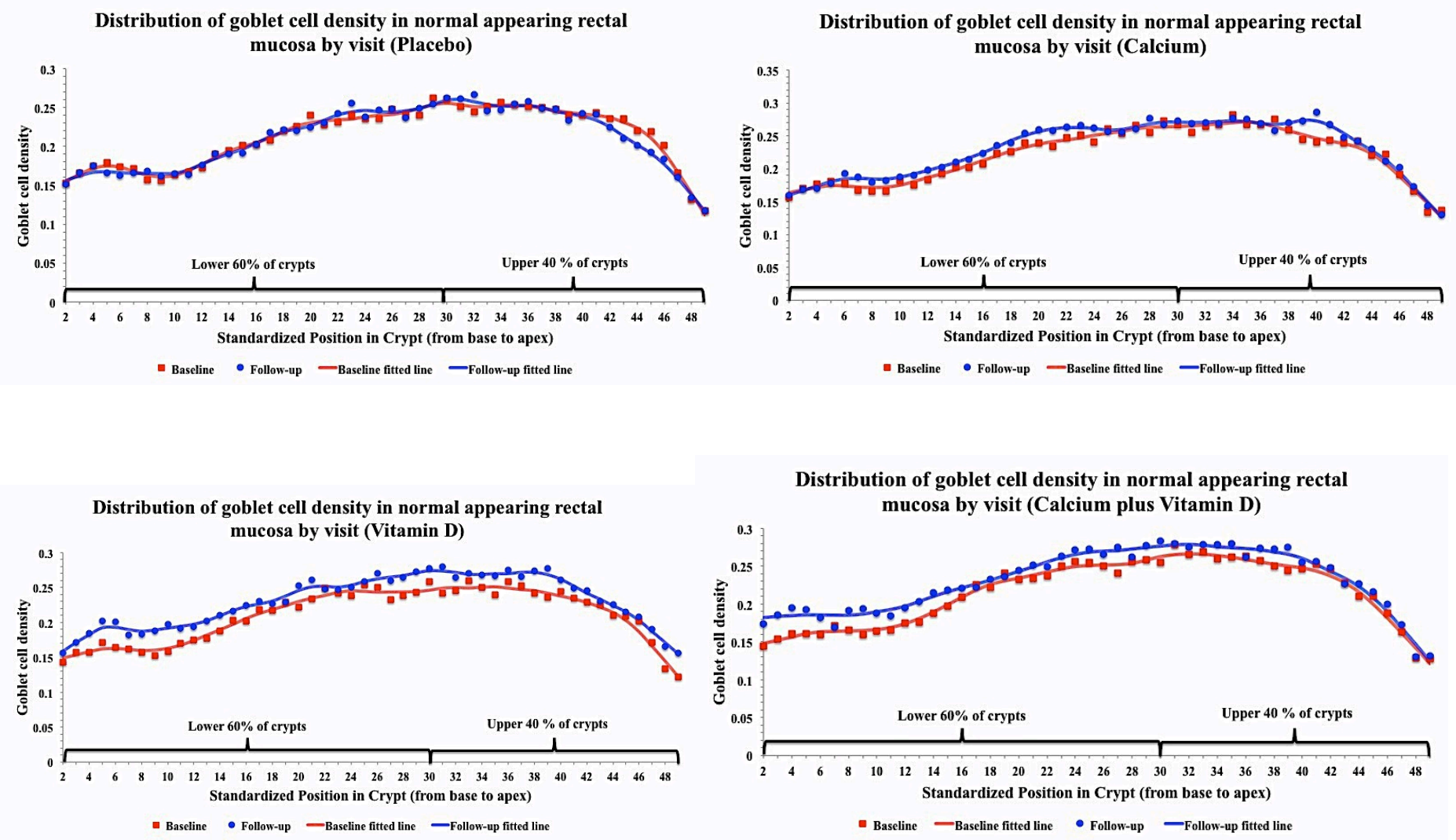
APPENDIX

SUPPLEMENTAL TABLES AND FIGURES



Appendix Figure 3. Goblet cell density along normal colorectal crypts at baseline and 6-month follow-up, by treatment group.

