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Effects of Supplemental Vitamin D and Calcium Alone or in Combination on Expression of Claudin-1 in the Normal Colorectal Mucosa of Colorectal Adenoma Patients.

By

Ferdous Akter Jahan

Masters of Public Health

Epidemiology

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Veronika Fedirko, PhD

Committee Chair

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An abstract of
A thesis submitted to the Faculty of the
Rollins School of Public Health of Emory University
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Effects of Supplemental Vitamin D and Calcium Alone or in Combination on Expression of Claudin-1 in the Normal Colorectal Mucosa of Colorectal Adenoma Patients.

Abstract

Background: Dysfunction of the tight junction proteins (TJs) is considered to be one of the initial events in colorectal carcinogenesis. Evidence from animal, cell culture, and human studies strongly support calcium and vitamin D as being promising chemopreventive agents against colorectal neoplasms. Therefore, the aim of our study was to test whether calcium and/or vitamin D has an effect on claudin-1 (CLDN1) expression in the normal colorectal mucosa.

Methods: We tested calcium (1200 mg daily) and/or vitamin D3 (1000 IU daily) effects on the expression of CLDN1 in the normal-appearing colorectal mucosa of 105 sporadic colorectal adenoma patients, nested within a large randomized, double-blind, placebo controlled, partial 2 x 2 factorial chemopreventive clinical trial. We assessed CLDN1 expression at baseline and 1-yr follow up using standardized, automated immunohistochemistry and quantitative image analysis.

Results: Following 1-yr of treatment, in the calcium only group CLDN1 expression was increased by 14% (p=0.171) in the full length of the crypts compared to placebo. There was no change in expression of CLDN1 in the vitamin D only group and minimal non-significant increase in the combination group compared to placebo. Treatment effects of calcium were suggestively stronger among women (54%, p=0.01) compared to men, among individuals with positive family history of CRC (4-fold, p=0.02) compared to those without family history of CRC, and among individuals who had baseline serum 25(OH)D levels below median (29%, p=0.04) compared to those who had at or above median level of serum 25(OH)D. Baseline CLDN1 expression was 34% higher (p=0.02) in obese compared to normal weight, 18% higher (p=0.06) in regular aspirin users compared to aspirin non-users, and 26% lower (p=0.09) in participants with family history of CRC compared to those without family history of CRC.

Conclusion: Calcium may modify the expression of CLDN1 in the normal-appearing colorectal epithelium in humans. This finding further supports calcium as a chemopreventive agent against colorectal neoplasms.

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Table of Contents

Background

Colorectal cancer (CRC) is the third most common cancer in the world and second most common cause of cancer-related death in both men and women in the United States [1]. Overall, the lifetime risk of developing CRC is about 1 in 21 (4.7%) for men and 1 in 23 (4.4%) for women in U.S.[1, 2]. There are large geographical differences in the distribution of CRC throughout the world. It has been much more common in high income countries but is now increasing in middle and low income countries. Rate of CRC incidence increases with industrialization and urbanization [3]. Furthermore, migrants from lower- to higher-risk countries tend to acquire the CRC risk of their adopted countries within 1-2 generations which indicates that CRC is highly sensitive to environmental factors [4]. CRC is one of the major cancers for which modifiable causes may be readily identified, and a large proportion of cases are theoretically preventable [5]. Existing epidemiological studies have reported that among modifiable risk factors, red and processed meat, alcohol, smoking, obesity and type-2 diabetes were associated with an increased risk of CRC, whereas folate, calcium, vitamin D, aspirin, and physical activity were associated with decreased risk of CRC [6, 7]. Non-modifiable risk factors showing a positive association include age, family history of CRC or adenomatous polyp, personal history of adenomatous polyp, history of inflammatory bowel disease, having an inherited syndrome (e.g. Familial adenomatous polyposis, Lynch syndrome, Turcot syndrome), and racial or ethnic background [7]. It is estimated that about 47% of the US CRC cases can be prevented by diet and lifestyle modifications [8]. There is strong human observational evidence for an association between diet and CRC [9-11]. In a large pooled analysis, consisting of 13 large prospective cohort studies found that dietary fiber intake was inversely associated with the risk of colorectal cancer in age-adjusted analyses. However, after accounting for other dietary risk factors, high dietary fiber intake was not associated with a reduced risk of CRC [12]. Total fat, saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids, and cholesterol

were not found to be associated with CRC risk [13] and one meta-analysis conducted recently found no relationship between polyunsaturated fatty acid intake and colon cancer risk [14]. Until 2011, 263 published articles from cohort and randomized controlled clinical trials (RCT) investigating dietary factors (*e.g.* dietary fiber, sources of fiber, fruit intake, folate) in relation to CRC risk were identified by Continuous Update Project (CPU) of World Cancer Research Fund (WCRF). Though several modifiable risk factors have been identified for CRC but data related to chemopreventive agents for CRC is not well established until now.

Adenomas are gland like growths which develop on the mucous membrane that lines the large intestine, and are recognized as the precursor lesions in majority of cases of CRC [15]. In 2016, adenomas of the colon were estimated to be present in 20 to 53% of the U.S. population older than 50 years of age, with a prevalence of 3.4 to 7.6% for advanced histological features and 0.2 to 0.6% for adenocarcinoma. An individual with a history of adenomas has an increased risk of developing CRC compared to individuals with no previous history of adenomas. A long latency period, estimated at 5 to 10 years, is usually required for the development of malignancy from adenomas [16]. CRC develops through an ordered series of events beginning with the transformation of normal colonic epithelium to an adenomatous intermediate and then ultimately adenocarcinoma, the so-called “adenoma-carcinoma sequence”[17].

Vitamin D and Colorectal Neoplasms Risk

The mammalian form of vitamin D3 (cholecalciferol), a fat-soluble prohormone, is generated endogenously in the skin by ultraviolet light-mediated metabolism of the precursor sterol 7-dehydrocholesterol or is obtained, to a lesser extent, from nutritional sources, Vitamin D2 (ergocalciferol) [18]. Both forms are converted to 25-hydroxyvitamin D [25(OH)D] in the liver. 25(OH)D then travels through the blood to the kidneys, where it is further modified to 1,25-

dihydroxyvitamin D, or calcitriol, the active form of vitamin D in the body. The most accurate method of evaluating a person's vitamin D status is to measure the level of 25(OH)D in the blood [19]. Whereas the primary function of 1,25-(OH)2D is to regulate calcium absorption and maintain mineral homeostasis, upon binding to its cognate receptor (vitamin D receptor [VDR]), 1,25-(OH)2D3 was shown to decrease proliferation of colonic epithelial cells and enhanced the differentiation of CRC cells as well as altered the transcription of a large number of genes involved in inhibiting carcinogenesis [20]. Stress-activated protein kinase JNKs physically and functionally interacted with VDR and positively regulated VDR expression at transcriptional and translational levels, which influenced calcitriol mediated inhibition of colon cancer cell proliferation [21]. Other possible roles of vitamin D in reducing the risk of CRC include inhibition of cellular inflammation (both local and systemic) and oxidative stress and promotion of cell differentiation, apoptosis, and immunomodulation [22, 23].

Over two decades ago, researchers first recognized the importance of vitamin D from sunlight in preventing CRC. They observed significantly higher mortality rates from CRC in the northern and northeastern United States, compared to the southwest, Hawaii and Florida. They showed that people who had higher levels of serum vitamin D, had lower rates of colon cancer [24]. Since then multiple observational and laboratory studies have investigated the association of serum vitamin D levels and CRC risk [24]. Numerous epidemiological studies have shown that high exposure to vitamin D is associated with a lower risk of CRC. In 2011, in a meta-analysis [25] of 10 prospective cohort studies, the highest levels of circulating 25(OH)D compared with the lowest levels of circulating 25(OH)D were associated with 34% lower risk of CRC. The inverse association was found to be stronger for rectal cancer than colon cancer, although a formal test for difference between colon and rectal cancers was not statistically significant (*P* for difference = 0.20). The pooled multivariate OR was 0.77 (95% CI: 0.56–1.07) for colon cancer and 0.50 (95% CI: 0.28–0.88) for rectal cancer, when compared the highest quantile with the lowest quantile of circulating 25(OH)D levels. Another meta-analysis [26] including almost all

studies from the previous analysis conducted a dose-response analysis and showed a 4% lower CRC risk for serum 25(OH)D (RR per 100 IU/L vitamin D intake = 0.96, 95% CI: 0.94-0.97; with range of intake (midpoints) = 200-1,800 IU/L), but not with total vitamin D. Similar findings were observed in Nurses' Health Study. They found a significant inverse linear association between plasma 25(OH)D and risk of CRC (P = 0.02). Among women in the highest quintile, the OR (95% confidence interval) was 0.53 (95% CI: 0.27-1.04) compared with the lowest quintile [27]. Results of a meta-analysis conducted to evaluate the overall relationship between circulating (plasma or serum) 25(OH)D, and colorectal adenoma incidence supports the role of vitamin D in prevention of colorectal adenoma incidence and recurrence, OR= 0.70 (95% CI: 0.56-0.87) for high versus low circulating 25(OH)D levels [28]. In a prospective study, associations were investigated (followed for 11.3 years) between vitamin D status and incidence of specific types of cancers. Result were described in terms of hazard ratio (HR), and 95% CI per 10 nmol/L higher baseline vitamin D level. For CRC, there was no statistically significant association between vitamin D status and incidence of cancer (HR = 0.95; 95% CI: 0.88-1.02) [29]. There were multiple observational studies on dietary intake of vitamin D and CRC but findings were inconsistent, may be because of issues with measuring diet, low amount of vitamin D usually obtain from normal diet and main source of vitamin D is exposure to sun or supplements [30, 31].

