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Effects of Supplemental Calcium and Vitamin D on the APC/β-Catenin Pathway in the Normal Colorectal Mucosa of Colorectal Adenoma Patients

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Master of Science in Public Health

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2013

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Abstract

Effects of Supplemental Calcium and Vitamin D on the APC/β-Catenin Pathway in the Normal Colorectal Mucosa of Colorectal Adenoma Patients

By Siyu Liu

Malfunctioning of the APC/β-catenin pathway is a common and early event in colorectal carcinogenesis. To assess the effects of calcium and vitamin D on the APC/β-catenin pathway in the normal-appearing colorectal mucosa of sporadic colorectal adenoma patients, we conducted a randomized, double-blind, placebo-controlled, modified 2 ×2 factorial chemoprevention clinical trial (n = 104) of supplemental calcium (1,200 mg daily) and vitamin D (1,000 IU daily), alone and in combination versus placebo. APC, β-catenin, and E-cadherin expression and distributions in colon crypts in normal-appearing rectal mucosa biopsies were detected using standardized automated immunohistochemistry and quantified using image analysis. For vitamin D vs. no vitamin D, the ratio of APC expression to β-catenin expression in the upper 40% of crypts (APC/β-catenin score) increased by 28% (P = 0.02), for calcium vs. no calcium it increased by 1% (P = 0.88), and for vitamin D + calcium vs. calcium by 35% (P = 0.01). Total E-cadherin expression increased by 7% (P = 0.35) for vitamin D vs. no vitamin D, 8% (P = 0.31) for calcium vs. no calcium, and 12% (P = 0.21) for vitamin D + calcium vs. calcium. These results support (i) that vitamin D, alone or in combination with calcium, may modify APC, β-catenin, and E-cadherin expression in humans in directions hypothesized to reduce risk for colorectal neoplasms, (ii) vitamin D as a potential chemopreventive agent against colorectal neoplasms, and (iii) the potential of APC, β-catenin, and E-cadherin expression as treatable, pre-neoplastic risk biomarkers for colorectal neoplasms.

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Literature Review

Colorectal Cancer Epidemiology

Despite advances in screening and treatment, colorectal cancer (CRC) remains the third most commonly diagnosed cancer and the second leading cause of cancer death in men and women combined in the US.[1] Approximately 5%, or 1 in 20, Americans will be diagnosed with CRC in their lifetime.[2] The American Cancer Society estimates that 132,700 people will be diagnosed with CRC and 49,700 people will die from the disease in 2015.[1] In the past decade, there has been unprecedented progress in reducing CRC incidence and mortality rates in the US, large due to early detection of CRC through screening tests among adults 50 years and older, which allows for the detection and removal of colorectal polyps before they progress to cancer.[2] From 2007 to 2011, incidence rates decreased by 4.3% per year among adults 50 years and older, but increased by 1.8% per year among adults younger than age 50. During the same period, the overall mortality rate for CRC declined by 2.5% per year, reflecting decreasing incidence rates and improvements in early detection and treatment.[1]

CRC incidence and mortality rates are about 30% to 40% higher in males than females.[2] Incidence rates for colon cancer are similar in both sexes, but there is a male predominance for rectal cancer.[3] CRC rates are highest among blacks and lowest among Asian/Pacific Islanders (APIs). During 2006 - 2010, CRC incidence rates in blacks were about 25% higher than those in whites and about 50% higher than those in APIs. CRC mortality rates are about 50% higher than those in whites and double those in APIs.[2] CRC is uncommon before age 40, but the incidence rises progressively from age 40, rising sharply after age 50. Overall, 90% of CRC cases and 93% of deaths occur in people 50 years and older.[3]

There is a large geographical difference in the global distribution of CRC. CRC incidence rates vary by up to 25-fold throughout the world, with the highest rates documented in Australia and New Zealand, North America, and Western Europe and the lowest rates in Africa and South-Central Asia.[4] Migrant populations from low-risk to high-risk countries tend to acquire the CRC incidence rates of their adopted country instead of preserving those of their country of origin, emphasizing the importance of environmental exposures and suggesting that CRC is preventable through environmental modifications.[4]

The 5- and 10-year relative survival rates following diagnosis of CRC are 65% and 58%, respectively. Only 40% of CRC is detected at a localized stage, for which the 5-year survival rate is 90%. For patients diagnosed with regional and distant stages, the 5-year survival rates drop to 71% and 13%, respectively.[1]

Molecular Basis of Colorectal Cancer

Colorectal carcinogenesis was understood as a multistep process that involved accumulation of tumor suppressor genes and oncogenes mutations, such as *APC*, *KRAS*, and *p53*.[5] There are two main pathways in the development of CRC.[6] The first, the APC/ β-catenin/WNT pathway, accounts for 80% to 90% of sporadic CRCs and is the cause of the familial adenomatous polyposis (FAP). The *APC* tumor suppressor gene, mapped to chromosome 5q, is a gatekeeper for the colorectal adenoma and encodes a huge protein consisting of 2,843 amino acids that has been involved in various cellular functions. APC protein functions to degrade β-catenin, the effector of the WNT signaling pathway that controls the coordinated expansion and differentiation of the intestinal crypt stem cells. The WNT signaling pathway is normally inactive, but impaired APC expression can result in WNT signaling through stabilization of nuclear β-catenin, thus promoting cell proliferation and inhibiting differentiation.[5,6] The second pathway, the Mismatch Repair (MMR) pathway, accounts for about 15% of sporadic CRCs and

Hereditary Non-polyposis Colorectal Cancer (HNPCC). The DNA mismatch repair genes functions to repair mismatches in paired DNA strands post replication. Mismatches can be propagated when DNA repair genes are impaired, leading to the accumulation of DNA errors which eventually hamper the ability of the affected gene to function properly.[7]

Modifiable Risk Factors for Colorectal Cancer

CRC, like other cancers, is the result of the interplay of many risk factors. The marked geographic variation and the effect of migration on CRC incidence suggest that environmental or lifestyle factors are major contributions to the etiology of this disease.

Dietary factors

In the 2007 World Cancer Research Fund and American Institute of Cancer Research (WCRF/AICR) report "Food, Nutrition, Physical Activity, and the Prevention of Cancer: a Global Perspective", it was concluded that high intake of red and processed meat is a convincing risk factor for CRC.[4] The most recent meta-analysis of 19 cohort, 2 case-control, and 3 nested case-control studies (up to March 2011) assessed the association of red and processed meat intakes with CRC incidence. Intake of red and processed meat was found to be associated with a 22% higher risk of CRC (RR = 1.22, 95% CI = 1.11 - 1.34) when comparing the highest versus the lowest category, and the RR for every 100 g/day increase was 1.14 (95% CI = 1.04 - 1.24). Non-linear dose-response meta-analyses found that CRC risk increased approximately linearly with increasing intake of red and processed meats up to approximately 140 g/day, above which the risk increase was less pronounced. Similar to the overall association, statistically significant associations were found for both colon (RR = 1.19, 95% CI = 1.06 - 1.34) and rectal cancer (RR = 1.51, 95% CI = 1.31 - 1.75).[8] There are several potential underlying mechanisms for a positive association of red and processed meat consumption with CRC. Cooking meat at high temperature

may result in the formation of mutagenic and carcinogenic heterocyclic amines and polycyclic aromatic hydrocarbons. A second mechanism is that the abundant presence of heme in red meat and the high levels of salt and nitrite in processed meat may contribute to the generation of potentially carcinogenic N-nitroso compounds. Also, high-fat diets could lead to increased levels of bile acids, which have mitogenic and mutagenic effects.[4,8]

The WCRF/AICR report stated that there was limited suggestive evidence for risk reduction by fruits and non-starchy vegetables.[4] A recent meta-analysis of 19 cohort studies (up to May 2010) found weak but statistically significant inverse associations of intakes of fruit and non-starchy vegetables with CRC risk. The summary RR for the highest vs. the lowest intake was 0.92 (95% CI = 0.86 – 0.99) for fruit and vegetables combined, 0.90 (95% CI = 0.86 – 0.99) for fruit, and 0.91 (95% CI = 0.86 – 0.96) for vegetables.[9]

Alcohol

According to the WCRF/AICR report, alcohol was a convincing risk factor for CRC in men, but only a probable risk factor in women.[4] The most recent meta-analysis of 27 cohort and 34 case-control studies (up to May 2010) investigated the association of alcohol drinking with CRC risk. It was found that the RRs were 1.21 (95% CI = 1.13 – 1.28) and 1.52 (95% CI = 1.27 – 1.81) for moderate drinkers (2 - 3 drinks/day) and heavy drinkers (≥ 4 drinks/day), compared to non-/occasional drinkers (≤ 1 drink/day). Men were at statistically significantly higher risk than women among any drinkers (P = 0.001) and moderate drinkers (P = 0.02).[10] There were some potential explanations for the stronger association among men than among women, including the generally higher consumption of alcohol among men, the difference in the preference of alcohol types, and hormone-related differences in alcohol metabolism.[4]