Appendix Table 3. Goblet cell density in the normal-appearing colorectal mucosa at baseline and 6-month follow-up during the clinical trial

	Baseline				6-month follow-up				Absolute treatment effect*				Relative effect
	N	Mean	(SE)	*P-value*	N	Mean	(SE)	*P-value*	N	Mean	(SE)	*P-value*	
(A) Whole crypts													
Placebo	23	0.21	(0.01)	--	21	0.21	(0.01)	--	21	--	--	--	1.00
Calcium	23	0.22	(0.01)	0.53	21	0.24	(0.01)	0.15	21	0.01	(0.02)	0.46	1.07
Vitamin D_3	23	0.20	(0.01)	0.48	22	0.23	(0.01)	0.31	22	0.02	(0.02)	0.21	1.15
Calcium + Vitamin D_3	23	0.22	(0.01)	0.69	21	0.23	(0.01)	0.45	21	0.00	(0.02)	0.92	1.03
(B) Upper 40% of crypts													
Placebo	23	0.23	(0.01)	--	21	0.23	(0.01)	--	21	--	--	--	1.00
Calcium	23	0.23	(0.01)	0.74	21	0.25	(0.02)	0.28	21	0.02	(0.02)	0.46	1.08
Vitamin D_3	23	0.21	(0.01)	0.49	22	0.24	(0.01)	0.41	22	0.02	(0.02)	0.27	1.14
Calcium + Vitamin D_3	23	0.24	(0.01)	0.67	21	0.24	(0.02)	0.54	21	0.00	(0.02)	0.96	1.02
(C) Lower 60% of crypts													
Placebo	23	0.20	(0.01)	--	21	0.20	(0.01)	--	21	--	--	--	1.00
Calcium	23	0.21	(0.01)	0.40	21	0.23	(0.01)	0.10	21	0.01	(0.02)	0.50	1.06
Vitamin D_3	23	0.19	(0.01)	0.49	22	0.22	(0.01)	0.27	22	0.02	(0.02)	0.20	1.14
Calcium + Vitamin D_3	23	0.21	(0.01)	0.76	21	0.22	(0.01)	0.41	21	0.00	(0.02)	0.80	1.04
(D) Upper 20% of crypts													
Placebo	23	0.20	(0.02)	--	21	0.19	(0.01)	--	21	--	--	--	1.00
Calcium	23	0.19	(0.02)	0.95	21	0.21	(0.02)	0.33	21	0.02	(0.02)	0.32	1.13
Vitamin D_3	23	0.18	(0.01)	0.53	22	0.21	(0.01)	0.43	22	0.03	(0.02)	0.25	1.18
Calcium + Vitamin D_3	23	0.20	(0.01)	0.88	21	0.20	(0.01)	0.64	21	0.00	(0.02)	0.91	1.04
(E) Lower 20% of crypts													
Placebo	23	0.16	(0.01)	--	21	0.16	(0.01)	--	21	--	--	--	1.00
Calcium	23	0.18	(0.01)	0.20	21	0.18	(0.01)	0.20	21	0.00	(0.02)	0.94	1.01
Vitamin D_3	23	0.15	(0.01)	0.54	22	0.18	(0.01)	0.13	22	0.03	(0.02)	0.10	1.22
Calcium + Vitamin D_3	23	0.16	(0.01)	0.83	21	0.18	(0.01)	0.23	21	0.01	(0.02)	0.49	1.09
(F) Upper 40% /whole crypt													
Placebo	23	1.06	(0.02)	--	21	1.06	(0.02)	--	21	--	--	--	1.00
Calcium	23	1.04	(0.02)	0.52	21	1.04	(0.02)	0.22	21	0.00	(0.03)	0.98	1.00
Vitamin D_3	23	1.06	(0.02)	0.93	22	1.05	(0.02)	0.62	22	-0.01	(0.03)	0.70	0.99
Calcium + Vitamin D_3	23	1.08	(0.02)	0.49	21	1.06	(0.02)	0.93	21	-0.02	(0.03)	0.56	0.98