Calcium and Colorectal Neoplasms risk

According to the World Cancer Research Fund (WCRF), among the dietary factors, calcium is a probable risk reducing agent. From an epidemiological standpoint, there have been many studies assessing the association between calcium intake and CRC risk. There are generally consistent prospective cohort evidence on inverse association between dietary calcium, total calcium (dietary and supplemental) and calcium supplements and CRC risk. The effects were apparent in both men and women. There were 17 studies investigating dietary calcium and risk of

CRC and 11 studies for dietary calcium and colon cancer. The WCRF panel found that 16 of 17 cohort studies reported decreased risk with increasing dietary calcium intake. Pooling relevant cohort studies using CRC or colon cancer as the outcome of interest gave a summary RR of 0.77 (95% CI: 0.71–0.81). The results based on pooled analysis of 10 studies using colon cancer alone were similar [RR = 0.76 (95% CI: 0.69–0.84); p = 0.70)] [30]. Using rectal cancer as the outcome of interest, in the same cohort studies, examining dietary/total calcium intake also showed an approximately 30% reduction in CRC risk [RRs = 0.72 (95% CI: 0.60–0.86)]. The evidence on milk from cohort studies is reasonably consistent, supported by stronger evidence from dietary calcium as a marker [32]. The CUP also found overall lower risk with calcium supplements and CRC from 7 studies among them 3 was cohort studies [32]. A randomized, double-blinded clinical trial tested the effects of supplemental calcium on the recurrence of colorectal adenoma [33]. Results of this study demonstrated that calcium supplementation caused a 15% reduction in the risk of recurrent colorectal adenomas (95% CI: 0.74-0.98; p=0.03). The classical hypothesis for the beneficial effects of calcium derived from an original physiochemical hypothesis by Newmark and colleagues in which they suggested that free fatty acids and bile acids in the colon may be detrimental to the colonic epithelium and is important in the initial steps of colorectal carcinogenesis. Calcium could bring bile acids and fatty acids out of solution in the colonic lumen and, thus, can reduce the cytotoxicity of these agents [34]. Supplemental calcium in the diet or drinking water has been reported to decrease the colonic epithelial hyperproliferation induced by exposure to free bile and fatty acids, enteric resection, a nutritional stress diet, and also suppressed the induction of the tumor-promotion enzyme ornithine decarboxylase. Calcium has also demonstrated an inhibitory effect on colon carcinogenesis in experimental animals [35].

Martin Lipkin's group had evaluated the effects of a Western style diet (WD) relatively high in fat contents (20%) and relatively low in vitamin D and calcium upon carcinogenic changes in the colon of mice. This represented the first demonstration of diet-induced colorectal tumor formation in the absence of a carcinogen [36]. High intakes of calcium and exposure to

vitamin D decreased oxidative stress and oxidative DNA damage in the colon, and, thus reduced risk of colorectal neoplasm in a randomized clinical trial [37]. Although calcium and vitamin D work together metabolically and both are possible protective agents against colorectal cancer, it is unknown whether they interact in colorectal carcinogenesis.

Gut Barrier, Tight Junction Proteins, and Colorectal Neoplasms Risk

Tight junctions (TJs) are multi-protein complexes that form a selectively permeable seal between adjacent epithelial cells and demarcate the boundary between apical and basolateral membrane domains of epithelium [38]. TJs limit the passage of molecules and ions through the spaces between the cells [39].They consist of a network of occludins, claudins and other proteins. The modifications of TJ barrier function and paracellular permeability are dynamically regulated by various extracellular stimuli and are closely associated with our health and susceptibility to diseases [40, 41]. Cancer cells frequently exhibit alteration in the TJ proteins. Claudins (CLDNs) are the major integral membrane proteins of TJ. CLDNs are necessary for the formation and maintenance of TJs. Altered expression of several CLDN proteins, in particular CLDN-1, -3, -4 and -7, have been linked to the development of various cancers. Recent studies have shown that they play a role in epithelial to mesenchymal transition (EMT), in the formation of cancer stem cells or tumor-initiating cells, and also in chemoresistance, suggesting that CLDNs are promising targets for the treatment of chemoresistant and recurrent tumors. Epithelial tumor cells lose TJ function, leading to the loss of cell polarity and impairment of epithelial integrity during tumorigenesis. Accordingly, loss of CLDN expression was assumed to contribute to tumor progression in association with the loss of cell adhesion [42]. CLDNs show type-specific differential expression in epithelial tumors and helpful for distinguishing different epithelial tumors from each other. CLDN overexpression or loss of expression varies among different types of cancer [43]. There is still controversy whether overexpression or loss of expression of CLDN

contributes to colorectal carcinogenesis. CLDN1 appeared to be restricted to the epithelial cells and stronger immunoreactivity indicated increased synthesis of CLDN1 in the neoplastic cells [43]. The loss of CLDN1 expression was found to be related to lymphovascular invasion, histological grade, decreased disease-free survival, and overall survival in stage 2 and 3 CRC, indicated that CLDN1 can be used as an independent predictor for recurrence and loss of CLDN1 expression and might play a role in the distortion of immune response against carcinogenesis [44]. Some studies including one *in vitro* study reported that CLDN1 mRNA levels were upregulated by matrix metalloproteinase activation in CRC, demonstrated that CLDN1 was increased in CRC [45, 46]. Adenocarcinoma tissue and paired normal mucosa specimens were resected from surgical specimens of CRC patients and analyzed to determine whether the expression of CLDN1 correlated with the clinicopathological factors and to determine the role of CLDN1 in the alteration of TJs during tumorigenesis. Results of gene cloning and construction of recombinant gene followed by cell culture and transinfection suggested that CLDN1 was significantly up-regulated in the CRC tissues [47]. The mRNA level of CLDN1 expression significantly correlated with the depth of cancer and CLDN1 mRNA level was higher in the distal site of the colon than the proximal site since the incidence of colon cancer is much higher in the former group. CLDN1 expression may have significant clinical relevance and therefore it may become a potential useful marker and therapeutic target in CRC [47]. Genetic manipulations of CLDN1 expression in colon cancer cell lines induced changes in cellular phenotype, with structural and functional changes in markers of epithelial-mesenchymal transition. Changes in CLDN1 expression demonstrated significant effects on growth of xenografted tumors and metastasis in athymic mice [48]. Increase mucosal permeability due to the adenomas and the inherent barrier defect in these mice further facilitated bacterial translocation into the mucosa to induce inflammation, which in turn promoted the tumorigenesis [49]. Gene expression profiles were used to identify CLDN1 as a promising early CRC target and results showed a 2.5-fold increase in gene expression for CLDN1 in human colonic adenomas compared to normal in cell

culture study [50]. All the colon cancer cell lines with increased expression of CLDN1 are known to carry an APC mutation, a well-known initiating factor of carcinogenesis in the colon. Supplemental Vitamin D, alone or in combination with calcium, may increase APC and, to a lesser extent, E-cadherin, expression in the normal appearing colorectal mucosa of sporadic colorectal adenoma patients [51]. 1,25(OH)2D_3 also induces the expression of the TJ protein CLDN1 in animal model [52]. With a calcium- and vitamin D-deficient diet, TJ gene expression was significantly decreased in the duodenum of the CaBP-9k (a critical transcellular protein) knockout mice. Decreased CLDN2 and CLDN15 expression in the intestine indicated that a lack of calcium absorption due to calcium and/or vitamin D deficiencies could lead to reduce TJ gene expression in mice [53]. Vitamin D was thought to be critical for the regulation of tight junction, because the levels of tight junction proteins, such as claudin, occludin and zona occludens-1, were decreased by vitamin D deprivation in animal study [53].

Vitamin D status strongly modified the effects of calcium supplementation on adenoma recurrence [54]. Calcium supplements were found to lower adenoma risk only among individuals with 25(OH)D levels above the overall median. Equally, 25(OH)D was associated with a reduced risk only among individuals randomly assigned to receive calcium. Vitamin D and calcium supplementation appeared largely to act jointly, not separately, on colorectal carcinogenesis [54]. However, the independent and or combined effects of calcium and vitamin D in human colorectal mucosa on expression of CLDN1, is not well understood.

Despite evidence from previous animal and cell culture studies along with basic science there is no reported human study regarding the effects of calcium and vitamin D alone or in combination on the expression of CLDN1 in the colorectal mucosa. Based on the previously reported studies on evidence of level of CLDN1 expression in colorectal adenoma and colonic carcinoma [55, 56] along with biological plausibility, we hypothesized that calcium and vitamin D alone or in combination, would increase CLDN1 expression in normal appearing colorectal

mucosa of previously diagnosed patients with colorectal adenoma. To address this issue, we herein report the results of a randomized, double-blind placebo-controlled, partial 2x2 factorial chemoprevention clinical trial which was designed to test the efficacy of 1-year supplemental Vitamin D and calcium, alone or in combination, to assess the expression of CLDN1 in the colon crypts of normal-appearing colorectal mucosa. The aim of our research is to identify a "treatable" phenotypic pre-neoplastic biomarker for the risk of CRC. Phenotypic biomarkers are important targets for chemoprevention since they "summarize" the results of complex interactions among genotypes, gene-gene interactions, epigenetic phenomenon, environmental exposures, and gene-environment interactions [37].

Materials and Methods

Participant Population

The Participants in this study ("adjunct biomarker study") were all participating in a large 11-center, randomized, placebo-controlled, partial 2 x 2 factorial chemoprevention clinical trial ("parent study") which was designed to test the efficacy of supplemental calcium and vitamin D, alone or in combination, over 3-5 yr on adenoma recurrence in colorectal adenoma patients [57]. Eligible participants were 45-75 yr of age and in general good health; within 4 months of study entry had a complete, clean colonoscopy during which all visible polypoid lesions were removed, at least one of which was a histologically verified neoplastic polyp ≥ 2mm in diameter; and were scheduled for a follow-up colonoscopy 3 or 5 year after the index colonoscopy. Exclusion from participation included invasive carcinoma in any colonic polyp removed, familial colonic polyposis syndrome, inflammatory bowel diseases, malabsorption syndromes, history of large bowel resection, narcotic or alcohol dependence, serum calcium outside normal range, creatinine greater than 20% above the upper limit of normal, serum 25-hydroxy vitamin D levels, [25(OH)D] <12 ng/ml or >90 ng/ml, history of kidney stones or

hyperparathyroidism, and history of osteoporosis or other medical condition that may require supplemental vitamin D or calcium. For participation in the adjunct biomarker study, additional exclusions were being unable to be off aspirin 7 d, history of bleeding disorders, or current use of an anticoagulant medication.

Clinical Trial Protocol

Details of the parent clinical trial protocol, including the recruitment yields, were previously published [57]. Briefly, for the parent study, between May 2004 and July 2008, 19,038 apparently eligible patients were identified through initial screening of colonoscopy and pathology reports; of these, 2,259 met final eligibility criteria, consented to participate, and were randomized. After the parent study was underway, funding was received for the adjunct biomarker study. For the adjunct biomarker study, near the end of the placebo run-in period, without knowledge of treatment assignment, a total of 231 apparently eligible parent study participants at two clinical centers (South Carolina and Georgia) were offered participation in the biomarker study; of these, 109 met final eligibility, signed consent, and had baseline rectal biopsied taken, and of these, sufficient rectal biopsy tissue for biomarker measurements was obtained at baseline and 1-yr follow up on 105. All participants signed a consent form at enrollment; the Institutional Review Boards at each clinic center approved the research.