Physical Activity

There is a convincing inverse association between physical activity (PA) and CRC risk.[4] The most recent meta-analysis done, which included 21 cohort and case-control studies on the association between PA and CRC (up to 2010), found a RR of 0.88 per 2 standard score (95% CI = 0.86 – 0.91), which was a PA score assigned to each activity category with 1 as the lowest and 5 as the highest.[11] There were several potential underlying mechanisms for this inverse association. PA raises the metabolic rate and increases maximal oxygen uptake. Regular periods of PA may increase the body's metabolic efficiency and capacity, and reduce blood pressure and insulin resistance.[4]

Obesity

There is abundant and consistent epidemiological evidence on the association between general and central obesity with CRC.[4] A meta-analysis of 54 prospective studies (up to January 2012) found significant RRs of CRC for the obese vs. normal category of body mass index (BMI) (RR = 1.33, 95% CI = 1.25 – 1.42), and the highest vs. lowest category of waist circumference (RR = 1.46, 95% CI = 1.33 – 1.60). Stronger associations were observed among males than females.[12] Consistently, another meta-analysis, which included 23 studies on BMI and CRC, found a 29% higher risk for CRC per 8 kg/m^2 among males (95% CI = 1.26 – 1.34), and a 15% higher risk per 8 kg/m^2 among females (95% CI = 0.98 – 1.34).[11]

Obesity was found to be associated with levels of many circulating hormones, such as insulin, insulin-like growth factors, and estrogens that lead to an environment that encourages carcinogenesis and discourages apoptosis. Moreover, body fatness could stimulate the body's inflammatory response, which may be related to the initiation and progression of several cancers.[4,12]

Others

According to the WCRF/AICR report, other probable protective factors for CRC included garlic, milk, and foods containing vitamin D and calcium.[4]

Calcium and Colorectal Cancer

Calcium is an important micronutrient that is key for the maintenance of proper structure of many components of the cell and controls a large number of intracellular and extracellular processes. Intracellular calcium is a pervasive second messenger that carries signals from the plasma membrane and other intracellular stores to activate a large number cellular functions, such as fertilization, secretion, muscle contraction, growth and memory. The dynamics of calcium entry into cells and intracellular release from the endoplasmic reticulum are closely regulated, and the changes in the flux are signals for many cell functions. Therefore, the dynamics of the flux are well regulated to achieve calcium balance and ensure normal cell function. All calcium in bodily fluids originates from the diet.[13,14]

There is strong biological plausibility and generally consistent evidence from observational studies and trials for a protective effect of calcium against CRC.

Twenty cohort studies investigated associations between calcium and CRC risk and 18 of them reported inverse associations, of which 8 were statistically significant.[15] A recent meta-analysis, which included 9 prospective cohort studies on calcium supplements, found a statistically significant lower CRC risk for use of calcium supplements versus no use (RR = 0.86, 95% CI = 0.79 – 0.95) and for the highest category versus the lowest (RR = 0.80, 95% CI = 0.70, 0.92). Moreover, an increase of 100 mg/day of supplemental calcium was found to be associated with a 4% lower risk of CRC (RR = 0.96, 95% CI = 0.94 – 0.99).[16]

The most compelling evidence supporting a protective effect of calcium against CRC comes from randomized controlled trials (RCTs) of supplemental calcium. The most recent meta-analysis of

three trials in individuals with a history of adenomas (1,279 participants) found a statistically significant reduction in adenoma recurrence (RR = 0.80, 95% CI = 0.69 – 0.94) for those receiving calcium 1,200 to 2,000 mg/d, alone or in combination with other micronutrients. A statistically non-significant effect was found against advanced adenoma (RR = 0.77, 95% CI = 0.50 – 1.71). Furthermore, a meta-analysis of two trials in individuals with no known increased risk for CRC (37,461 participants) found a statistically non-significant effect of supplemental calcium, with or without vitamin D (RR = 0.62, 95% CI = 0.11 - 3.40).[17]

There are several proposed underlying mechanisms for how calcium may protect against CRC. Calcium may protect the colorectal mucosa by binding free bile acids and ionized fatty acids to form insoluble soaps of these toxic compounds. A second mechanism is that calcium could lower cellular proliferation in the colorectal mucosa given that calcium was found to have a direct growth-restraining, and differentiation- and apoptosis- inducing action on normal and tumor cells *in vitro*.[14]

Vitamin D and Colorectal Cancer

As an essential nutrient, in addition to its well-known role in mineral and skeletal homeostasis, vitamin D exerts various physiological functions. 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the most active molecular form of vitamin D, can restrain cell proliferation and induce differentiation and apoptosis in a large variety of normal and tumor cells. Many cell types, including colorectal epithelial cells, express the vitamin D receptor, and are therefore capable of converting circulating 25-hydroxyvitmain D (25(OH)D) into $1,25(OH)_2D_3$. Activation of these receptors by $1,25(OH)_2D_3$ has anti-cancerous effects, including increasing cell differentiation and apoptosis and inhibiting proliferation, invasiveness, angiogenesis, and metastatic potential.[18] Therefore, vitamin D deficiency may increase the risk of CRC.

Numerous case-control and cohort studies have examined the association between vitamin D with CRC and have generally found an inverse association. In a meta-analysis of 17 cohort and case-control studies (up to 2010) on vitamin D and CRC risk, significant inverse associations were found for both vitamin D intake (RR = 0.88, 95% CI = 0.80 – 0.96) and 25(OH)D (RR = 0.67, 95% CI = 0.54 – 0.80) when comparing the highest category to the lowest. Furthermore, a 10ng/mL increment in serum 25(OH)D level was found to be associated with a 26% lower risk of CRC (RR = 0.74, 95% CI = 0.63 – 0.89).[19] Consistent with these findings, a meta-analysis of four observational studies on the association between serum 25(OH)D and CRC mortality found that patients in the highest category of 25(OH)D had 37% lower mortality from CRC compared to those in the lowest category (OR = 0.63, $P < 0.0001$).[20]

Results for RCTs could provide more compelling evidence, but the current data are sparse. To date, the largest related trial is the Women's Health Initiative (WHI), which didn't support a strong protective effect of vitamin D on CRC risk during 7 years of follow-up. [21] However, this study had several important limitations, including the relatively low dose of vitamin D supplementation (400 IU/d) which corresponded to 2 – 3 ng/mL increase in serum 25(OH)D, whereas in most observational studies that found statistically significant associations the difference between the high and low categories was no less than 20 ng/mL. Moreover, the pill taking compliance was not ideal: only 50 – 60% of participants took 80% of assigned supplements. Thus, the results from the WHI should be interpreted with caution.[21,22,23]

Biomarkers and Colorectal Cancer

Despite the rapidly growing insights into the molecular biology of CRC, there is no generally accepted pre-neoplastic molecular marker of risk for CRC. CRC, like ischemic heart disease (IHD), is a complex, multi-factorial disease. The identification and utilization of modifiable biomarkers of risk for IHD has led to an approximately 70% decline in mortality since 1975.

Thus, the development of pre-neoplastic biomarkers of risk for CRC is highly desirable for managing risk and mortality for CRC.[24]

The APC protein, β-catenin, and E-cadherin are appealing candidates for being potentially treatable pre-neoplastic biomarkers of risk for colorectal adenomas. Impaired APC expression, which occurs in approximately 80% to 90% of sporadic CRCs, results in an increased potential of β-catenin to translocate to the nucleus, bind with T-cell factor (TCF) transcription factors, and activate target genes responsible for promoting cell proliferation and inhibiting differentiation.[6] Also, β-catenin, together with α-catenin, can bind to the cytoplasmic tail of the calcium-dependent cell adhesion protein E-cadherin, linking E-cadherin to actin filaments and promoting cell adhesion and differentiation. E-cadherin may antagonize the APC/β-catenin pathway by sequestering β-catenin at cell adhesion junctions.[25]

The Markers of Adenomatous Polyps II (MAP II) study, a colonoscopy-based case-control study, investigated potentially modifiable biomarkers of risk for incident, sporadic colorectal adenomas, including APC, β-catenin, and E-cadherin. It was found that APC expression was 3.2% lower in cases compared to controls, β-catenin expression was 3.0% higher, and E-cadherin expression was 0.7% lower; but these differences were not statistically significant. Moreover, having a higher proportion of APC expression in the upper 40% (differentiation zone) of colorectal crypts was found to be consistently, but not statistically significantly, greater among controls than cases at all colon sites. The ratio of the proportion of APC expression in the upper 40% of crypts to total β-catenin expression (APC/β-catenin score) was 14.3% higher among controls than cases (OR = 0.40, 95% CI = 0.14 - 1.14), and this score statistically significantly differed according to categories of plausible risk factors for CRC. [25] These results were consistent with previous reports that the colorectal crypt proliferation zone (the lower 60%) expanded into the differentiation zone in CRC cases.[25]

A randomized clinical trial investigated the separate and joint effects of calcium and vitamin D supplementation on the expression of potential pre-neoplastic molecular biomarkers of risk for colorectal neoplasms, including APC, β-catenin, and E-cadherin. In this study, 92 colorectal adenoma patients were randomly assigned, stratified on sex and non-steroidal anti-inflammatory drug use, to four treatment groups receiving daily over 6 months: placebo (n = 23), 2.0 g elemental calcium supplementation (n = 23), 800 IU vitamin D supplementation (n = 23), and 2.0 g elemental calcium plus 800 vitamin D supplementation (n = 23). It was found that the proportion of APC expression in the upper 40% of crypts increased 21% (P = 0.01), β-catenin decreased 12% (P = 0.18), E-cadherin increased 72% (P = 0.03), and the APC/β-catenin score increased 31% (P = 0.02) in the vitamin D supplemented group. In the calcium supplemented group, β-catenin decreased 15% (P = 0.08), and the APC/β-catenin score increased 41% (P = 0.01). In the vitamin D plus calcium supplemented group, E-cadherin increased 51% (P = 0.08), and the APC/ β-catenin score increased 16% (P = 0.26)[26] Results from this study provided the first and the only human, *in vivo* evidence that supplemental calcium and vitamin D, alone in combination, may increase APC and E-cadherin expression and decrease β-catenin expression in the normal colorectal mucosa of sporadic adenoma patients.