* Treatment 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. Overall crypt area in the normal-appearing colorectal mucosa at baseline and 6-month follow-up during the clinical trial

	Baseline				6-month follow-up				Absolute treatment effect*				Relative effect
	N	**Mean**	**(SE)**	***P-value***	**N**	**Mean**	**(SE)**	***P-value***	**N**	**Mean**	**(SE)**	***P-value***	
Whole crypt													
Placebo	23	4830.0	178.7	--	21	5193.9	206.0	--	21	--	--	--	1.00
Calcium	23	4787.7	150.8	0.86	21	5080.0	140.8	0.62	21	-84.0	269.51	0.76	0.99
Vitamin D_3	23	4591.5	188.8	0.32	22	5008.6	139.0	0.41	22	17.4	267.4	0.95	1.01
Calcium + Vitamin D_3	23	5023.5	156.7	0.42	21	5275.1	141.8	0.72	21	-156.4	269.5	0.56	0.98

* Treatment 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 5. Whole crypt goblet cell mucin area in the normal-appearing colorectal mucosa at baseline and 6-month follow-up during the clinical trial, stratified by selected risk factors for colorectal neoplasms

	Baseline				Follow-up				Absolute treatment effect*				Relative effect[§]
	N	Mean	SE	*P-value*	*N*	Mean	SE	*P-value*	N	Mean	SE	*P-value*[**]	
(A) Males													
Placebo	16	940.6	71.4	--	14	941.1	71.6	--	14	--	--	--	1.00
Calcium	16	967.8	68.8	0.77	11	1032.1	85.7	0.43	11	67.8	116.8	0.56	1.07
Vitamin D_3	16	938.5	68.9	0.98	14	1130.5	63.5	0.08	14	157.9	111.4	0.16	1.20
Calcium + Vitamin D_3	16	969.5	58.1	0.76	14	1058.9	88.8	0.28	14	49.5	111.4	0.66	1.09
(B) Females													
Placebo	7	847.8	102.3	--	6	998.0	103.1	--	6	--	--	--	1.00
Calcium	7	997.0	98.5	0.35	6	1200.1	107.3	0.20	6	19.3	146.6	0.90	1.02
Vitamin D_3	7	665.9	97.8	0.25	6	857.5	73.6	0.37	6	4.2	146.6	0.98	1.09
Calcium + Vitamin D_3	7	974.6	137.1	0.42	7	1025.3	126.0	0.86	7	-105.0	142.3	0.47	0.89
(C) Age < 59 yrs.													
Placebo	14	874.3	76.6	--	12	926.7	71.0	--	12	--	--	--	1.00
Calcium	10	1011.4	76.3	0.26	6	1066.7	108.6	0.23	6	-70.7	120.1	0.83	1.00
Vitamin D_3	11	864.3	95.7	0.93	11	864.3	95.7	0.03	11	210.8	121.9	0.09	1.09
Calcium + Vitamin D_3	10	943.2	94.4	0.57	10	945.9	60.3	0.85	10	-70.7	120.1	0.56	0.94
(D) Age ≥ 59 yrs.													
Placebo	14	874.3	76.6	--	12	926.7	71.0	--	12	--	--	--	1.00
Calcium	10	1011.4	76.3	0.26	6	1066.7	108.6	0.23	6	-70.7	120.1	0.83	1.00
Vitamin D_3	11	864.3	95.7	0.93	11	864.3	95.7	0.03	11	210.8	121.9	0.09	1.09
Calcium + Vitamin D_3	10	943.2	94.4	0.57	10	945.9	60.3	0.85	10	-70.7	120.1	0.56	0.94
(E) NSAID users													
Placebo	10	886.3	99.4	--	10	963.9	68.1	--	10	--	--	--	1.00
Calcium	13	944.5	81.7	0.63	10	1117.6	94.4	0.25	10	109.4	133.3	0.42	1.09
Vitamin D_3	7	808.9	95.5	0.58	7	1017.9	93.6	0.71	7	131.4	149.2	0.38	1.16
Calcium + Vitamin D_3	15	954.5	69.5	0.56	13	1104.1	98.4	0.26	13	55.5	126.4	0.66	1.06
(F) Non-NSAID users													
Placebo	10	886.3	99.4	--	10	963.9	68.1	--	10	--	--	--	1.00
Calcium	13	944.5	81.7	0.63	10	1117.6	94.4	0.25	10	109.4	133.3	0.42	1.09
Vitamin D_3	7	808.9	95.5	0.58	7	1017.9	93.6	0.71	7	131.4	149.2	0.38	1.16
Calcium + Vitamin D_3	15	954.5	69.5	0.56	13	1104.1	98.4	0.26	13	55.5	126.4	0.66	1.06