At enrollment, the coordinator collected information from each parent study participant on medical history, medication and nutritional supplement use, and diet and lifestyle. Diet was assessed using the semi-quantitative Block Brief 2000 food frequency questionnaire (Nutritionquest, Berkeley, CA). After the subsequent placebo run-in period, subjects were randomly assigned to the following four treatment groups, 1200 mg/d calcium supplementation (as calcium carbonate in equal doses twice daily), 1000 IU/d vitamin D3 supplementation (500 IU

twice daily), and 1200 mg/d elemental calcium plus 1000 IU/d vitamin D supplementation ("full factorial randomization"). Women who declined to forego calcium supplementation were randomized to calcium or calcium plus vitamin D3 ('2-arm randomization"). Participants agreed to avoid taking vitamin D or calcium supplements outside the trial, although personal supplements up to 1000 IU vitamin D and/or 400mg elemental calcium were permitted from April 2008 onwards. Randomization was conducted using computer-generated random numbers with permitted blocks, and stratified by sex, clinical center, scheduled colonoscopic follow-up of 3 or 5-yr, and 4-versus 2-arm participation. Participants and all clinical coordinators, and laboratory staff were blinded to the treatment assignment.

Adjunct Biomarker Study

During the treatment period, every 4 months, bottles of study tablets were mailed to participants who 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. During the first year of follow-up (the period that is relevant to the adjunct biomarker study) blood levels of calcium, creatinine, 25(OH)D, and 1,25(OH)D were obtained at baseline and 1 year after randomization.

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 one follow-up visit. Six approximately 1 mm thick rectal biopsy specimens were taken from the rectal mucosa 10 cm above the level of external anal aperture through a short rigid proctoscope using a jumbo cup flexible biopsy forceps mounted on a semi-rigid rod. All biopsies were taken at least 4 cm from any polypoid lesions to avoid possible field affect from them. Biopsies were placed onto a strip of bibulous paper and immediately placed in normal saline, oriented, transferred to 10% normal-buffered formalin for 24 h, and then transferred to 70% ethanol. Then within a week, the biopsies were processed and embedded in paraffin blocks (two

blocks of three biopsies per participant, per biopsy visit). CLDN1 expression was measured in the biopsies using automated immunohistochemistry with image analysis.

Immunohistochemistry Protocol

Five slides with three levels of 3 µm-thick biopsy section taken 40 µm apart were prepared for CLDN1 biomarker, yielding a total of 15 levels. To uncover the epitope, heat mediated antigen retrieval was used: the slides were placed in a preheated Pretreatment Module (Lab Vision Corp., Fremont, CA) with 100 x Citrate Buffer pH 6.0 (Thermo Fisher, TA 250-Premix Waltham, MA) and steamed for 40 min. Then the slides were placed in a DakoCytomation Autostainer Plus System automated immunostainer and immunohistochemically processed using a labeled streptavidin-biotin method (UltraVision System, thermofisher) [TP-125-HL]) and Rabbit Polyclonal Antibody to CLDN1 (Abcam15 Cambridge MA02139) at a concentration of 1:200. 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, which were not counterstained with hematoxylin, were glass covered with Leica CV5000 Coverslipper (Leica Microsystems, Inc., Buffalo Grove, IL). Positive and negative control slides were included in each slide staining batch.

Protocol for Quantifying Labeling Densities of Immunohistochemically Detected Biomarkers in Normal Colonic Crypts ("Scoring")

A quantitative image analysis method ("scoring") was used to measure detected levels of the biomarkers in colon crypts. The major equipment and software for the image analysis procedures were computer, digital drawing board, Matlabsoftware (Mathworks, Inc., Natick, MA), CellularEyes Image Analysis Suite (DivEyes LLC, Atlanta, GA), and MySQL (Sun Microsystems Inc., Redwood Shores CA). First, slides were scanned with Panoramic SCAN BF

(Perkin Elmer), then, electronic images were retrieved in the CellularEyes program to identify colon crypts acceptable for analysis. A “scorable” crypts 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 procedure. Two or three biopsies with 8-20 “scorable” hemicrypts (one half of the crypt) per biopsy were blindly selected to the treatment assignment. Using the digital drawing board, the border 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 (upper 40% and lower 60% of each hemicrypt). All resulting data were automatically transferred into the MySQL database. Then, the next scorable hemicrypt was identified and previously described analysis steps were repeated. A reliability control sample previously analyzed by the reader was reanalyzed during the trial to determine intra-reader “scoring” reliability for CLDN1 which was >0.90.

Statistical Analysis

Our analyses were to assess changes in the expression of CLDN1 after randomization into 4-arm (placebo, calcium only, vitamin D only, calcium + vitamin D) and 2-arm (calcium versus calcium + vitamin D) treatment groups. We were also looking for the changes in the CLDN1 expression within the treatment groups who received (i) calcium relative to those who did not (“calcium vs. no calcium”), (ii) vitamin D relative to those who did not (“vitamin D vs. no vitamin D”), (iii) calcium plus vitamin D relative to those who received only calcium (“calcium plus vitamin D vs. calcium). In addition to evaluating biomarker changes in the whole crypts, we evaluated changes within crypt functional zones, including the upper 40% of the crypts (the

canonical differentiation zone), the lower 60% of the crypts (the canonical proliferation zone), and the proportion of the upper 40% of the crypts to the whole crypts (ϕh).

We used Chi-square test for categorical variable and ANOVA or t-test for continuous variables to assess the comparability of characteristics at baseline and at 1-yr follow-up. Treatment effects were evaluated by assessing the differences in CLDN1 expression from baseline to 1-yr follow-up between participants in the treatment group of interest and those in the comparison group using a repeated measures MIXED linear model. The model included the intercept, study center, batch ID, follow-up visit effects, time, treatment group, and the interaction of treatment with time. The calcium analyses included only participants randomized to calcium, and vitamin D analyses included only participants randomized to vitamin D (i.e. none of the 2-arm study participants were included). Potential confounders were selected because of suggestive imbalances in their distribution across treatment groups at baseline, included current smoking status, multivitamin use, physical activity measured as metabolic equivalent of task (MET)-minutes, total calorie intake and total dietary fiber intake. Exploratory analyses to assess potential treatment effect modification were conducted by stratifying the above analyses on age, sex, family history of colorectal cancer, NSAIDs (non-steroidal anti-inflammatory drugs) use, aspirin use, total calcium intake, serum 25(OH)D level, dietary fiber intake, and red and processed meat intake. For these analysis age was categorized as below median and at or above median. NSAID and aspirin use was categorized as taking at least once in a week vs. non-users. Total calcium (diet and supplements), serum 25(OH)D, intakes of dietary fiber, and red and processed meat intake were categorized as below and at or above the sex-specific medians. Because all biomarker measurements were in optical density, to provide perspective on the magnitudes of the estimated effects, relative treatment effects were calculated (relative effect= [(treatment group follow-up) / (treatment group baseline)] / [(control group follow-up) / (control group baseline)]. The interpretation of the relative effect is similar to that of an odds ratio. In all analyses of the

randomized treatments, participants were retained in their originally assigned treatment group, regardless of adherence to study treatment and procedures. We used generalized logistic model to assess the expression of CLDN1 at baseline among selected risk factors. To detect the differences in the levels of expression of CLDN1 within each selected risk factor group at baseline we calculated proportional differences (proportional difference= [(mean expression of CLDN1 at baseline in the risk group at baseline) – (mean expression of CLDN1 in the reference group at baseline)] / (mean expression of CLDN1 in the reference group at baseline)]. In sensitivity analyses, we also analyzed data without 1 patient who had less than 8 scorable hemicrypts adjusted for batch ID, study center and visit number. The results from these analyses did not differ materially from those reported. All statistical analyses were conducted using SAS 9.4 statistical software (SAS Institute Inc.). A *p*-value 0.05 (two-sided) was considered statistically significant.

Results

Selected baseline characteristics of 105 participants in the adjunct biomarker study are shown in Table 1. The mean age of the study participants was 59 years, 47% were men, 79% were white and 82% had received at least some college degrees or higher education. Most of the participants were overweight, non-diabetic, currently non-smokers, and were consumed on average less than 1 drink per day. Regarding medical history, 9% had family history of colorectal cancer in first degree relatives and nineteen participants had advanced adenomas at baseline. Almost two third of the participants reported taking NSAIDs and aspirin and 90% reported taking either one. There were statistically significant differences in physical activity and dietary fiber intake among the treatment groups.

For this adjunct biomarker study, during the first year after randomization, 76% of the participants reported taking 80% or more of their study tablets. There was a significant mean increase in serum 25(OH) D of 11 ng/ml (95% CI: 7-14 ng/ml) among participants who were taking vitamin D compared to those who were not, and of 12 ng/ml mean increase (95% CI: 7-16 ng/ml) among participants who were taking calcium plus vitamin D compared to placebo at 1 year after randomization (Table 2).

The overall expression of CLDN1 in colorectal crypt epithelium by treatment assigned at base line and 1-yr follow up with associated treatment effects are presented in Table 3. Adjusting for factors that differed at baseline did not change the estimates of the treatment effects for treatment groups. (Supplemental tables 1 & 2, see Appendix), therefore only unadjusted results are presented. When considering full length of the crypts the estimated expression of CLDN1 was decreased by 14% (p=0.310) in the vitamin D only group compared with placebo group with minimal increase in calcium only and calcium plus vitamin D group compared to placebo in the 4-arm treatment group. There was no change in the expression of CLDN1 in the 2-arm treatment group when compared calcium plus vitamin D with placebo. (Table 3). For the upper 40% of the crypt (differentiation zone), in the 4-arm treatment group the expression was decreased in the vitamin D group by 23% (p=0.153) while no change in expression of CLDN1 was observed in the calcium only and, in calcium plus vitamin D combination group relative to the placebo group. In the 2-arm treatment group there was almost no change in expression in the upper 40% of the crypts in the group that received vitamin D relative to the placebo group. The findings for the expression of CLDN1 in the lower 60% of the crypts (proliferation zone), showed 10% increased expression in the calcium only and calcium plus vitamin D combination group within the 4-arm treatment group with p-values 0.579 and 0.597 respectively and no change in expression of CLDN1 in the vitamin D only group compared to placebo. In the 2-arm treatment group the expression was slightly increased by 5% (p=0.716) in the lower 60% of the crypts in the group

that received vitamin D relative to the placebo group. For the proportion of expression of CLDN1 in the differentiation zone, that were in the upper 40% of the crypts, ϕh, there was a 11% decreased expression in the vitamin D group in the 4-arm treatment group and no change in the calcium alone and calcium plus vitamin D group. There was no change in expression of CLDN1 in the vitamin D group compared to placebo in the 2-arm treatment group for the ϕh of the crypts.