Taken together, these two studies provided support that APC, β-catenin, and E-cadherin may be modifiable, pre-neoplastic biomarkers of risk for colorectal adenomas, and the clinical trial also provided potential human *in vivo* mechanistic evidence of the possible protective effects of calcium and vitamin D against CRC. Further investigation in larger population is needed to evaluate APC, β-catenin, and E-cadherin as potentially treatable, pre-neoplastic biomarkers of risk for colorectal neoplasms.

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Effects of Supplemental Calcium and Vitamin D on the APC/β-Catenin Pathway in the Normal Colorectal Mucosa of Colorectal Adenoma Patients

Abstract

Malfunctioning of the APC/β-catenin pathway is a common and early event in colorectal carcinogenesis. To assess the effects of calcium and vitamin D on the APC/β-catenin pathway in the normal-appearing colorectal mucosa of sporadic colorectal adenoma patients, we conducted a randomized, double-blind, placebo-controlled, modified 2 ×2 factorial chemoprevention clinical trial (n = 104) of supplemental calcium (1,200 mg daily) and vitamin D (1,000 IU daily), alone and in combination versus placebo. APC, β-catenin, and E-cadherin expression and distributions in colon crypts in normal-appearing rectal mucosa biopsies were detected using standardized automated immunohistochemistry and quantified using image analysis. For vitamin D vs. no vitamin D, the ratio of APC expression to β-catenin expression in the upper 40% of crypts (APC/β-catenin score) increased by 28% (P = 0.02), for calcium vs. no calcium it increased by 1% (P = 0.88), and for vitamin D + calcium vs. calcium by 35% (P = 0.01). Total E-cadherin expression increased by 7% (P = 0.35) for vitamin D vs. no vitamin D, 8% (P = 0.31) for calcium vs. no calcium, and 12% (P = 0.21) for vitamin D + calcium vs. calcium. These results support (i) that vitamin D, alone or in combination with calcium, may modify APC, β-catenin, and E-cadherin expression in humans in directions hypothesized to reduce risk for colorectal neoplasms, (ii) vitamin D as a potential chemopreventive agent against colorectal neoplasms, and (iii) the potential of APC, β-catenin, and E-cadherin expression as treatable, pre-neoplastic risk biomarkers for colorectal neoplasms.

Introduction

Despite advances in screening and treatment, colorectal cancer (CRC) remains the third most commonly diagnosed cancer and the second leading cause of cancer death in men and women combined in the US.[1] Approximately 5%, or 1 in 20, Americans will be diagnosed with CRC in their lifetime.[2] There is international variation in CRC rates, with the highest rates in Australia and New Zealand, North America, and Western Europe, and the lowest in Africa and South-Central Asia.[3] Also, international ecologic and migration studies show that immigrants from lower-risk to higher-risk countries tend to acquire the CRC incidence rates of their adopted country instead of preserving those of their country of origin, emphasizing the importance of environmental exposures, especially diet and lifestyle, and suggesting that CRC is preventable through environmental modifications.[3]

There is strong biological plausibility and animal experimental and human observational evidence for a protective effect of calcium and vitamin D against CRC.[4] Proposed mechanisms of calcium against CRC include protection of the colorectal mucosa against free bile and fatty acids, direct effects on the cell cycle, and modulation of the APC colon carcinogenesis pathway.[5,6] Besides its important role in maintaining calcium balance, vitamin D promotes bile acid degradation, regulates cell cycle events, and modulates growth factor signaling, DNA repair, and more than 200 responsive genes.[6] Moreover, results from numerous epidemiologic case-control and prospective cohort studies suggest that calcium intake is inversely associated with risk of colorectal neoplasms, and in large randomized controlled trials calcium supplementation reduced adenoma recurrence.[4,7] Higher vitamin D intake has been found to be associated with lower risk of CRC, and stronger associations were seen when supplemental or total (dietary plus supplemental) vitamin D intake was considered.[4,8] Also, higher serum levels of 25-hydroxyvitamin-D (25(OH)D) have been associated with lower risk of colorectal adenoma.[4,8]

The adenomatous polyposis coli (APC) protein, β-catenin, and E-cadherin are potentially treatable pre-neoplastic biomarkers of risk for colorectal adenomas. Impaired APC expression,

which occurs in approximately 80% to 90% of sporadic CRCs, results in an increased potential of β-catenin to translocate to the nucleus, bind with T-cell factor (TCF) transcription factors, and activate target genes responsible for promoting cell proliferation and inhibiting differentiation.[9] Also, β-catenin, together with α-catenin, can bind to the cytoplasmic tail of the calcium-dependent cell adhesion protein E-cadherin, linking E-cadherin to actin filaments and promoting cell adhesion and differentiation. E-cadherin may antagonize the APC/β-catenin pathway by sequestering β-catenin at cell adhesion junctions.[10] In the normal colorectal mucosa, APC, β-catenin, and E-cadherin are all strongly expressed—APC primarily in the cytoplasm, and β-catenin and E-cadherin primarily at the cell membrane. During the adenoma-carcinoma sequence, APC and E-cadherin expression markedly decreases, β-catenin expression increases and translocates from the membrane to the cytoplasm and eventually into the nucleus.[11]

Despite the basic science evidence, there is only one reported human *in vivo* investigation on the effects of calcium and vitamin D on the expression of APC, β-catenin, and E-cadherin in the normal colorectal mucosa.[12] We hypothesized that calcium and vitamin D, alone and in combination, would increase the expression of APC and E-cadherin and decrease the expression of β-catenin in the normal-appearing colorectal mucosa of sporadic colorectal adenoma patients.

Materials and Methods

Participant population

The participants in this study ("adjunct biomarker study") were all participating in a larger multi-center, randomized, placebo-controlled, 2 × 2 factorial chemoprevention clinical trial ("parent study") which was designed to test the efficacy of supplemental calcium and vitamin D, alone and in combination, over 3-5 years on adenoma recurrence in colorectal adenoma patients. The parent study participants were recruited from gastrointestinal and surgical services of the 11 PPSG

(Polyp Prevention Study Group) clinical centers. Eligible participants were 45 to 75 years of age, in general good health, with a history of at least one histologically-verified neoplastic polyp ≥ 2 mm in diameter removed from the large bowel within 4 months of study entry. Exclusions from participation included invasive carcinoma in any colonic polyp removed, familial colonic polyposis syndromes, ulcerative colitis or Crohn's disease, malabsorption syndrome, history of large bowel restriction for any reason, diagnosed narcotic or alcohol dependence, elevated serum calcium or creatinine or supraphysiologic levels of serum 25(OH)D at study entry, history of kidney stones or hyperparathyroidism, and history of osteoporosis or other medical condition that may require supplemental vitamin D. For participation in the adjunct biomarker study, additional exclusions were being unable to be off aspirin for 7 days, history of a bleeding disorder, or current use of an anticoagulant medication.

Clinical trial protocol

Between May 2004 and July 2008, 19,083 apparently eligible patients were identified through initial screening of colonoscopy and pathology reports for the parent study; of these, 17,270 (85.3%) were excluded (n = 8,409 refused, n = 863 ineligible, n = 6,802 unable to contact, n = 196 enrollment ended). Of the enrolled 2,813 (14.7%) participants, we excluded 446 (15.9%) individuals who did not meet inclusion criteria (n = 97 with serum 25(OH)D levels < 12 ng/ml, n = 63 with serum calcium outside the normal range, n = 14 with serum creatinine > 20% above the upper limit of normal, n = 211 with < 80% pill-taking adherence during the placebo run-in period, n = 61 who were found ineligible), 103 (3.7%) who refused to participate, and 5 (0.2%) who were not randomized within the 84-day randomization window. Ultimately, 2,259 (80.3%) participants were randomized. For the adjunct biomarker study, near the end of the placebo run-in period, a total of 104 willing parent study participants from 2 clinical centers (South Carolina and Georgia) were recruited. All participants signed a consent form at enrollment; the research was approved by the Institutional Review Boards at each clinical center.