(Continued)

	Baseline				Follow-up				Absolute treatment effect*				Relative effect§
	N	Mean	SE	*P-value*	*N*	Mean	SE	*P-value*	N	Mean	SE	*P-value* **	
(G) With history of colorectal cancer or polyps in first-degree relative (%)													
Placebo	11	852.4	92.2	--	10	928.2	74.6	--	10	--	--	--	1.00
Calcium	13	1027.4	75.6	0.14	10	1108.3	100.2	0.16	10	-0.3	132.2	1.00	0.99
Vitamin D_3	8	741.5	89.4	0.40	7	972.9	77.0	0.75	7	104.3	146.8	0.48	1.20
Calcium + Vitamin D_3	4	1231.3	151.5	0.03	4	1411.8	199.7	0.01	4	78.9	178.4	0.66	1.05
(H) Without history of colorectal cancer or polyps in first-degree relative (%)													
Placebo	12	967.3	72.5	--	10	936.6	86.1	--	10	--	--	--	1.00
Calcium	10	910.9	80.1	0.62	7	1067.2	91.5	0.54	7	127.8	135.6	0.35	1.21
Vitamin D_3	12	968.5	87.9	0.99	10	1149.4	80.9	0.17	10	136.0	124.7	0.28	1.23
Calcium + Vitamin D_3	18	909.0	56.6	0.55	16	943.4	60.5	0.67	16	-5.7	112.8	0.96	1.07
(I) Non-obese (BMI < 30, median)													
Placebo	12	854.3	70.3	--	9	898.2	86.2	--	9	--	--	--	1.00
Calcium	12	897.3	53.8	0.74	9	1047.3	85.7	0.38	9	57.5	132.6	0.67	1.11
Vitamin D_3	16	765.2	54.4	0.67	14	1005.6	51.6	0.54	14	139.0	121.0	0.26	1.25
Calcium + Vitamin D_3	11	1035.5	65.3	0.05	11	1048.7	107.5	0.35	11	-80.5	129.3	0.54	0.96
(J) Obese (BMI ≥ 30, median)													
Placebo	11	975.6	93.9	--	10	979.7	80.8	--	10	--	--	--	1.00
Calcium	11	1063.4	95.6	0.91	8	1140.9	111.5	0.27	8	75.2	119.1	0.53	1.07
Vitamin D_3	7	1061.9	134.8	0.68	6	1149.0	145.7	0.29	6	24.9	130.4	0.85	1.08
Calcium + Vitamin D_3	12	912.0	88.9	0.16	10	1046.5	96.4	0.63	10	73.1	112.9	0.52	1.14
(K) Serum 25-(OH)-vitamin D concentration < median¶													
Placebo	10	786.0	70.7	--	10	831.2	62.7	--	10	--	--	--	1.00
Calcium	14	949.2	48.6	0.04	10	1053.8	58.8	0.02	10	65.1	129.4	0.62	1.05
Vitamin D_3	10	899.9	48.1	0.19	16	1034.9	56.3	0.21	16	85.9	127.1	0.50	1.09
Calcium + Vitamin D_3	10	975.4	62.9	0.03	12	1140.2	110.3	0.01	12	137.6	131.7	0.30	1.11
(L) Serum 25-(OH)-vitamin D concentration ≥ median¶													
Placebo	11	971.8	83.7	--	11	1075.6	73.7	--	11	--	--	--	1.00
Calcium	9	1019.5	123.0	0.75	11	1116.6	100.1	0.06	11	-115.6	114.8	0.33	0.99
Vitamin D_3	12	797.3	109.0	0.22	5	985.2	154.2	0.06	5	-17.8	123.3	0.89	1.12
Calcium + Vitamin D_3	13	967.7	89.3	0.98	6	974.5	68.2	0.01	6	-72.9	117.2	0.54	0.91

* 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 is 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).