We also assessed the expression of CLDN1 in the normal-appearing rectal mucosa at baseline and at 1-yr follow-up by treatment received (Table 4). Following 1-yr of treatment, for the calcium versus no calcium group, the biomarker expression was increased by an estimated 14% (p=0.171) in the full length of the crypts, 16% (p=0.161) in the upper 40% of the crypts, 13% both in the lower 60% of the crypts and ϕh of the crypts with p-values 0.224 and 0.503 respectively. For the vitamin D only group, there was no change in CLDN1 expression in the full length of the crypts, upper 40% of the crypts, lower 60% of the crypts, and ϕh of the crypts compared to placebo. For the treatment group who received calcium plus vitamin D there was 4% estimated increase in CLDN1 expression in the full length (p=0.787) of the crypts, upper 40% (p=0.874) of the crypts, and ϕh (p=0.359) of the crypts and 5% increase in the lower 60% of the crypts (p=0.697) compared to placebo.

CLDN1 and Baseline Risk Factors

Baseline CLDN1 expression differed by selected demographic and lifestyle factors, race, obesity status, regular aspirin use and family history of colorectal cancer (Table 5, 6). Obese individuals had 34% higher expression (p-trend=0.019) of CLDN1 in the whole crypts, 39% higher expression in the upper 40% of crypts (p-trend=0.020), 30% higher expression in the lower 60% of crypts (p-trend=0.029) and 0.6% higher expression (p-trend=0.321) in the ϕh of crypts relative to the normal weight individuals. Participants who were taking aspirin regularly had 18% higher expression (p=0.061) of CLDN1 in the whole crypts, 21% higher expression (p=0.060) in the upper 40% of crypts, 16% higher expression (p=0.092) in the lower 60% of the

crypts and no change in expression of CLDN1 in the ϕh of crypts (p= 0.744) relative to aspirin non-users. Individuals with family history of colorectal cancer had 26% lower expression (p=0.085) of CLDN1 in the whole crypts, 32% lower expression (p= 0.063) in the upper 40% of crypts, 24% lower expression (p=0.100) in the lower 60% of crypts and 17% lower expression (p= 0.009) in the ϕh of the crypts compared to those without family history of colorectal cancer (Table 5,6). Though the result was statistically significant, there were only 2 individuals in other race category (other than white or black) made the interpretation unreliable.

Stratified Analysis

For calcium versus no calcium group, CLDN1 expression tended to be higher along the full length of the crypts in women, participants with family history of colorectal cancer and participants with baseline serum 25(OH)vitamin D level below median (Table 7). CLDN1 expression from crypts base to crypts lumen didn't differ significantly among treatment groups from baseline to 1-yr follow-up. For that reason, we considered to assess CLDN1 expression along the full length of the crypts for stratified analysis (Figure 1).

Discussion

The results of our study suggest that supplemental calcium alone could increase the overall expression of CLDN1 in the normal appearing colorectal mucosa compared to placebo. Supplemental vitamin D alone had no substantial effect on CLDN1 expression while vitamin D taken along with calcium had statistically non-significant increased expression of CLDN1 in the colorectal epithelium compared to placebo. In secondary analyses, sex, family history of CRC and serum 25(OH)D levels were identified as potential effect modifiers of calcium treatment effects. The treatment effects of calcium were suggestively stronger among women, and participants with positive family history of CRC and serum 25(OH)D below median at baseline.

CLDN1 expression at baseline differed among participants who were obese vs. normal weight, taking aspirin regularly vs. aspirin non-users, and had family history of CRC vs. those without family history of CRC. Obesity and taking aspirin regularly were associated with higher expression of CLDN1 while positive family history showed lower expression of CLDN1 compared to their reference group.

CLDNs are major integral membrane proteins of TJ, necessary for formation and maintenance of TJ. They act as a barrier protein and regulate permeability of blood vessels and epithelium in various types of tissues. Altered expression of CLDNs has been linked to development of various cancers. To date, 23 distinct members are identified in CLDN family [58]. Due to their role in the formation of cancer stem cells or tumor-initiating cells, CLDNs are promising target for cancer treatment and prevention of recurrence.

Decreased CLDN1 expression was found in patients with colonic adenocarcinoma [55], but the mechanism was not clear. However, it has been proposed that decreased expression of CLDN1 with loss of cell polarity is followed by an abnormal influx of different growth factors, which give rise to auto- and paracrine stimulations of neoplastic epithelium and thus provides nutrition factors and other factors necessary for tumor cell growth [59, 60]. Higher CLDN1 expression in the calcium only group compared to placebo in our study supports the role of calcium as a chemopreventive agent against colorectal neoplasms.

Evidence is accumulating for dietary and lifestyle factors as the modifiable risk factors for CRC. Dietary and lifestyle interventions are regarded as promising preventive measures against CRC development and calcium and vitamin D are two convincing chemopreventive agents that could potentially be used against colorectal neoplasms. Calcium protects the colonic epithelial cells from cytotoxic effects of free fatty acids and bile acids by binding with them and bringing them out to the colonic lumen. Calcium also suppresses the induction of tumor-promoting enzyme ornithine decarboxylase and exhibits inhibitory effects on colon

carcinogenesis [35]. Vitamin D decreases proliferation of colonic epithelial cells, enhances the differentiation of CRC cells and alters the transcription of a large number of genes involved in inhibitory carcinogenesis [20]. Other possible mechanisms by which vitamin D may reduce the risk of CRC include: inhibition of cellular inflammation (both local and systemic), reduction of oxidative stress, apoptosis and immunomodulation [22, 23]. With calcium and vitamin D deficient diet TJs gene expression were significantly reduced in mice, supporting the role of supplemental calcium and vitamin D in CLDN1 expression [53]. Supplemental vitamin D induced the expression of TJs protein in colonic epithelium in animal model [52].

Results from our study are supportive of the hypothesis that calcium alone and to a lesser extend when combined with vitamin D increases the expression of CLDN1 in the normal appearing colorectal mucosa of previously diagnosed colorectal adenoma patients. When taking calcium only, there was 14% increased expression of CLDN1 in the whole crypts, 16% increased expression of CLDN1 in the differentiating zone and 13% increased expression of CLDN1 in both the proliferative zone and ϕh of crypts compared to placebo, which is in line with the evidence that calcium is a probable risk reducing agent in colorectal carcinogenesis. Our data supports that calcium may induce the expression of CLDN1 in human colorectal epithelium which was previously found in animal model and human cell culture model [53, 61]. However, none of these associations were statistically significant. Participants taking vitamin D in addition to calcium showed a minimal higher expression of CLDN1 compared to placebo, 4% increased expression in whole crypts, 4% increased expression in the differentiating zone and 5% increased expression in the proliferative zone. Vitamin D alone did not change the overall expression of CLDN1 in the colonic epithelial crypts. This finding from our human study contradicts with the concept that vitamin D enhances the expression of CLDN1 in animals [52].

In our stratified analysis CLDN1 tended to be expressed higher in women compared to men among calcium vs. no calcium group. Sex-dependent and tissue specific CLDN1 expression

was observed in animal study [62]. CLDN1 expression was increased in patients with family history of CRC compared to those without family history of CRC. Though the result was statistically significant, due to very small sample size, the findings may be due to chance alone. In this analyses, calcium increased the CLDN1 expression when Serum 25(OH)D was below median, which supports calcium as a probable chemopreventive agent against CRC and conflicts with the previous evidence of positive relationship of serum 25(OH)D and CRC.

In our analysis of baseline risk factors for colorectal cancer that could potentially be associated with CLDN1 expression, we found that the CLDN1 levels tended to be higher in obese compared to participants with normal BMI. Obesity is considered to be a state of chronic low-grade inflammation and cause increased expression of TNF-alpha. Intestinal epithelial cells treated with increased doses of TNF-alpha showed increase in CLDN1 expression [63], which supports our finding of increased CLDN1 expression in obese individuals. Furthermore, the gut microbiome of obese individuals was found to be different compared to normal weight individuals, and have a higher abundance of gram negative bacteria [64]. From the membrane of Gram-negative bacteria, lipopolysaccharides (LPS) penetrate the blood stream, *via* impaired permeability of the intestinal mucosa and induce inflammation [65]. So, it is possible to have redistribution of TJ proteins [65] along with compensatory increase in TJ proteins in obese individuals. On the other hand, increased oxidative stress in obese individuals was shown to disrupts intestinal TJs and decreased expression of CLDN1 in animal models [66] which contradicts with our finding. A high-fat diet can cause obesity associated inflammation through changes in the gut microbiota, endotoxemia, and increased gut permeability. Use of probiotics suppress the production of proinflammatory LPS and ameliorates high fatty diet induced inflammation by increasing the expression of colon TJ proteins in mice [67]. We also found that participants who took aspirin regularly at baseline tended to have higher CLDN1 expression compared to aspirin non-users. Aspirin causes dose dependent uncoupling of oxidative

phosphorylation which disrupts the calcium homeostasis, and TJ function [68] that may be a possible explanation of increased expression of CLDN1 in aspirin users compared to aspirin non-users in our study. We also found lower expression of CLDN1 at baseline in participants with positive family history of CRC compared to those without family history of CRC. Family history of CRC is a well-established risk factor for CRC, and relatives of CRC cases may have genetic determinants that can cause loss of cell adhesion by decreased expression of TJ proteins. But the sample size was small for this result to be reliable.

The study has several strengths and limitations. The strengths of the study include: high protocol adherence by study participants, automated immunohistochemistry staining and novel image analysis software to quantify crypt biomarker distributions and the consequent high biomarker scoring reliability. This study is the only randomized, double-blind, placebo-controlled trail to have assess the independent and combined effects of supplemental calcium and vitamin D on expression of CLDN1 in the normal colorectal epithelium.