At enrollment, the coordinator obtained from each subject in the parent study a general medical history, medications, nutritional supplements, and behavioral factors such as tobacco use, alcohol consumption, sun exposure, and exercise. Diet was assessed using the semi-quantitative Block Brief 2000 food frequency questionnaire (Nutritionquest, Berkeley, CA). Subsequently, enrolled subjects entered a placebo run-in period of 56-84 days to exclude subjects unlikely to adhere to study procedures, including those who reported taking < 80% of their study tablets. After the run-in period, subjects were randomly assigned to the following 4 treatment groups: placebo (n = 415), 1,200 mg/day calcium supplementation (as calcium carbonate in equal doses twice daily, n = 419), 1,000 IU/day vitamin D_3 supplementation (500 IU twice daily, n = 420), and 1,200 mg/day elemental calcium plus 1,000 IU/day vitamin D_3 supplementation (n = 421) ("4-arm study"). Participants agreed to avoid taking vitamin D or calcium supplements outside the trial, although personal supplements up to 1,000 IU vitamin D and/or 400 mg elemental calcium were permitted from April 2008 onwards. Women who declined to forego calcium supplementation were randomized to calcium (n = 295) or calcium plus vitamin D_3 (n = 289) ("2-arm study"). Randomization was conducted using computer-generated random numbers with permuted blocks, and stratified by sex, clinical center, scheduled colonoscopic follow-up of 3 or 5 years, and 4- vs. 2-arm participation. Participants and all clinical and coordination staff were blinded. Study treatment was scheduled to continue until the anticipated 3- or 5-year colonoscopy. Bottles of study tablets were mailed to subjects every 4 months and a specially formulated preparation lacking calcium and vitamin D was offered to subjects who wished to take a multivitamin. Study treatment ended August 31, 2013, and follow-up ended November 30, 2013.

During the treatment period, colonoscopic visualization of the entire large bowel was performed in each subject 3 or 5 years after the qualifying colonoscopy, as set out before randomization by each patient's clinician. Participants were interviewed via telephone every 6 months regarding their adherence to study treatment, illnesses, use of medications and supplements, and colorectal

endoscopic or surgical procedures. Records for all hospitalizations and major medical events, and all colorectal surgical procedures and endoscopic examinations were collected. Two blinded physicians adjudicated adverse event diagnoses. Blood levels of calcium, creatinine, 25(OH)D, and $1,25(OH)_2D$ were obtained at baseline (during the run-in period) and 1 year after randomization, and 3 years after randomization for subjects with a 5-year surveillance cycle.

Participants in the adjunct biomarker study underwent "non-prep" (i.e., with no preceding bowel-cleansing preparation or procedure) biopsies of normal-appearing rectal mucosa at baseline and at a year 1 follow-up visit. Six approximately 1 mm thick biopsy specimens were taken from the mucosa of a valve or fold in the rectum 10 cm above the level of the external anal aperture through a short rigid proctoscope using a jumbo cup flexible biopsy forceps mounted on a semi-rigid rod. Biopsies were placed onto a strip of bibulous paper and immediately placed in normal saline, oriented, transferred to 10% normal buffered formalin for 24 hours, and then transferred to 70% ethanol. Then, within a week, the biopsies were processed and embedded in paraffin blocks (2 blocks of 3 biopsies per participant, per biopsy visit). A total of 10 potential pre-neoplastic molecular phenotypic biomarkers of risk for colorectal neoplasms, including APC, β-catenin, and E-cadherin, were measured in the biopsies using automated immunohistochemistry with image analysis.

Immunohistochemistry protocol

Five slides with 3 levels of 3 μm-thick biopsy sections taken 40 μm apart were prepared for each antigen, yielding a total of 15 levels for each antigen. To uncover the epitope, heat-mediated antigen retrieval was used: slides were placed in a preheated Pretreatment Module (Lab Vision Corp., CA) with 100x Citrate Buffer pH 6.0 (DAKO S1699, DAKO Corp., Carpinteria, CA) and steamed for 40 minutes. Then, slides were placed in a DakoCytomation Autostainer Plus System automated immunostainer and immunohistochemically processed using a labeled streptavidin-

biotin method (LSAB2 Detection System [DAKO K0675] and a monoclonal antibody to each biomarker (for APC, Oncogene OP80 at a concentration of 1:50; for β-catenin, BD Pharmingen [formerly Transduction Laboratories 610154], at a concentration of 1:300; for E-cadherin, Zymed 33-4000 at a concentration of 1:50). For each participant, baseline and follow-up biopsy slides were stained in the same batch, and each staining batch included a balance of participants from each treatment group. The slides were not counterstained. After staining, the slides were coverslipped with a Leica CV5000 Coverslipper (Leica Microsystems, Inc., IL). In each staining batch of slides, positive and negative control slides were included. The control tissues used were normal colon tissue for APC, normal tonsil for β-catenin, and breast adenocarcinoma for E-cadherin.

Protocol for quantifying labeling densities of immunohistochemically-detected biomarkers in normal colon crypts ("scoring")

The basic scoring method used to describe and quantify various characteristics of the labeled antigens in the colon crypts was an image analysis scoring procedure for antigens that were labeled with a wide range of intensities in gradient distributions along the crypt axis. A scorable crypt was defined as an intact crypt extending from the muscularis mucosa to the colon lumen.

A quantitative image analysis method ("scoring") was used to evaluate detected levels of the biomarkers in colon crypts, as depicted in Figure 1. The major equipment and software for the image analysis procedures were: Scanscope CS digital scanner (Aperio Technologies, Inc., CA), computer, 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, then, electronic images were 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. 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 equally spaced segments with the average widths of normal colonocytes. Finally, the program measured the background-corrected optical density of the biomarker labeling across the entire hemicrypt as well as within each segment. All resulting data were automatically transferred into the MySQL database. 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 > 0.90 for APC, β-catenin, and E-cadherin.

Statistical analysis

Our main analyses were to assess changes in APC, β-catenin, and E-cadherin expression after randomization in the treatment groups that received vitamin D relative to those that did not ("vitamin D vs. no vitamin D"), in the treatment groups that received calcium relative to those that did not ("calcium vs. no calcium"), and in those that received calcium plus vitamin D relative to those that received only calcium ("calcium + vitamin D vs. calcium"). To evaluate distinct functional zones of crypts, measures of crypt biomarkers included the whole crypt, the upper 40% of the crypts (differentiation zone), the lower 60% of the crypts (proliferation zone), and the ratio of the upper 40% of crypts to the whole crypt (Φh). An APC/β-catenin score was calculated by dividing an individual's APC expression in the upper 40% of crypts by their β-catenin expression in the upper 40% of crypts.

Treatment groups were assessed for comparability of characteristics at baseline and at final follow-up by chi square test for categorical variables and ANOVA or t-test for continuous variables. Treatment effects were evaluated by assessing the differences in the transformed APC, β-catenin, and E-cadherin expression from baseline to the final follow-up between participants in the treatment group of interest and those in the comparison group using a general MIXED linear model. The model included the intercept, follow-up visit effects, time, treatment group, and the interaction of treatment with time. The calcium analyses included only participants randomized to calcium (i.e., none of the 2-arm study participants were included). Potential confounders included current smoking status, non-aspirin non-steroidal anti-inflammatory drug (NSAID) use, multi-vitamin use, and physical activity measured as metabolic equivalent of task (MET). The analyses were also conducted stratified on sex, body mass index (BMI), age, NSAID use, and total fat intake, where NSAID use was categorized as infrequent (less than once a week) and frequent (at least once a week) and BMI, age, and total fat intake were categorized as below and above the sex-specific medians. Because all measurements were in optical density, to provide perspective on the magnitudes of the estimated treatment effects, relative treatment effects were calculated (relative effect = [(treatment group follow-up)/(treatment group baseline)]/[('placebo' group follow-up)/('placebo' group baseline)]. The interpretation of a relative effect is similar to that for OR; for example, a relative effect of 1.3 would indicate that the biomarker of interest increased about 30% more in the active treatment group relative to 'placebo' group. In all analyses of randomized treatments, participants were retained in their originally assigned treatment group, regardless of adherence to study treatment and procedures. All statistical analyses were conducted using SAS 9.4 statistical software (SAS Institute Inc.). A P value ≤ 0.05 (2-sided) was considered statistically significant.

Results

Selected baseline characteristics of the adjunct biomarker study participants are presented in Table 1. The mean age of study participants was 59 years, 46% were men, and 79% were white. Most participants were high school graduates, non-current smokers, and overweight. There were significant differences in physical activity, which was measured in metabolic equivalent of task (MET) minute, and dietary fiber intake among the treatment groups.

For the adjunct biomarker study, during the first year after randomization, 76% of participants reported taking 80% or more of their study tablets. There was a net increase in serum 25(OH)D of 10.87 (SD = 9.57) ng/ml at year 1 for subjects randomized to vitamin D vs. no vitamin D.

The estimated effects of the study interventions on the expression of the three biomarkers are presented in Table 2 and described below. Adjustment for factors on which the treatment groups differed at baseline did not materially affect the estimated treatment effects, so only the unadjusted results are shown.