Note: Seven patients were lost to follow-up at their last visit.

¶ The median serum 25-(OH)-vitamin D concentration at baseline was 23.2 ng/mL.

Appendix Table 6. Goblet cell mucin area in the lower 60% of normal-appearing colorectal mucosa at baseline and 6-month follow-up during the clinical trial, stratified by selected risk factors for colorectal neoplasms

	Baseline				Follow-up				Absolute treatment effect*				Relative effect[§]
	N	Mean	SE	*P-value*	*N*	Mean	SE	*P-value*	N	Mean	SE	*P-value*[**]	
(A) Males													
Placebo	16	538.6	38.8	--	14	546.8	42.6	--	14	--	--	--	1.00
Calcium	16	558.0	35.5	0.71	16	558.0	35.5	0.42	16	37.2	66.2	0.58	0.99
Vitamin D_3	16	543.6	37.8	0.92	14	640.4	34.9	0.13	14	73.2	63.2	0.25	1.20
Calcium + Vitamin D_3	16	554.2	34.6	0.76	14	605.4	50.8	0.34	14	23.0	63.2	0.72	1.09
(B) Females													
Placebo	7	482.4	52.9	--	6	553.9	53.9	--	6	--	--	--	1.00
Calcium	7	574.9	54.1	0.28	6	697.0	62.1	0.11	6	-25.1	78.7	0.75	1.06
Vitamin D_3	7	371.9	55.5	0.20	6	502.9	43.6	0.55	6	37.2	85.5	0.67	1.18
Calcium + Vitamin D_3	7	533.3	72.4	0.55	7	583.7	67.4	0.72	7	-27.1	83.1	0.75	0.95
(C) Age < 59 yrs.													
Placebo	14	494.4	41.9	--	12	527.2	40.7	--	12	--	--	--	1.00
Calcium	10	579.9	43.1	0.22	6	604.1	51.5	0.24	6	-29.0	135.0	0.83	0.98
Vitamin D_3	11	497.0	55.4	0.97	9	647.0	44.4	0.04	9	103.5	71.1	0.16	1.22
Calcium + Vitamin D_3	10	535.7	56.3	0.55	10	544.4	34.8	0.76	10	-36.2	70.2	0.61	0.95
(D) Age ≥ 59 yrs.													
Placebo	9	563.7	45.4	--	8	581.6	57.4	--	8	--	--	--	1.00
Calcium	13	550.3	40.4	0.83	11	649.9	54.2	0.42	11	87.8	70.8	0.22	1.14
Vitamin D_3	12	486.1	45.9	0.23	11	560.0	40.3	0.80	11	31.8	71.0	0.66	1.12
Calcium + Vitamin D_3	13	557.2	37.7	0.92	11	647.0	67.3	0.44	11	48.0	70.8	0.50	1.13
(E) NSAID users													
Placebo	10	496.9	52.2	--	10	548.5	37.9	--	10	--	--	--	1.00
Calcium	13	544.0	41.4	0.47	10	649.2	57.1	0.18	10	60.8	75.4	0.43	1.08
Vitamin D_3	7	465.6	58.5	0.68	7	595.3	54.5	0.57	7	78.2	84.6	0.36	1.16
Calcium + Vitamin D_3	15	539.1	37.5	0.50	13	641.0	52.5	0.19	13	41.8	71.6	0.56	1.08
(F) Non-NSAID users													
Placebo	13	540.5	39.1	--	10	549.4	56.6	--	10	--	--	--	1.00
Calcium	10	588.0	40.7	0.48	8	626.9	47.1	0.29	8	1.8	67.1	0.98	1.05
Vitamin D_3	16	502.6	44.1	0.52	13	601.2	38.6	0.42	13	48.8	59.5	0.42	1.18
Calcium + Vitamin D_3	8	564.3	61.5	0.74	8	528.5	54.9	0.77	8	-69.3	67.9	0.31	0.92