The primary limitation of the study was small sample size. We only assessed the rectal mucosa and therefore treatment effects on other parts of the colon are unknown. Most of our study participants were white, limiting our ability to see any racial/ethnic differences in CLDN1 expression in colorectal mucosa. There were only 2 participants in the racial group other than white or black. Though we found statistically significant relationship with race at baseline CLDN1 expression (70% higher expression in participants with race other than white or black, ($p=0.023$), we are not considering this result to be reliable due to very small sample size. We didn't see any significant effects of vitamin D on CLDN1 expression in our study, which may be related to the dose of vitamin D that we used, which warrants further analysis with higher dose of vitamin D.

In conclusion, the results from this clinical trial suggests that, calcium could increase the expression of CLDN1, a critical tight junction protein in the normal colorectal epithelium, and is

consistent with the hypothesis that calcium could reduce the risk for colorectal neoplasms. Our results also found minimal increase in CLDN1 expression when vitamin D was taken along with calcium. This study identified several biological plausible risk factors that could be associated with CLDN1 expression and risk for CRC, including being obese, regular aspirin use, and having family history of CRC, supporting further research of these factors and their association with CRC risk.

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Tables

Table 1. Selected Baseline Characteristics of the Adjunct Biomarker Study Participants (n=105), According to Treatment Assigned[a]

Characteristics	Treatment Assigned: Randomization to vitamin D and to calcium (4-arm) Placebo (n=12)	Calcium (n=16)	Vitamin D (n=17)	Calcium + Vitamin D (n=18)	p value[b]	Randomization to vitamin D only (2-Arm) 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)	58.0 (7.1)	0.857	58.2 (5.3)	59.2 (7.3)	0.598
Men (%)	75	81	71	83	0.833	0	0	--
White (%)	83	75	71	94	0.286	70	84	0.305
≥College[d] (%)	92	63	88	83	0.227	91	74	0.214
Family history of colorectal cancer[ef] (%)	0	13	20**	6	0.367	4	11*	0.573
Take non-aspirin NSAID[g] regularly[h] (%)	33	44	24	28	0.665	26	32	0.742
Regular Aspirin Use[h](%)	10	17	11	13	0.404	17	14	1.000
Current smoker (%)	25	6	0	6	0.103	0	16	0.084
Alcohol intake, drinks/day	0.7(0.7)	0.8(1.0)	0.9(0.9)	0.9(0.9)	0.950	0.5(1.0)	0.3(0.5)	0.422
Diabetes (%)	8	19	12	11	0.878	17	16	1.000
Had advanced adenoma (%)	36*	7*	24	28	0.295	9*	16	0.649
Take multivitamin (%)	42	81	47	67	0.102	70	89	0.149
Physical activity, MET-min/wk[i]	1620 (1195)	2128 (2378)	2782 (2764)	4042 (2456)	0.033	1458 (1235)	3021 (3469)*	0.051
Body Mass Index (BMI), kg/m^2	29.4 (4.9)	32.3 (7.6)	28.7 (5.5)	30.2 (4.4)	0.313	29.7 (5.6)	27.5 (4.7)	0.178
Dietary Intakes								
Total energy intake, kcal/d	1314 (381)	1737 (556)	1437 (527)	1569 (565)	0.213	1254 (549)	1429 (595)	0.327
Total fat, gm/d	57.1 (22.3)	68.9 (25.6)	60.5 (27.3)	61.6 (26.8)	0.688	50.3 (25.9)	61.5 (36.1)	0.249
Dietary fiber, gm/d	9.5 (4.1)**	15.8 (5.6)*	13.7 (6.2)	15.1 (5.7)	0.043	13.8 (5.4)	17.2 (5.0)	0.043
Red or Processed meat, servings/day	1.2(0.9)	1.0(0.7)	0.9(0.8)	1.0(0.7)	0.740	0.6(0.5)	0.7(0.6)	0.595
Total Vitamin D[j], IU/d	354.0 (306.6)	457.5 (189.2)	313.0 (278.4)	420.6 (295.6)	0.485	521.4 (354.2)	633.6(275.6)	0.341
Total Calcium[k], mg/dl	715.3 (455.4)	894.5 (263.9)	671.3(278.3)	667.1(254.7)	0.143	995.6 (497.6)	1232.3 (562.9	0.198
Serum levels								
25-OH-vitamin D, ng/mL	22.4 (8.2)	24.5 (13.4)	23.1 (8.7)	22.7 (6.4)	0.934	24.8 (8.9)	26.5 (9.6)	0.543
Vitamin D deficient (<20 ng/mL) (%)	58	50	47	39	0.781	43	32	0.530
Calcium, mg/dL	9.2 (0.2)	9.3 (0.3)	9.3 (0.3)	9.4 (0.3)	0.249	9.5 (0.3)	9.4 (0.3)	0.516

[a]Data are given as mean (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-tests for continuous variables

[d]Receive some college degree

[e] in first degree relatives

[f]3 missing information, 2 in the placebo+vitamin D (4-arm) and 1 in the placebo+vitaminD (2-arm)

[g]Non-steroidal anti-inflammatory drug

[h] At least once a week

[i]Metabolic equivalent of task

[j]Dietary vitamin D plus supplemental vitamin D. Missing information for 3 placebo patients, 2 calcium, 2 vit D, one combined (4-arm), 6 placebo and 5 vit D (2-arm)

[k]Dietary calcium plus supplemental calcium. Missing information for 2 placebo, 1 calcium, 1 vit D, one combined (4-arm), 6 placebo (2-arm), and one vit D (2-arm)

*Missing one patient's information

**Missing two patients' information

Table 2: Serum levels of 25(OH)D* by Treatment Arm/Treatment Agent (n=105)

		Baseline		1-Year Follow-up		Treatment Effect		
						Absolute Effect[c]		Relative
Treatment Arm/Treatment Agent	n	Mean(95% CI)	*p*-value[b]	Mean(95% CI)	*p*-value[b]	Mean(95%CI)	*p*-value[b]	Effect[d]
Treatment Arm								
4-Arm								
Placebo	12	22 (17 - 28)		22 (17 - 27)				
Calcium	16	24 (20 - 29)	0.549	24 (19 - 28)	0.583	-0.17 (-7.36 - 7.01)	0.962	0.99
Vitamin D	17	23 (19 - 27)	0.841	31 (27 - 35)	0.010	8.33 (1.24 - 15.43)	0.022	1.37
Calcium + Vitamin D	18	23 (18 - 27)	0.933	34 (29 - 38)	0.001	11.28 (4.27 - 18.29)	0.002	1.51
2-Arm								
Calcium	23	25 (21 - 29)		22 (18 - 26)				
Calcium + Vitamin D	19	27 (22 - 31)	0.564	35 (30 - 39)	<.0001	11.43 (5.14 - 17.72)	0.001	1.50
Treatment Agent								
Vitamin D Vs. No vitamin D								
No Vitamin D	51	24 (22 - 27)		22 (22 - 27)				
Vitamin D	54	24 (22 - 27)	0.981	33 (31 - 36)	<.0001	10.71 (7.01 - 14.41)	<.0001	1.48
Calcium Vs. Calcium + Vitamin D								
Calcium	39	25 (22 - 28)		23 (20 - 26)				
Calcium + Vitamin D	37	25 (22 - 28)	0.997	34 (31 - 37)	<.0001	11.62 (7.26 - 15.98)	<.0001	1.51
Calcium Vs No Calcium								
No Calcium	29	23 (19 - 26)		27 (24 - 31)				
Calcium	34	24 (20 - 27)	0.764	29 (26 - 32)	0.473	1.01 (-4.34 - 6.35)	0.708	1.03

* per ng/ml

CI, Confidence Interval

[b] *p*-value for the difference between each treatment group and placebo group from repeated-measures Mixed model

[c] Absolute effect=[(treatment group follow-up)-(treatment group baseline]- [(placebo group follow-up)-(placebo group baseline)]

[d] Relative effect=[(treatment group follow-up)/(tratment group baseline)]/[(placebo group follow-up)/(placebo group baseline)]

Table 3. Expression of CLDN1 in the Normal-Appearing Colorectal Mucosa of Study participants (n=105) by Treatment Assigned.

		Baseline		1-Yr Follow-Up		Treatment Effect		
						Absolute Effect[c]		Relative Effect[d]
Treatment Group	n	Mean (95% CI)	*p* -value[b]	Mean (95% CI)	*p* -value[b]	Mean (95% CI)	*p* -value[b]	
Whole crypts								
4-Arm								
Placebo	12	2,899 (1,728-4,070)		3,203 (2,032 - 4,375)				
Calcium	16	2,849 (1,834-3,863)	0.949	3,281 (2,266 - 4,295)	0.921	128 (-728 , 983)	0.766	1.04
Vitamin D	17	2,396 (1,411-3,380)	0.513	2,267 (1,283 - 3,251)	0.226	-433 (-1,277 , 412)	0.310	0.86
Calcium+Vitamin D	18	2,639 (1,682-3,595)	0.732	3,085 (2,128 - 4,041)	0.876	142 (-693 , 977)	0.735	1.06
2-Arm								
Placebo	23	4,140 (3,402-4,877)		4,123 (3,386 - 4,861)				
Vitamin D	19	3,314 (2,503-4,126)	0.136	3,400 (2,588 - 4,211)	0.190	102 (-950 , 1,153)	0.846	1.03
Upper 40% of crypts								
4-Arm								
Placebo	12	902 (461-1,343)		1,042 (601 - 1,483)				
Calcium	16	1,066 (685-1,448)	0.574	1,215 (833 - 1,597)	0.557	-7 (-331 , 346)	0.965	0.99
Vitamin D	17	873 (503-1,244)	0.921	772 (402 - 1,143)	0.352	-242 (-576 , 92)	0.153	0.76
Calcium+Vitamin D	18	963 (603-1,323)	0.830	1,130 (770 - 1,490)	0.760	26 (-304 , 356)	0.875	1.01
2-Arm								
Placebo	23	1,474 (1,194-1,753)		1,505 (1,225 - 1,784)				
Vitamin D	19	1,117 (809-1,424)	0.090	1,157 (850 - 1,465)	0.099	9 (-429 , 447)	0.966	1.01
Lower 60% of crypts								
4-Arm								
Placebo	12	1,829 (1,159-2,499)		1,959 (1,289 - 2,629)				
Calcium	16	1,575 (995-2,156)	0.570	1,848 (1,268 - 2,429)	0.803	143 (-368 , 653)	0.579	1.10
Vitamin D	17	1,356 (793-1,919)	0.284	1,372 (809 - 1,935)	0.184	-115 (-619 , 389)	0.650	0.94
Calcium+Vitamin D	18	1,479 (932-2,027)	0.422	1,742 (1,195 - 2,290)	0.618	133 (-366 , 631)	0.597	1.10
2-Arm								
Placebo	23	2,399 (1,967-2,831)		2,339 (1,907 - 2,771)				
Vitamin D	19	1,981 (1,506-2,456)	0.196	2,028 (1,553 - 2,503)	0.334	107 (-485 , 700)	0.716	1.05
ϕh [e]								
4-Arm								
Placebo	12	30 (26-33)		32 (28 - 36)				
Calcium	16	37 (34-40)	0.004	37 (34 - 41)	0.040	-2.16 (-7.20 , 2.88)	0.395	0.93
Vitamin D	17	35 (32-38)	0.042	33 (30 - 36)	0.605	-3.81 (-8.79 , 1.17)	0.131	0.89
Calcium+Vitamin D	18	36 (33-39)	0.017	38 (35 - 41)	0.020	-0.16 (-5.08 , 4.76)	0.950	0.98
2-Arm								
Placebo	23	36 (34-38)		36 (34 - 38)				
Vitamin D	19	33 (30-35)	0.050	33 (31 - 36)	0.136	0.76 (-2.97 , 4.50)	0.682	1.02

CI, confidence interval.