APC

Following 1 year of treatment, APC expression increased in subjects who were randomized to vitamin D relative to those who were not by 12% (P = 0.21) in the full length of crypts, 21% (P = 0.03) in the upper 40% of crypts, 5% (P = 0.65) in the lower 60% crypts, and 4% (P = 0.16) in the Φh of crypts (Table 2). For those in the treatment groups that received calcium relative to those that did not, there were minimal non-statistically significant estimated increases in APC expression in all of the crypt parameters. For those in the treatment groups that received calcium plus vitamin D relative to those that received only calcium, APC increased by 19% (P = 0.12) in the full length of crypts, 27% (P = 0.03) in the upper 40% of crypts, 13% (P = 0.32) in the lower 60% of crypts, and 4% (P = 0.31) in the Φh of crypts.

β-catenin

For vitamin D vs. no vitamin D, β-catenin expression decreased by 3% (P = 0.41), 4% (P = 0.28), and 2% (P = 0.58) in the full length, the upper 40%, and the lower 60% of the crypts, respectively (Table 2). The estimated treatment effects for vitamin D + calcium vs. calcium on the three crypt parameters were identical to those for vitamin D vs. no vitamin D. For calcium vs. no calcium there were non-statistically significant increases in β-catenin expression of 6 – 7% in the three crypt parameters. None of the treatments appeared to materially affect the Φh of crypts.

E-cadherin

For vitamin D vs. no vitamin D, E-cadherin expression increased by 7% (P = 0.35) in the full length of crypts, 3% (P = 0.66) in the upper 40% of crypts, and 10% (P = 0.22) in the lower 60% of crypts (Table 2). For vitamin D + calcium vs. calcium, E-cadherin expression increased by 12% (P = 0.21) in the full length of crypts, 9% (P = 0.38) in the upper 40% of crypts, and 15% (P = 0.14) in the lower 60% of crypts. For calcium vs. no calcium, E-cadherin expression increased by 8% (P = 0.31) in the full length of crypts, 12% (P = 0.14) in the upper 40% of crypts, and 5% (P = 0.55) in the lower 60% of crypts. None of the treatments appeared to materially affect the Φh of crypts.

APC/β-catenin score

For vitamin D vs. no vitamin D, the APC/β-catenin score increased by 28% (P = 0.02), for calcium vs. no calcium it increased by 1% (P = 0.88), and for vitamin D + calcium vs. calcium by 35% (P = 0.01) (Table 2).

Stratified analyses

The sample size for stratified analyses was small. However, for vitamin D vs. no vitamin D and vitamin D + calcium vs. calcium, APC expression and the APC/β-catenin score tended to be

higher among subjects who took a non-aspirin NSAID ≥ 1/week and among those with a higher total fat intake (Table 3).

Discussion

Our findings suggest that vitamin D, alone or in combination with calcium, may modify APC and E-cadherin, and to a lesser extent β-catenin, expression in the normal appearing colorectal mucosa of humans in directions hypothesized to reduce risk for colorectal neoplasms. Our findings also suggest that calcium may favorably modify (i.e., increase) E-cadherin expression, but that it may not materially affect APC or β-catenin expression. The results of our stratified analyses suggested that the effects of vitamin D, alone or in combination with calcium, on the expression of APC, β-catenin, and E-cadherin may be stronger in participants who regularly take an NSAID or have higher total fat intake.

APC, β-catenin, and E-cadherin are appealing candidates for being treatable pre-neoplastic biomarkers of risk for colorectal adenomas because malfunctioning of the APC/β-catenin pathway is a common and early event in colorectal carcinogenesis.[9] In the normal colorectal mucosa, APC protein functions to degrade β-catenin, the effector of the WNT signaling pathway that controls the coordinated expansion and differentiation of the intestinal crypt stem cell. The WNT signaling pathway is normally inactive, but impaired APC expression can result in WNT signaling through stabilization of nuclear β-catenin, thus promoting cell proliferation and inhibiting differentiation. E-cadherin may antagonize the APC/β-catenin pathway by sequestering β-catenin at cell adhesion junctions.[11]**Error! Bookmark not defined.** In the normal colorectal mucosa, APC, β-catenin, and E-cadherin are all strongly expressed; during the adenoma-carcinoma sequence, APC and E-cadherin expression markedly decreases and β-catenin expression increases.[11] An APC/β-catenin score has also been suggested to be a modifiable predictor of risk for colorectal adenomas because it may represent the potential of β-catenin to

translocate to the nucleus and promote proliferative signaling.[10] It was found that the APC/β-catenin score was statistically significant lower in the normal colorectal mucosa of sporadic colorectal adenoma patients than in the normal colorectal mucosa of healthy controls.[10] APC, β-catenin, and E-cadherin expression and the APC/β-catenin score may be modifiable because they are associated with lifestyle and dietary risk factors for colorectal neoplasms.[12]

The etiology of CRC is heavily influenced by modifiable lifestyle and dietary factors, and vitamin D and calcium are two promising chemopreventive agents against colorectal adenomas. Findings from CRC cell line studies indicate that calcium and $1,25(OH)_2D$ upregulate E-cadherin production and promote a shift in β-catenin distribution from the nucleus and cytoplasm to the cell membrane.[13,14] The typical "Western" diet was found to induce increased β-catenin expression and decreased APC expression when fed to wild-type mice; however, intake of a "Western" diet with increased dietary calcium and vitamin D decreased β-catenin expression, but had no significant effect on APC expression.[15]

The only previously reported clinical trial of the effects of calcium and/or vitamin D on the APC/β-catenin pathway was conducted by our group.[12] In that trial 92 sporadic colorectal adenoma patients were randomized to calcium 2,000 mg/day and/or vitamin D 800 IU/day over six months. In the vitamin D_3-supplemented group relative to placebo, the proportion of APC in the upper 40% of crypts (Φh APC) increased 21% (p=0.01), β-catenin decreased 12% (p=0.18), E-cadherin increased 72% (p=0.03), and the APC/β-catenin score increased 31% (p=0.02). In the calcium-supplemented group Φh APC increased 10% (p=0.12), β-catenin decreased 15% (p=0.08), and the APC/β-catenin score increased 41% (p=0.01). In the calcium/vitamin D_3 supplemented group β-catenin decreased 11% (p=0.20), E-cadherin increased 51% (p=0.08), and the APC/β-catenin score increased 16% (p=0.26). As can be seen, the results for the effects of vitamin D and/or calcium on APC, E-cadherin, and the APC/β-catenin score in the two trials were similar in most, but not all, respects. In contrast to the previous trial, the results in the present trial

in relation to β-catenin alone were close to the null. In addition, in the present study, unlike in the previous trial but consistent with our hypothesis and what has been reported in some studies,[16,17] the estimated treatment effects of vitamin D plus calcium was greater than that of either the calcium or vitamin D alone in increasing APC and the APC/β-catenin score. It was also previously reported that vitamin D together with calcium may enhance treatment effect of vitamin D and calcium alone on colorectal mucosa markers of apoptosis and differentiation.[18] The reason(s) for the differences in findings between the two studies in relation to β-catenin are unclear, but could be due to the different intervention agent doses, study durations, and study populations, and, considering the small sample sizes, may have been due to chance.

In our stratified analyses, we found that the estimated effects of supplemental vitamin D, alone or in combination with calcium, on APC expression and the APC/β-catenin score tended to be a little stronger among participants who regularly took an NSAID or who had higher intakes of total fat. The sample size for the stratified analyses was small and the results were not statistically significant and thus may have been strictly due to chance. However, there is some plausibility to the findings. NSAID use is consistently reported to reduce risk of colorectal neoplasms, presumably mostly via reducing COX-2 expression, which impacts the APC/ β-catenin pathway.[19] When vitamin D binds to its receptor, it can upregulate CYP3A4, which catabolizes the secondary bile acid, lithocholic acid, which can prevent its cytotoxicity and thus a secondary inflammatory response.[20] This suggests that vitamin D and NSAIDs together may reduce inflammation and increase APC expression. Higher intakes of total fat are directly associated with risk of colorectal neoplasms, presumably via increased production of cytotoxic, mitogenic bile acids.[21] This suggests that vitamin D may be most effective in circumstances in which fat intake is sufficiently high to produce the bile acids that vitamin D can affect via the mechanism described above.

This study had several limitations and strengths. The primary limitation of this study was the small sample size, which increased the role of chance observations. Despite our limited sample size, we found statistically significant effects of vitamin D plus calcium on APC expression and the APC/β-catenin score, although given the small sample size these results should be interpreted cautiously. We only examined the rectal mucosa and therefore treatment effects on other parts of the colon are unknown. Also, we only assessed the protein expression of selected biomarkers but not the protein activity and therefore could not correlate changes in expression with changes in protein activity. The strengths of the study include the following: (i) the high protocol adherence by study participants, and (ii) the automated immunostaining and novel image analysis software to quantify the crypt distribution of the expression of APC, β-catenin, and E-cadherin, which leads to high biomarker measurement reliability.

In summary, the results of this randomized, double-blind, placebo-controlled clinical trial provide human *in vivo* evidence that supplemental vitamin D, alone or in combination with calcium, may increase APC and E-cadherin expression and the APC/β-catenin score and decrease β-catenin expression in the normal colorectal mucosa of sporadic colorectal adenoma patients. These results provide further support that APC and β-catenin expression, the APC/β-catenin score, and E-cadherin expression may be modifiable pre-neoplastic biomarkers of risk for colorectal neoplasms and that further, larger investigations are needed. Our results also support further investigation of vitamin D as a chemopreventive agent against colorectal neoplasms.