(Continued)

	Baseline				Follow-up				Absolute treatment effect*				Relative effect[§]
	N	Mean	SE	*P-value*	*N*	Mean	SE	*P-value*	N	Mean	SE	*P-value*[**]	
(G) With history of colorectal cancer or polyps in first-degree relative (%)													
Placebo	11	486.5	49.9	--	10	535.4	38.9	--	10	--	--	--	1.00
Calcium	13	591.4	37.7	0.14	10	654.6	56.7	0.16	10	-0.3	132.2	1.00	1.01
Vitamin D_3	8	421.8	54.0	0.40	7	555.8	39.0	0.75	7	104.3	146.8	0.48	1.20
Calcium + Vitamin D_3	4	675.5	77.0	0.03	4	789.3	108.6	0.01	4	78.9	178.4	0.66	1.06
(H) Without history of colorectal cancer or polyps in first-degree relative (%)													
Placebo	12	553.7	38.3	--	10	562.5	55.6	--	10	--	--	--	1.00
Calcium	10	526.4	44.8	0.62	7	603.9	51.4	0.54	7	127.8	135.6	0.35	1.13
Vitamin D_3	12	558.3	48.0	0.99	10	645.4	46.0	0.17	10	136.0	124.7	0.28	1.14
Calcium + Vitamin D_3	18	515.4	33.8	0.55	16	543.8	36.3	0.67	16	-5.7	112.8	0.96	1.04
(I) Non-obese (BMI < 30, median)													
Placebo	12	493.1	37.6	--	10	545.0	50.5	--	10	--	--	--	1.00
Calcium	12	527.8	27.8	0.49	9	617.3	51.0	0.32	9	33.0	74.5	0.66	1.06
Vitamin D_3	16	442.8	31.8	0.28	14	579.2	28.6	0.60	14	73.9	67.9	0.28	1.18
Calcium + Vitamin D_3	11	590.5	37.6	0.06	11	586.8	61.8	0.55	11	-62.2	72.5	0.40	0.90
(J) Obese (BMI ≥ 30, median)													
Placebo	11	552.5	51.1	--	10	552.8	45.7	--	10	--	--	--	1.00
Calcium	11	572.9	50.8	0.52	10	604.3	52.9	0.22	8	48.6	68.8	0.49	1.05
Vitamin D_3	7	602.3	77.3	0.56	6	645.7	79.7	0.29	6	11.6	75.4	0.88	1.07
Calcium + Vitamin D_3	12	508.8	49.0	0.56	10	610.6	51.5	0.45	10	72.1	65.3	0.28	1.20
(K) Serum 25-(OH)-vitamin D concentration < median[¶]													
Placebo	10	454.4	35.7	--	10	468.4	33.2	--	10	--	--	--	1.00
Calcium	14	550.6	27.9	0.03	10	619.8	34.1	0.03	10	58.6	71.1	0.42	1.09
Vitamin D_3	10	513.0	24.3	0.20	16	592.2	32.4	0.05	16	64.5	69.8	0.36	1.12
Calcium + Vitamin D_3	10	551.3	32.1	0.04	12	633.0	62.7	0.01	12	78.6	72.4	0.29	1.11
(L) Serum 25-(OH)-vitamin D concentration ≥ median[¶]													
Placebo	11	547.5	46.2	--	11	624.9	41.7	--	11	--	--	--	1.00
Calcium	9	582.6	62.3	0.68	11	634.3	56.5	0.89	11	-76.9	67.4	0.27	0.95
Vitamin D_3	12	464.9	64.0	0.30	5	569.0	84.3	0.52	5	-29.8	72.5	0.69	1.07
Calcium + Vitamin D_3	13	545.3	51.7	0.98	6	588.9	43.3	0.66	6	-8.4	68.9	0.90	0.95

[*] 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 is 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).

Note: Seven patients were lost to follow-up at their last visit.

[¶] The median serum 25-(OH)-vitamin D concentration at baseline was 23.2 ng/mL.