[b] *p* -value for the difference between each treatment group and placebo group from repeated-measures MIXED model.

[c] Absolute effect= [(treatment group follow-up)-(treatment group baseline)]-[(placebo group follow-up)-(placebo group baseline)].

[d] Relative effect=[(treatment group follow-up)/(treatment group baseline)]/[(placebo group follow-up)/(placebo group baseline)]; Interpretation similar to odds ratio.

[e] ϕh , proportion (%) of CLDN1 density in the differentiating zone (upper 40%)

Table 4. Expression of CLDN1 in the Normal-Appearing Colorectal Mucosa of Study Participants (n=105) by Treatment Agent

		Baseline		1-Yr Follow-Up		Treatment Effect		
Treatment Agent	n	Mean (95% CI)	*p* value[b]	Mean (95% CI)	*p* value[b]	Absolute Effect[c] Mean (95% CI)	*p* value[b]	Relative effect[d]
Calcium Vs. no Calcium								
Whole crypts								
No Calcium	29	2,604 (1,857 - 3,351)		2,655 (1,907 - 3,402)				
Calcium	34	2,738 (2,048 - 3,428)	0.793	3,177 (2,487 - 3,867)	0.308	389 (-173 , 950)	0.171	1.14
Upper 40% of crypts								
No Calcium	29	885 (605 - 1,165)		884 (604 - 1,164)				
Calcium	34	1,012 (753 - 1,270)	0.509	1,170 (911 - 1,428)	0.139	159 (65 , 383)	0.161	1.16
Lower 60% of crypts								
No Calcium	29	1,552 (1,123 - 1,981)		1,615 (1,186 - 2,044)				
Calcium	34	1,525 (1,128 - 1,921)	0.926	1,792 (1,396 - 2,189)	0.546	205 (-128 , 538)	0.224	1.13
ϕh[e]								
No Calcium	29	33 (30 - 35)		33 (30 - 35)				
Calcium	34	36 (34 - 38)	0.031	38 (35 - 40)	0.005	1.14 (-2.23 , 4.50)	0.503	1.13
Vitamin D vs. no vitamin D								
Whole crypts								
No vitamin D	51	3,443 (2,902 - 3,983)		3,642 (3,102 - 4,183)				
Vitamin D	54	2,800 (2,275 - 3,325)	0.094	2,938 (2,413 - 3,464)	0.067	-61 (-590 , 467)	0.818	0.99
Upper 40% of crypts								
No vitamin D	51	1,211 (1,007 - 1,415)		1305 (1101 - 1,509)				
Vitamin D	54	989 (791 - 1,187)	0.124	1,027 (829 - 1,225)	0.055	-56 (-271 , 160)	0.610	0.96
Lower 60% of crypts								
No vitamin D	51	2,006 (1,694 - 2,318)		2,096 (1,784 - 2,408)				
Vitamin D	54	1,617 (1,314 - 1,920)	0.079	1,726 (1,423 - 2,030)	0.095	20 (-285 , 325)	0.897	1.02
ϕh[e]								
No vitamin D	51	35 (33 - 36)		35 (34 - 37)				
Vitamin D	54	34 (33 - 36)	0.735	35 (33 - 37)	0.685	-0.08(-2.54 , 2.38)	0.948	1.00
Vitamin D + Calcium Vs. Calcium								
Whole crypts								
Calcium	39	3,610 (2,981 - 4,240)		3,778 (3,148 - 4,407)				
vitamin D + Calcium	37	2,986 (2,340 - 3,632)	0.172	3,247 (2,600 - 3,893)	0.245	93 (-591 , 778)	0.787	1.04
Upper 40% of crypts								
Calcium	39	1,306 (1,068 - 1,545)		1,386 (1,147 - 1,624)				
vitamin D + Calcium	37	1,042 (797 - 1,287)	0.127	1,144 (899 - 1,389)	0.163	23 (-262 , 307)	0.874	1.04
Lower 60% of crypts								
Calcium	39	2,061 (1,698 - 2,424)		2,138 (1,775 - 2,500)				
vitamin D + Calcium	37	1,737 (1,365 - 2,109)	0.218	1,889 (1,517 - 2,262)	0.344	76 (-310 , 462)	0.697	1.05
ϕh[e]								
Calcium	39	36 (34 - 38)		36 (34 - 38)				
vitamin D + Calcium	37	34 (32 - 36)	0.119	36 (34 - 38)	0.564	1.37(-1.59 , 4.34)	0.359	1.04

CI, Confidence Interval

[b] *p*- value for difference between each active treatment group and placebo group from repeated-measures Mixed model

[c] Absolute effect=[(treatment group follow-up)-(treatment group baseline]- [(placebo group follow-up)-(placebo group baseline)]

[d]Relative effect=[(treatment group follow-up)/(tratment group baseline)]/[(placebo group follow-up)/(placebo group baseline)]

ϕhe, proportion (%) of hemicrypt biomarker density that is in the differentiation zone (upper 40%)

Table 5: Mean Baseline CLDN1 Expression by Selected Demographic and Lifestyle Factors (n=105)

Baseline Characteristics	n	Whole Crypt Mean (95% CI)	*p* -value[b]	*p* -trend	Proportional difference(%)[g]
Age(tertiles), years					
≤53	39	3,228 (2,756 - 3,699)			
54-63	38	2,937 (2,504 - 3,370)	0.346		-9.01
>63	28	3,288 (2,774 - 3,801)	0.868		1.87
Gender					
Male	49	3,241 (2,720 - 3,762)			
Female	56	3,068 (2,629 - 3,508)	0.667		-5.33
Race					
White	83	3,132 (2,847 - 3,417)			
Black	20	2,969 (2,212 - 3,725)	0.686		-5.21
Other	2	5,331 (3,434 - 7,229)	0.025		70.22
BMI, kg/m2					
<25	22	2,741 (2,158 - 3,324)			
≥25-<30	43	2,876 (2,482 - 3,270)	0.698		4.93
≥30	40	3,680 (3,255 - 4,105)	0.015	0.019	34.25
Take NSAID[h] regularly[d]					
No	73	3,114 (2,787 - 3,442)			
Yes	32	3,249 (2,743 - 3,756)	0.658		4.34
Take Aspirin regularly[d]					
No	60	2,923 (2,560 - 3,285)			
yes	45	3,456 (3,041 - 3,871)	0.062		18.25
Take NSAID[h]/Aspirin regularly[d]					
No	11	3,557 (2,754 - 4,360)			
Yes	94	3,110 (2,824 - 3,395)	0.292		-12.57
Number of adenoma at baseline					
1	76	3,163 (2,799 - 3,527)			
>1	29	3,140 (2,643 - 3,637)	0.944		-0.73
Family history of colorectal cancer[e]*					
No	93	3,219 (2,929 - 3,508)			
yes	9	2,381 (1,456 - 3,306)	0.085		-26.02
Alcohol intake, drinks/day[f]					
Non-drinker	36	3,310 (2,773 - 3,848)			
≤1 drink/day	53	3,171 (2,791 - 3,550)	0.701		-4.22
>1 drink/day	16	3,005 (2,257 - 3,752)	0.543	0.490	-9.23
Physical activity, MET-min/wk[f]					
Tertile 1	35	3,011 (2,536 - 3,486)			
Tertile 2	36	3,418 (2,973 - 3,862)	0.333		13.49
Tertile 3	34	3,099 (2,646 - 3,552)	0.794	0.785	2.91
Red or Processed meat, servings/day[f]					
Tertile 1	37	3,207 (2,733 - 3,682)			
Tertile 2	38	2,964 (2,538 - 3,390)	0.446		-7.58
Tertile 3	30	3,303 (2,819 - 3,787)	0.781	0.755	2.98
Dietary fiber, gm/d[f]					
Tertile 1	35	3,058 (2,615 - 3,501)			
Tertile 2	37	3,059 (2,602 - 3,516)	0.997		0.04
Tertile 3	33	3,373 (2,877 - 3,869)	0.342	0.718	10.30
25(OH) vitamin D					
Below median	52	3,223 (2,854 - 3,592)			
At or above median	53	3,131 (2,754 - 3,508)	0.734	0.796	-2.84
Total Calcium		0 (0 - 0)			
Below median	58	3,057 (2,717 - 3,396)			
At or above median	47	3,350 (2,967 - 3,734)	0.274	0.898	9.60

CI, confidence interval

[b] *p* -value from generalized logistic model

[d] atleast once in a week

[e] in first degree relative

[f] Sex-Specific Value

[g] proportional difference (%) =([(mean expression of CLDN1 in the group) - (mean expression of CLDN1 in placebo)]/(mean expression of CLDN1 in placebo))*100

* 3 missing information, 2 vitamin D only group (4-arm) and 1 in vitamin D group (2-arm)

[h] Non-steroidal anti-inflammatory drug

Table 6: Mean Baseline CLDN1 Expression by Selected Demographic and Lifestyle Factors (n=105)