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Figure 1. Quantitative image analysis. A, finding and tracing the hemicrypt; B, automated sectioning and quantification of β-catenin labeling optical density.

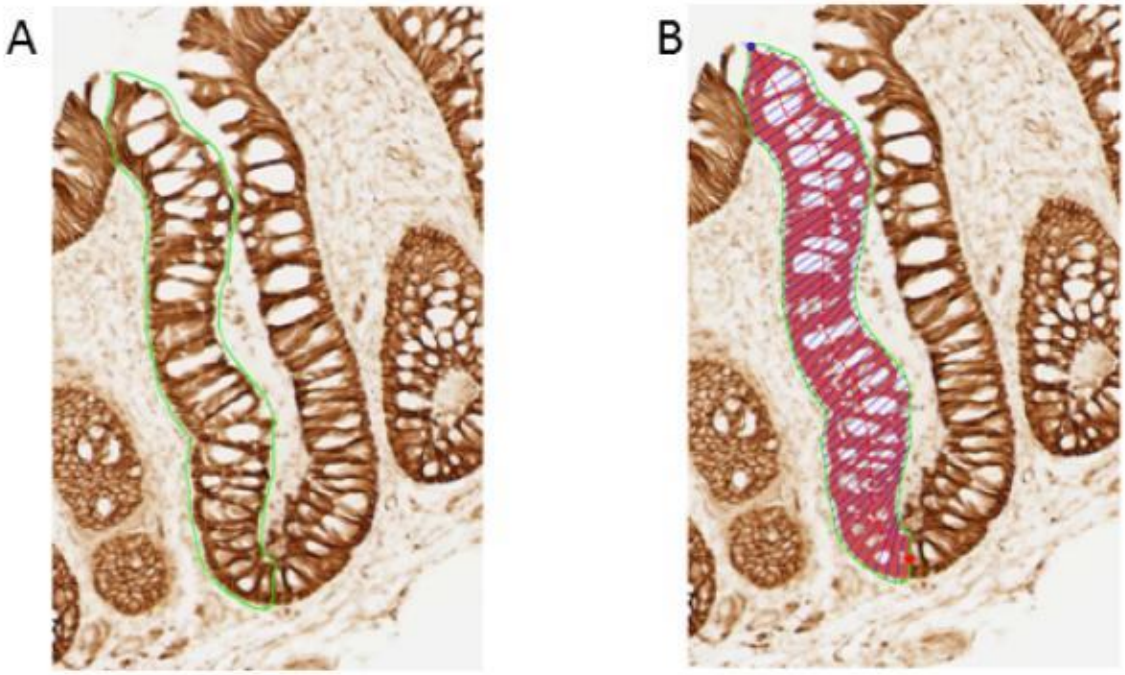

Table 1. Selected baseline characteristics of the adjunct biomarker study participants, according to treatment assignment (n = 104)[a]

Characteristics	Treatment Assignment							
	Randomization to Vitamin D and to Calcium					Randomization to Vitamin D only		
	Placebo (n = 12)	Calcium (n = 16)	Vitamin D (n = 17)	Calcium + Vitamin D (n = 17)	P value[b]	Placebo (n = 23)	Vitamin D (n = 19)	P value[c]
Demographics, medical history, habits, anthropometrics								
Age, years	59.9 (7.2)	59.9 (6.5)	59.2 (7.8)	57.7 (7. 1)	0.79	58.2 (5.3)	59.2 (7.3)	0.17
Male (%)	75	81	71	82	0.87	0	0	--
White (%)	83	75	71	94	0.42	70	84	0.57
≥ High school (%)	92	63	88	82	0.24	91	74	0.21
Take non-aspirin NSAID[d] regularly[e]	33	44	24	29	0.69	26	32	0.74
Current smoker (%)	25	6	0	6	0.11	0	16	0.08
Alcohol intake, drinks/day	0.7 (0.7)	0.8 (1.0)	0.9 (1.0)	0.9 (0.9)	0.92	0.5 (1.0)	0.3 (0.5)	0.01
Take multivitamin (%)	42	81	47	65	0.11	70	89	0.15
Physical activity, MET[f] min./wk.[g]	1,620 (1,195)	2,128 (2,378)	2,782 (2,764)	3,875 (2,424)	0.06	1,458 (1,235)	3,021 (3,469)	< .0001
BMI, kg/m^2	29.4 (4.9)	32.3 (7.6)	28.7 (5.5)	30.0 (4.5)	0.32	29.7 (5.6)	27.5 (4.7)	0.46
Dietary intakes								
Total energy intake, kcal/d[h]	1,314 (381)	1,737 (556)	1,437 (527)	1,613 (550)	0.18	1,254 (549)	1,429 (595)	0.33
Total fat, gm/d[h]	57.1 (22.3)	68.9 (25.6)	60.5 (27.3)	62.6 (27.2)	0.70	50.3 (25.9)	61.5 (36.1)	0.25
Dietary fiber, gm/d[h]	9.5 (4.1)	15.8 (5.6)	13.7 (6.2)	15.6 (5.5)	0.03	13.8 (5.4)	17.2 (5.0)	0.04
Total calcium[i], mg/d[j]	715.3 (455.4)	894.5 (263.9)	671.3 (278.3)	667.1 (254.7)	0.14	995.6 (97.6)	1,232.3 (562.9)	0.20
Serum levels								
Circulating 25(OH)D, ng/ml	22.4 (8.2)	24.5 (13.4)	23.1 (8.7)	22.5 (6.5)	0.93	24.8 (8.9)	26.5 (9.6)	0.71

[a]Data are given as means (SD) unless otherwise specified.

[b]By Fisher's Exact test for categorical variables, and ANOVA for continuous variables.

[c]By Fisher's Exact test for categorical variables, and t-test for continuous variables.

[d]Nonsteroidal anti-inflammatory drug.

[e]At least once a week.

[f]Metabolic equivalent of task.

[g]One missing value in the vitamin D group, 2-arm study.

[h]Two missing values in the placebo group, 4-arm; 1 missing value in the calcium group, 4-arm.

[i]Dietary plus supplemental calcium intake.

[j]Two missing values in the placebo group, 4-arm; 1 missing value in the calcium group, 4-arm; 1 missing value in the vitamin D group, 4-arm; 6 missing values in the placebo group, 2-arm; 1 missing value in the vitamin D group, 2-arm.

Table 2. Expression of APC, β-catenin, E-cadherin, and the APC/β-catenin score[a] in the normal-appearing colorectal mucosa of the adjunct biomarker study participants (n=104)

Treatment group	Baseline				1-year follow-up				Absolute Rx effect			Relative effect[d]
	n	Mean	SE	P	n	Mean	SE	P	Rx effect[b]	SE	P[c]	
APC												
Whole crypts												
Vitamin D placebo	51	2,607	194		51	2,098	149					
Vitamin D	53	2,419	173	0.47	53	2,177	145	0.71	266	209	0.21	1.12
Calcium placebo	29	2,583	244		29	2,008	194					
Calcium	33	2,858	212	0.40	33	2,333	148	0.18	49	265	0.85	1.05
Calcium	39	2,524	224		39	2,041	164					
Vitamin D + Calcium	36	2,440	210	0.79	36	2,348	183	0.21	391	249	0.12	1.19
Upper 40% of crypts												
Vitamin D placebo	51	1,106	89		51	867	68					
Vitamin D	53	929	72	0.13	53	881	73	0.89	190	86	0.03	1.21
Calcium placebo	29	1,040	120		29	813	100					
Calcium	33	1,216	92	0.24	33	986	70	0.15	-2	115	0.99	1.04
Calcium	39	1,050	97		39	835	73					
Vitamin D + Calcium	36	959	88	0.49	36	966	88	0.25	221	101	0.03	1.27
Lower 60% of crypts												
Vitamin D placebo	51	1,339	110		51	1,101	88					
Vitamin D	53	1,356	108	0.91	53	1,171	77	0.55	53.3	118	0.65	1.05
Calcium placebo	29	1,395	140		29	1,073	104					
Calcium	33	1,462	131	0.73	33	1,201	88	0.35	60.9	145	0.68	1.07
Calcium	39	1,322	131		39	1,084	99					
Vitamin D + Calcium	36	1,338	131	0.93	36	1,244	99	0.26	144.5	143	0.32	1.13
Φh[e]												
Vitamin D placebo	51	0.42	0.02		51	0.41	0.02					
Vitamin D	53	0.37	0.02	0.03	53	0.38	0.02	0.16	0.02	0.01	0.16	1.04
Calcium placebo	29	0.38	0.02		29	0.37	0.03					
Calcium	33	0.43	0.02	0.07	33	0.43	0.02	0.05	0.01	0.02	0.49	1.03
Calcium	39	0.41	0.02		39	0.42	0.02					
Vitamin D + Calcium	36	0.38	0.02	0.15	36	0.39	0.02	0.34	0.01	0.01	0.31	1.04
β-catenin[h]												
Whole crypts												
Vitamin D placebo	51	10,517	341		51	10,990	356					
Vitamin D	52	10,728	366	0.68	52	10,868	370	0.81	-332	402	0.41	0.97
Calcium placebo	29	10,882	489		29	10,872	427					
Calcium	32	10,922	420	0.95	32	11,591	514	0.29	680	588	0.25	1.06
Calcium	39	10,477	376		39	11,080	398					
Vitamin D + Calcium	35	10,572	454	0.87	35	10,807	504	0.67	-368	469	0.43	0.97
Upper 40% of crypts												
Vitamin D placebo	51	3,805	128		51	4,011	139					
Vitamin D	52	3,915	140	0.56	52	3,955	137	0.78	-166	153	0.28	0.96
Calcium placebo	29	3,900	188		29	3,941	165					
Calcium	32	4,007	173	0.68	32	4,318	201	0.16	269	227	0.24	1.07
Calcium	39	3,818	140		39	4,056	154					
Vitamin D + Calcium	35	3,875	175	0.80	35	3,935	186	0.62	-178	185	0.34	0.96