		Upper 40%			Proportional	Lower 60%			Proportional	ϕh[a]			Proportional
Baseline Characteristics	n	Mean (95% CI)	*p*-value[b]	*p*-trend	difference (%)[g]	Mean (95% CI)	*p*-value[b]	*p*-trend	difference(%)[g]	Mean (95% CI)	*p*-value[b]	*p*-trend	difference(%)[g]
Age(tertiles), years													
≤53	39	1,163 (973 - 1,354)				1,842 (1,576 - 2,108)				36 (33 - 38)			
54-63	38	1,009 (834 - 1,183)	0.214		-13.30	1,735 (1,491 - 1,979)	0.536		-5.82	33 (31 - 35)	0.098		-6.90
>63	28	1,133 (926 - 1,340)	0.835		-2.61	1,943 (1,654 - 2,233)	0.620		5.50	34 (31 - 36)	0.241		-5.73
Gender													
Male	49	1,145 (935 - 1,355)				1,880 (1,585 - 2,174)				34 (32 - 37)			
Female	56	1,064 (887 - 1,241)	0.613		-7.13	1,801 (1,552 - 2,049)	0.728		-4.20	34 (32 - 36)	0.820		-1.28
Race													
White	83	1,094 (980 - 1,208)				1,826 (1,664 - 1,988)				34 (33 - 36)			
Black	20	1,028 (725 - 1,330)	0.680		-6.10	1,766 (1,335 - 2,196)	0.793		-3.30	34 (30 - 37)	0.798		-1.51
Other	2	2,050 (1,290 - 2,810)	0.016		87.33	2,955 (1,874 - 4,035)	0.043		61.80	38 (29 - 47)	0.421		11.40
BMI, kg/m2													
<25	22	952 (718 - 1,186)				1,615 (1,281 - 1,949)				35 (32 - 38)			
≥25-<30	43	983 (824 - 1,141)	0.827		3.22	1,707 (1,482 - 1,933)	0.643		5.72	33 (31 - 35)	0.393		-4.37
≥30	40	1,318 (1,147 - 1,489)	0.019	0.0203	38.48	2,107 (1,863 - 2,350)	0.026	0.0292	30.44	35 (33 - 37)	0.917	0.3209	0.58
Take NSAID[h] regularly[d]													
No	73	1,068 (937 - 1,199)				1,841 (1,655 - 2,026)				33 (32 - 35)			
Yes	32	1,191 (988 - 1,394)	0.315		11.53	1,840 (1,553 - 2,126)	0.995		-0.06	36 (34 - 38)	0.058		8.28
Take Aspirin regularly[d]													
No	60	1,010 (865 - 1,156)				1,722 (1,516 - 1,927)				34 (32 - 36)			
yes	45	1,227 (1,060 - 1,394)	0.060		21.44	1,995 (1,759 - 2,230)	0.092		15.86	34 (32 - 36)	0.744		1.35
Take NSAID[h]/Aspirin regularly[d]													
No	11	1,206 (881 - 1,531)				2,121 (1,669 - 2,573)				33 (29 - 37)			
yes	94	1,093 (978 - 1,209)	0.511		-9.33	1,809 (1,648 - 1,970)	0.1927		-14.70	34 (33 - 36)	0.491		4.31
Number of adenoma at baseline													
1	76	1,113 (966 - 1,259)				1,843 (1,637 - 2,049)	(Ref)			34 (33 - 36)			
>1	29	1,091 (891 - 1,291)	0.870		-1.95	1,836 (1,555 - 2,117)	0.972		-0.35	34 (31 - 36)	0.660		-2.04
Family history of colorectal cancer[i]*													
No	93	1,130 (1,014 - 1,246)				1,877 (1,713 - 2,041)				34 (33 - 36)			
yes	9	766 (395 - 1,137)	0.063		-32.20	1,425 (901 - 1,948)	0.100		-24.10	29 (24 - 33)	0.009		-17.27
Alcohol intake, drinks/day[f]													
Non-drinker	36	1,166 (947 - 1,384)				1,933 (1,630 - 2,235)				36 (33 - 38)			
≤1 drink/day	53	1,141 (986 - 1,295)	0.632		-2.13	1,813 (1,599 - 2,027)	0.780		-6.18	34 (32 - 36)	0.907		-4.40
>1 drink/day	16	1,057 (753 - 1,361)	0.594	0.654	-9.33	1,745 (1,324 - 2,166)	0.508	0.408	-9.70	34 (30 - 38)	0.461	0.496	-5.09
Physical activity, MET-min/wk[f]													
Tertile 1	35	1,092 (897 - 1,286)				1,728 (1,461 - 1,994)				35 (33 - 38)			
Tertile 2	36	1,197 (1,015 - 1,378)	0.504		9.63	2,007 (1,758 - 2,256)	0.182		16.16	34 (32 - 36)	0.426		-4.92
Tertile 3	34	1,107 (922 - 1,292)	0.911	0.715	1.39	1,760 (1,506 - 2,014)	0.864	0.787	1.87	35 (33 - 37)	0.789	0.959	-1.25
Red or Processed meat, servings/day[f]													
Tertile 1	37	1,145 (954 - 1,336)				1,849 (1,580 - 2,118)				35 (33 - 37)			
Tertile 2	38	1,020 (848 - 1,191)	0.3294		-10.95	1,748 (1,507 - 1,989)	0.5771		-5.44	34 (32 - 36)	0.382		-3.83
Tertile 3	30	1,152 (957 - 1,347)	0.9609	0.7214	0.59	1,931 (1,657 - 2,205)	0.6742	0.772	4.43	33 (31 - 36)	0.2861	0.7895	-5.06
Dietary fiber, gm/d[f]													
Tertile 1	35	1,049 (871 - 1,227)				1,807 (1,556 - 2,059)				34 (32 - 36)			
Tertile 2	37	1,070 (886 - 1,254)	0.876		1.97	1,792 (1,532 - 2,051)	0.934		-0.86	34 (32 - 36)	0.964		-0.22
Tertile 3	33	1,208 (1,008 - 1,407)	0.235	0.6416	15.09	1,931 (1,650 - 2,213)	0.508	0.8225	6.87	35 (32 - 37)	0.525	0.5981	3.01
25(OH) vitamin D													
Below median	52	1,133 (980 - 1,285)				1,895 (1,685 - 2,106)				34 (32 - 36)			
At or above median	53	1,137 (982 - 1,291)	0.971	0.519	0.38	1,782 (1,569 - 1,996)	0.486	0.988	-5.95	35 (33 - 37)	0.609	0.376	2.12
Total Calcium													
Below median	58	1,068 (931 - 1,206)				1,785 (1,592 - 1,977)				35 (33 - 36)			
At or above median	47	1,218 (1,063 - 1,373)	0.170	0.996	13.96	1,908 (1,691 - 2,125)	0.415	0.839	6.92	35 (33 - 37)	0.870	0.763	0.63

CI, confidence interval

[b] *p*-value from generalized logistic model

[d] atleast once in a week

[a] ϕh, proportion of hemicrypt density that is in the differentiation zone

[e] in first degree relatives

[f] Sex-Specific Value

[g] proportional difference(%)= ([(mean expression of CLDN1 in the group) - (mean expression of CLDN1 in placebo)]/(mean expression of CLDN1 in placebo))*100

[i] In first degree relative

* 3 missing information, 2 in vitamin D only group (4-arm) and 1 in vitamin D group (2-arm)

[h] Non-steroidal anti-inflammatory drug

Table 7. Expression of CLDN1 in the Normal Appearing Colorectal Mucosa of the Adjunct Biomarker Study Participants by Selected Categories of Pre-defined Risk Factors (n=105)

		Baseline		1-yr follow-up		Absolute Treatment Effect[c]		Relative[d]
Treatment group	n	Mean (95% CI)	*p*-value[b]	Mean (95% CI)	*p*-value[b]	Mean (95% CI)	*p*-value[b]	Effect
Age, years								
< 60								
Whole Crypts								
Calcium vs no calcium								
No calcium	14	2,675 (1,528 - 3,822)		2,942 (1,795 - 4,089)				
Calcium	16	3,588 (2,515 - 4,661)	0.244	4,101 (3,028 - 5,174)	0.142	246 (-648 - 1,139)	0.578	1.04
Vitamin D vs no vitamin D								
No vitamin D	27	3,454 (2,730 - 4,178)		3,698 (2,973 - 4,422)				
Vitamin D	29	3,268 (2,569 - 3,967)	0.713	3,446 (2,747 - 4,145)	0.618	-66 (-766 - 634)	0.851	0.98
calcium + vitamin D vs Calcium								
Calcium	22	3,677 (2,855 - 4,500)		3,826 (3,003 - 4,648)				
Calcium + vitamin D	20	3,721 (2,621 - 4,346)	0.744	3,721 (2,858 - 4,584)	0.860	89 (-767 - 945)	0.835	0.96
≥60								
Whole Crypts								
Calcium vs no calcium								
No calcium	15	2,537 (1,596 - 3,478)		2,386 (1,445 - 3,327)				
Calcium	18	1,982 (1,122 - 2,841)	0.381	2,356 (1,497 - 3,215)	0.962	526 (-222 - 1,273)	0.162	1.26
Vitamin D vs no vitamin D								
No vitamin D	24	3,431 (2,616 - 4,245)		3,580 (2,765 - 4,395)				
Vitamin D	25	2,257 (1,458 - 3,055)	0.044	2,349 (1,551 - 3,148)	0.035	-57 (-894 - 779)	0.891	1.00
calcium + vitamin D vs Calcium								
Calcium	17	3,523 (2,521 - 4,526)		3,716 (2,713 - 4,718)				
Calcium + vitamin D	17	2,400 (1,398 - 3,402)	0.116	2,689 (1,686 - 3,691)	0.150	96 (-1,079 - 1,272)	0.868	1.06
Gender								
Male								
Whole Crypts								
Calcium vs no calcium								
No calcium	21	2,746 (1,820 - 3,671)		2,770 (1,844 - 3,696)				
Calcium	28	2,947 (2,145 - 3,749)	0.742	3,248 (2,446 - 4,050)	0.436	276 (-421 - 974)	0.429	1.09
Vitamin D vs no vitamin D								
No vitamin D	22	2,908 (2,002 - 3,815)		3,231 (2,325 - 4,138)				
Vitamin D	27	2,822 (2,004 - 3,640)	0.888	2,890 (2,071 - 3,708)	0.576	-255 (-950 - 439)	0.463	0.92
calcium + vitamin D vs Calcium								
Calcium	13	3,082 (1,743 - 4,420)		3,400 (2,062 - 4,738)				
Calcium + vitamin D	15	2,831 (1,585 - 4,077)	0.780	3,116 (1,870 - 4,362)	0.752	-34 (-1,057 - 990)	0.947	1.00
Female								
Whole Crypts								
Calcium vs no calcium								
No calcium	8	2,232 (960 - 3,504)		2,352 (1,080 - 3,624)				
Calcium	6	1,759 (291 - 3,228)	0.606	2,847 (1,378 - 4,315)	0.589	967 (256 - 1,678)	0.012	1.54
Vitamin D vs no vitamin D								
No vitamin D	29	3,848 (3,185 - 4,512)		3,954 (3,291 - 4,618)				
Vitamin D	27	2,778 (2,090 - 3,465)	0.029	2,987 (2,299 - 3,675)	0.047	103 (-706 - 912)	0.800	1.05
calcium + vitamin D vs Calcium								
Calcium	26	3,874 (3,186 - 4,563)		3,966 (3,278 - 4,655)				
Calcium + vitamin D	22	3,091 (2,342 - 3,840)	0.128	3,336 (2,587 - 4,084)	0.219	152 (-791 - 1,096)	0.746	1.05