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Treatment group	Baseline				1-year follow-up				Absolute Rx effect			Relative effect[d]
	n	Mean	SE	P	n	Mean	SE	P	Rx effect[b]	SE	P[c]	
Lower 60% of crypts												
Vitamin D placebo	51	6,355	207		51	6,585	212					
Vitamin D	52	6,423	219	0.82	52	6,514	227	0.82	-138	248	0.58	0.98
Calcium placebo	29	6,616	295		29	6,533	261					
Calcium	32	6,520	237	0.80	32	6,840	302	0.45	403	359	0.27	1.06
Calcium	39	6,298	229		39	6,631	241					
Vitamin D + Calcium	35	6,303	269	0.99	35	6,473	304	0.68	-164	283	0.56	0.98
Φh[e]												
Vitamin D placebo	51	0.36	0.00		51	0.36	0.00					
Vitamin D	52	0.36	0.00	0.55	52	0.36	0.00	0.89	0.00	0.01	0.65	0.99
Calcium placebo	29	0.36	0.01		29	0.36	0.01					
Calcium	32	0.37	0.00	0.26	32	0.37	0.00	0.15	0.00	0.01	0.77	1.01
Calcium	39	0.36	0.00		39	0.37	0.00					
Vitamin D + Calcium	35	0.37	0.00	0.88	35	0.36	0.00	0.84	0.00	0.01	0.77	1.00
E-cadherin[i]												
Whole crypts												
Vitamin D placebo	46	4,700	210		46	4,603	193					
Vitamin D	50	4,665	264	0.92	50	4,888	275	0.41	320	343	0.35	1.07
Calcium placebo	27	5,165	398		27	4,753	254					
Calcium	31	4,568	233	0.19	31	4,533	238	0.53	376	365	0.31	1.08
Calcium	34	4,571	217		34	4,562	229					
Vitamin D + Calcium	35	4,417	273	0.66	35	4,933	364	0.39	525	411	0.21	1.12
Upper 40% of crypts												
Vitamin D placebo	46	1,727	83		46	1,771	74					
Vitamin D	50	1,740	91	0.92	50	1,842	105	0.59	59	133	0.66	1.03
Calcium placebo	27	1,918	139		27	1,794	88					
Calcium	31	1,708	89	0.20	31	1,784	100	0.94	200	134	0.14	1.12
Calcium	34	1,697	91		34	1,776	89					
Vitamin D + Calcium	35	1,627	92	0.59	35	1,850	142	0.67	144	163	0.38	1.09
Lower 60% of crypts												
Vitamin D placebo	46	2,792	126		46	2,641	117					
Vitamin D	50	2,734	169	0.79	50	2,840	162	0.33	257	209	0.22	1.10
Calcium placebo	27	3,043	255		27	2,770	168					
Calcium	31	2,678	141	0.20	31	2,550	134	0.31	144	240	0.55	1.05
Calcium	34	2,694	124		34	2,590	134					
Vitamin D + Calcium	35	2,611	176	0.70	35	2,875	209	0.26	368	244	0.14	1.15
Φh[e]												
Vitamin D placebo	46	0.37	0.01		46	0.39	0.01					
Vitamin D	50	0.38	0.01	0.14	50	0.38	0.01	0.18	-0.02	0.01	0.03	0.95
Calcium placebo	27	0.37	0.01		27	0.38	0.01					
Calcium	31	0.37	0.01	0.99	31	0.39	0.01	0.20	0.01	0.01	0.33	1.03
Calcium	34	0.37	0.01		34	0.39	0.01					
Vitamin D + Calcium	35	0.37	0.01	0.66	35	0.37	0.01	0.03	-0.02	0.01	0.08	0.95

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Treatment group	Baseline				1-year follow-up				Absolute Rx effect			Relative effect[d]
	n	Mean	SE	P	n	Mean	SE	P	Rx effect[b]	SE	P[c]	
APC/β-catenin score[a,h]												
Vitamin D placebo	51	0.30	0.03		51	0.19	0.01					
Vitamin D	52	0.26	0.02	0.19	52	0.21	0.02	0.66	0.06	0.03	0.02	1.28
Calcium placebo	29	0.29	0.04		29	0.22	0.03					
Calcium	32	0.32	0.03	0.60	32	0.24	0.02	0.56	-0.01	0.03	0.88	1.01
Calcium	39	0.28	0.03		39	0.21	0.02					
Vitamin D + Calcium	35	0.27	0.03	0.71	35	0.26	0.03	0.07	0.07	0.03	0.01	1.35

[a]APC/β-catenin score = APC expression in the upper 40% of crypts/β-catenin expression in the upper 40% of crypts.
[b]Rx effect (treatment effect) = [(treatment group follow-up) – (treatment group baseline)] – [(placebo group follow-up) – (placebo group baseline)].
[c]P value for difference between each active treatment group and placebo group from repeated-measures MIXED model.
[d]Relative effect = [(treatment group follow-up)/(treatment group baseline)]/[(placebo group follow-up)/(placebo group baseline)]; interpretation similar to that for OR.
[e]Φh = proportion of expression in the distribution zone (i.e., ratio of expression in upper 40% to expression in whole crypt).
[h]One subject was excluded due to missing values for the measurement of β-catenin expression.
[i]Eight subjects were excluded due to missing values for the measurement of E-cadherin expression.

Table 3. Expression of APC, β-catenin, E-cadherin, and the APC/β-catenin score[a] in the normal-appearing colorectal mucosa of the adjunct biomarker study participants according to categories of selected risk factors (n=104)

Treatment group	Baseline				1-year follow-up				Absolute Rx effect			Relative effect[d]
	n	Mean	SE	P	n	Mean	SE	P	Rx effect[b]	SE	P[c]	
Non-aspirin NSAID[e] use < 1/week												
APC												
Upper 40% of crypts												
Vitamin D placebo	34	1,127	104		34	891	73					
Vitamin D	38	946	82	0.17	38	832	89	0.61	122	103	0.24	1.11
Calcium placebo	21	894	127		21	618	91					
Calcium	21	1,347	89	0.01	21	1,106	74	0.00	36	137	0.80	1.19
Calcium	26	1,136	115		26	925	84					
Vitamin D + Calcium	25	1,038	102	0.53	25	995	110	0.62	167	123	0.18	1.18
β-catenin												
Upper 40% of crypts												
Vitamin D placebo	34	3,715	165		34	4,000	189					
Vitamin D	37	3,801	150	0.70	37	3,956	175	0.86	-130	174	0.46	0.97
Calcium placebo	21	3,634	181		21	3,797	206					
Calcium	20	3,986	201	0.20	20	4,451	282	0.07	302	258	0.25	1.07
Calcium	26	3,834	187		26	4,100	206					
Vitamin D + Calcium	24	3,791	208	0.88	24	4,003	248	0.76	-54	226	0.81	0.99
E-cadherin												
Whole crypts												
Vitamin D placebo	30	4,551	258		30	4,614	256					
Vitamin D	35	4,777	287	0.57	35	5,057	370	0.34	218	439	0.62	1.04
Calcium placebo	19	4,900	383		19	4,461	280					
Calcium	20	4,725	302	0.72	20	4,831	310	0.38	545	402	0.18	1.12
Calcium	22	4,578	284		22	4,702	325					
Vitamin D + Calcium	24	4,579	351	1.00	24	5,300	503	0.33	597	560	0.29	1.13
APC/β-catenin score[a]												
Vitamin D placebo	34	0.32	0.04		34	0.23	0.02					
Vitamin D	37	0.27	0.03	0.29	37	0.23	0.03	0.87	0.04	0.03	0.16	1.15
Calcium placebo	21	0.28	0.05		21	0.18	0.04					
Calcium	20	0.36	0.03	0.19	20	0.27	0.02	0.05	0.01	0.04	0.87	1.15
Calcium	26	0.30	0.03		26	0.23	0.02					
Vitamin D + Calcium	24	0.30	0.04	1.00	24	0.28	0.03	0.22	0.05	0.03	0.17	1.21
Non-aspirin NSAID[e] use ≥ 1/week												
APC												
Upper 40% of crypts												
Vitamin D placebo	17	1.063	174		17	819	145					
Vitamin D	15	887	154	0.46	15	1,005	122	0.34	362	160	0.03	1.47
Calcium placebo	8	1,424	243		8	1,323	177					
Calcium	12	987	187	0.17	12	776	123	0.02	-110	221	0.62	0.85
Calcium	13	878	176		13	655	131					
Vitamin D + Calcium	11	779	169	0.69	11	900	148	0.23	345	184	0.07	1.55

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Treatment group	Baseline				1-year follow-up				Absolute Rx effect			Relative effect[d]
	n	Mean	SE	P	n	Mean	SE	P	Rx effect[b]	SE	P[c]	
β-catenin												
Upper 40% of crypts												
Vitamin D placebo	17	3,983	198		17	4,032	185					
Vitamin D	15	4,197	309	0.55	15	3,952	208	0.78	-294	298	0.33	0.93
Calcium placebo	8	4,598	411		8	4,320	221					
Calcium	12	4,043	330	0.30	12	4,096	261	0.55	331	448	0.47	1.08
Calcium	13	3,787	201		13	3,969	219					
Vitamin D + Calcium	11	4,060	330	0.47	11	3,787	250	0.59	-455	314	0.16	0.89
E-cadherin												
Whole crypts												
Vitamin D placebo	16	4,980	359		16	4,582	290					
Vitamin D	15	4,405	584	0.40	15	4,493	296	0.83	485	542	0.38	1.10
Calcium placebo	8	5,794	1,005		8	5,446	482					
Calcium	11	4,283	362	0.13	11	3,991	314	0.02	55	782	0.94	0.99
Calcium	12	4,558	343		12	4,306	257					
Vitamin D + Calcium	11	4,064	415	0.37	11	4,133	267	0.65	321	516	0.54	1.08
APC/β-catenin score[a]												
Vitamin D placebo	17	0.27	0.04		17	0.20	0.03					
Vitamin D	15	0.22	0.04	0.38	15	0.26	0.03	0.28	0.11	0.04	0.02	1.57
Calcium placebo	8	0.34	0.06		8	0.32	0.05					
Calcium	12	0.25	0.05	0.29	12	0.19	0.02	0.02	-0.04	0.06	0.47	0.79
Calcium	13	0.24	0.05		13	0.16	0.03					
Vitamin D + Calcium	11	0.19	0.03	0.41	11	0.24	0.04	0.15	0.13	0.05	0.01	1.88
Total fat intake												
< median[f]												
APC												
Upper 40% of crypts												
Vitamin D placebo	26	1,124	124		26	898	96					
Vitamin D	24	959	105	0.32	24	746	95	0.27	13	110	0.91	0.97
Calcium placebo	15	1,097	164		15	748	139					
Calcium	14	1,325	112	0.27	14	1,044	96	0.10	68	147	0.65	1.16
Calcium	19	1,030	131		19	875	102					
Vitamin D + Calcium	16	1,012	143	0.93	16	838	120	0.82	-19	136	0.89	0.97
β-catenin												
Upper 40% of crypts												
Vitamin D placebo	26	3,705	186		26	4,000	219					
Vitamin D	23	3,883	140	0.46	23	4,148	231	0.64	-30	244	0.90	0.99
Calcium placebo	15	3,541	228		15	3,755	268					
Calcium	13	4,160	182	0.05	13	4,874	351	0.02	500	357	0.17	1.10
Calcium	19	3,728	208		19	4,081	250					
Vitamin D + Calcium	15	4,111	145	0.16	15	4,370	302	0.46	-94	328	0.78	0.97
E-cadherin												
Whole crypts												
Vitamin D placebo	24	4,412	308		24	4,615	254					
Vitamin D	22	4,771	341	0.44	22	4,862	260	0.50	-112	379	0.77	0.97
Calcium placebo	13	5,071	614		13	4,923	438					
Calcium	14	4,302	234	0.24	14	4,689	334	0.67	534	488	0.28	1.12
Calcium	17	4,219	270		17	4,419	275					
Vitamin D + Calcium	16	4,575	315	0.40	16	4,913	245	0.19	138	409	0.74	1.03

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Treatment group	Baseline				1-year follow-up				Absolute Rx effect			Relative effect[d]
	n	Mean	SE	P	n	Mean	SE	P	Rx effect[b]	SE	P[c]	
APC/β-catenin score[a]												
Vitamin D placebo	26	0.33	0.05		26	0.24	0.03					
Vitamin D	23	0.25	0.03	0.14	23	0.19	0.02	0.17	0.03	0.04	0.44	1.02
Calcium placebo	15	0.35	0.07		15	0.22	0.05					
Calcium	13	0.33	0.03	0.82	13	0.24	0.03	0.80	0.03	0.05	0.47	1.13
Calcium	19	0.29	0.04		19	0.22	0.03					
Vitamin D + Calcium	15	0.24	0.04	0.40	15	0.20	0.03	0.68	0.03	0.04	0.45	1.10
Total fat intake ≥ median[f]												
APC												
Upper 40% of crypts												
Vitamin D placebo	22	1,077	139		22	805	96					
Vitamin D	29	905	101	0.31	29	993	104	0.20	360	137	0.01	1.47
Calcium placebo	12	961	187		12	868	142					
Calcium	18	1,127	144	0.48	18	927	103	0.74	-108	193	0.58	0.91
Calcium	19	1,057	154		19	774	110					
Vitamin D + Calcium	20	916	113	0.46	20	1,069	124	0.08	435	145	0.00	1.59
β-catenin												
Upper 40% of crypts												
Vitamin D placebo	22	3,836	187		22	3,999	192					
Vitamin D	29	3,941	227	0.73	29	3,801	162	0.43	-303	192	0.12	0.93
Calcium placebo	12	4,247	295		12	4,172	199					
Calcium	18	3,879	279	0.39	18	3,897	211	0.38	92	297	0.76	1.02
Calcium	19	3,881	201		19	4,000	199					
Vitamin D + Calcium	20	3,698	283	0.60	20	3,608	213	0.19	-209	205	0.31	0.95
E-cadherin												
Whole crypts												
Vitamin D placebo	19	5,007	315		19	4,569	341					
Vitamin D	28	4,582	393	0.44	28	4,908	451	0.58	764	603	0.21	1.17
Calcium placebo	12	5,325	626		12	4,633	320					
Calcium	16	4,739	398	0.42	16	4,341	356	0.56	293	580	0.62	1.05
Calcium	16	4,883	343		16	4,661	392					
Vitamin D + Calcium	19	4,284	434	0.30	19	4,950	647	0.72	888	722	0.23	1.21
APC/β-catenin score[a]												
Vitamin D placebo	22	0.28	0.03		22	0.20	0.02					
Vitamin D	29	0.26	0.04	0.76	29	0.28	0.03	0.07	0.09	0.04	0.02	1.47
Calcium placebo	12	0.24	0.05		12	0.22	0.04					
Calcium	18	0.31	0.05	0.28	18	0.24	0.03	0.63	-0.05	0.05	0.33	0.85
Calcium	19	0.27	0.04		19	0.19	0.02					
Vitamin D + Calcium	20	0.27	0.05	0.82	20	0.31	0.04	0.01	0.11	0.04	0.01	1.57

[a]APC/β-catenin score = APC expression in the upper 40% of crypts/β-catenin expression in the upper 40% of crypts.
[b]Rx effect (treatment effect) = [(treatment group follow-up) – (treatment group baseline)] – [(placebo group follow-up) – (placebo group baseline)].
[c]P value for difference between each active treatment group and placebo group from repeated-measures MIXED model.
[d]Relative effect = [(treatment group follow-up)/(treatment group baseline)]/[(placebo group follow-up)/(placebo group baseline)]; interpretation similar to that for OR.
[e]Nonsteroidal anti-inflammatory drug.
[f] Sex-specific median for total fat intake: 61.25 gm/d for men, and 47.00 gm/d for women.

Summary, Public Health Implication, Possible Future Directions

Overall, the results from this clinical trial suggest that supplemental vitamin D, alone or in combination with calcium, may modify APC and E-cadherin, and to a lesser extent β-catenin, expression in the normal appearing colorectal mucosa of humans in directions hypothesized to reduce risk for colorectal neoplasms; calcium may favorably modify E-cadherin expression; and the effects of vitamin D and calcium on the expression of APC, β-catenin, and E-cadherin may be stronger in those who frequently take a NSAID or who have higher total fat intakes.

Taken together with our previous findings, this study supports further investigation of (i) APC, β-catenin, E-cadherin, and the APC/β-score as potential treatable biomarkers of risk for colorectal neoplasms, and (ii) vitamin D and calcium as chemopreventive agents against colorectal neoplasms. Further proposed research includes a clinical trial with biopsies of normal-appearing colorectal mucosa taken from multiple levels of colon; a dose-response trial to investigate a possible dose-response relationship between vitamin D and calcium supplementation with APC, β-catenin, and E-cadherin expression; a larger clinical trial to investigate the effects of supplemental calcium and/or vitamin D on APC, β-catenin, and E-cadherin expression and whether this modulation is associated with decreased recurrence of sporadic colorectal adenomatous polyps; and a trial to investigate whether biomarker responses to treatments vary according to vitamin D receptor genotype.