Table 7. (Continued)

Treatment group	n	Baseline Mean (95% CI)	*p* -value[b]	1-yr follow-up Mean (95% CI)	*p* -value[b]	Absolute Treatment Effect[c] Mean (95% CI)	*p* -value[b]	Relative[d] Effect
Family history of colorectal cancer*								
No								
Whole Crypts								
Calcium vs no calcium								
No calcium	24	2,829 (2,003 - 3,656)		2,918 (2,092 - 3,744)				
Calcium	31	2,625 (1,898 - 3,352)	0.711	2,959 (2,232 - 3,686)	0.941	245 (-350 - 840)	0.412	1.09
Vitamin D vs no vitamin D								
No vitamin D	48	3,430 (2,869 - 3,991)		3,597 (3,036 - 4,158)				
Vitamin D	45	2,953 (2,374 - 3,533)	0.244	3,086 (2,506 - 3,665)	0.211	-35 (-567 - 498)	0.897	1.00
calcium + vitamin D vs Calcium								
Calcium	36	3,607 (2,945 - 4,269)		3,729 (3,067 - 4,390)				
Calcium + vitamin D	33	3,024 (2,333 - 3,715)	0.228	3,251 (2,560 - 3,942)	0.322	105 (-574 - 784)	0.758	1.04
Yes								
Whole Crypts								
Calcium vs no calcium								
No calcium	3	1,050 (-797 - 2,896)		336 (-1,510 - 2,182)				
Calcium	3	3,901 (2,055 - 5,748)	0.039	5,429 (3,582 - 7,275)	0.006	2,241 (610 - 3,872)	0.019	4.35
Vitamin D vs no vitamin D								
No vitamin D	3	3,647 (859 - 6,436)		4,365 (1,577 - 7,154)				
Vitamin D	6	1,623 (-349 - 3,595)	0.204	2,099 (127 - 4,070)	0.161	-243 (-3,416 - 2,931)	0.862	1.08
calcium + vitamin D vs Calcium								
Calcium	3	3,647 (215 - 7,080)		4,365 (933 - 7,797)				
Calcium + vitamin D	3	2,197 (-1,235 - 5,629)	0.453	3,861 (429 - 7,294)	0.788	947 (-3,556 - 5,449)	0.591	1.47
NSAIDs[e]								
No								
Whole Crypts								
Calcium vs no calcium								
No calcium	21	2,659 (1,860 - 3,458)		2,747 (1,948 - 3,546)				
Calcium	22	2,434 (1,653 - 3,215)	0.687	2,862 (2,081 - 3,643)	0.837	340 (-329 - 1,008)	0.311	1.14
Vitamin D vs no vitamin D								
No vitamin D	34	3,432 (2,786 - 4,078)		3,672 (3,026 - 4,318)				
Vitamin D	39	2,709 (2,105 - 3,312)	0.107	2,883 (2,280 - 3,487)	0.080	-66 (-735 - 604)	0.846	0.99
calcium + vitamin D vs Calcium								
Calcium	26	3,642 (2,896 - 4,389)		3,814 (3,067 - 4,561)				
Calcium + vitamin D	26	2,761 (2,014 - 3,508)	0.100	3,094 (2,347 - 3,840)	0.177	161 (-720 - 1,042)	0.715	1.07
Yes[f]								
Whole Crypts								
Calcium vs no calcium								
No calcium	8	2,459 (659 - 4,259)		2,411 (611 - 4,212)				
Calcium	12	3,294 (1,824 - 4,763)	0.460	3,755 (2,285 - 5,224)	0.240	509 (-662 - 1,679)	0.374	1.16
Vitamin D vs no vitamin D								
No vitamin D	17	3,465 (2,411 - 4,519)		3,584 (2,530 - 4,638)				
Vitamin D	15	3,037 (1,915 - 4,159)	0.575	3,082 (1,959 - 4,204)	0.511	-75 (-968 - 819)	0.866	0.98
calcium + vitamin D vs Calcium								
Calcium	13	3,546 (2,290 - 4,802)		3,705 (2,448 - 4,961)				
Calcium + vitamin D	11	3,517 (2,151 - 4,883)	0.974	3,608 (2,242 - 4,974)	0.915	-68 (-1,219 - 1,083)	0.904	0.98
Aspirin								
No								
Whole Crypts								
Calcium vs no calcium								
No calcium	16	2,756 (1,811 - 3,701)		3,108 (2,162 - 4,053)				
Calcium	15	2,127 (1,151 - 3,103)	0.352	2,977 (2,001 - 3,953)	0.846	498 (-239 - 1,236)	0.178	1.24
Vitamin D vs no vitamin D								
No vitamin D	27	3,788 (3,127 - 4,450)		3,936 (3,275 - 4,598)				
Vitamin D	33	2,476 (1,878 - 3,074)	0.005	2,909 (2,311 - 3,507)	0.025	285 (-437 - 1,007)	0.433	1.13
calcium + vitamin D vs Calcium								
Calcium	21	3,893 (3,189 - 4,596)		3,964 (3,261 - 4,668)				
Calcium + vitamin D	23	2,528 (1,856 - 3,200)	0.007	3,014 (2,342 - 3,686)	0.055	414 (-533 - 1,361)	0.383	1.17
Yes[f]								
Whole Crypts								
Calcium vs no calcium								
No calcium	13	2,416 (1,201 - 3,632)		2,097 (881 - 3,313)				
Calcium	19	3,220 (2,214 - 4,225)	0.307	3,335 (2,329 - 4,341)	0.120	435 (-396 - 1,266)	0.294	1.19
Vitamin D vs no vitamin D								
No vitamin D	24	3,054 (2,147 - 3,962)		3,312 (2,404 - 4,219)				
Vitamin D	21	3,309 (2,339 - 4,279)	0.701	2,984 (2,014 - 3,954)	0.622	-583 (-1,362 - 197)	0.139	0.83
calcium + vitamin D vs Calcium								
Calcium	18	3,281 (2,134 - 4,427)		3,560 (2,413 - 4,706)				
Calcium + vitamin D	14	3,737 (2,437 - 5,038)	0.594	3,629 (2,329 - 4,929)	0.936	-388 (-1,425 - 649)	0.451	0.89

Table 7. (Continued)

Treatment group	n	Baseline Mean (95% CI)	*p* -value[b]	1-yr follow-up Mean (95% CI)	*p* -value[b]	Absolute Treatment Effect[c] Mean (95% CI)	*p* -value[b]	Relative[d] Effect
Dietary fiber[a]								
Below median								
Whole Crypts								
Calcium vs no calcium								
No calcium	19	2,694 (1,804 - 3,583)		2,890 (2,001 - 3,780)				
Calcium	13	2,338 (1,262 - 3,413)	0.606	2,846 (1,770 - 3,921)	0.948	311 (-419 - 1,042)	0.391	1.13
Vitamin D vs no vitamin D								
No vitamin D	31	3,498 (2,811 - 4,186)		3,578 (2,890 - 4,266)				
Vitamin D	21	2,590 (1,754 - 3,425)	0.098	2,946 (2,111 - 3,782)	0.247	277 (-430 - 984)	0.435	1.11
calcium + vitamin D vs Calcium								
Calcium	21	3,841 (3,039 - 4,643)		3,749 (2,947 - 4,551)				
Calcium + vitamin D	12	2,583 (1,522 - 3,644)	0.063	3,262 (2,201 - 4,323)	0.460	770 (-242 - 1,782)	0.131	1.29
At or above median								
Whole Crypts								
Calcium vs no calcium								
No calcium	10	2,433 (1,041 - 3,824)		2,206 (815 - 3,598)				
Calcium	21	2,985 (2,025 - 3,945)	0.509	3,382 (2,422 - 4,342)	0.166	624 (-348 - 1,596)	0.200	1.25
Vitamin D vs no vitamin D								
No vitamin D	20	3,356 (2,451 - 4,262)		3,742 (2,836 - 4,648)				
Vitamin D	33	2,934 (2,228 - 3,639)	0.463	2,933 (2,228 - 3,638)	0.163	-386 (-1,227 - 454)	0.360	0.90
calcium + vitamin D vs Calcium								
Calcium	18	3,341 (2,331 - 4,351)		3,811 (2,801 - 4,821)				
Calcium + vitamin D	25	3,179 (2,322 - 4,036)	0.806	3,239 (2,382 - 4,096)	0.389	-409 (-1,391 - 572)	0.405	0.89
Red or processed meat[a]								
Below median								
Whole Crypts								
Calcium vs no calcium								
No calcium	16	2,319 (1,181 - 3,457)		2,192 (1,054 - 3,330)				
Calcium	17	3,088 (1,984 - 4,192)	0.330	3,579 (2,475 - 4,683)	0.084	618 (-181 - 1,416)	0.125	1.23
Vitamin D vs no vitamin D								
No vitamin D	26	3,463 (2,660 - 4,266)		3,616 (2,813 - 4,419)				
Vitamin D	27	2,695 (1,907 - 3,483)	0.177	2,541 (1,753 - 3,329)	0.061	-308 (-980 - 364)	0.362	0.90
calcium + vitamin D vs Calcium								
Calcium	21	3,763 (2,827 - 4,700)		3,896 (2,960 - 4,832)				
Calcium + vitamin D	16	2,916 (1,844 - 3,989)	0.235	2,858 (1,786 - 3,931)	0.148	-190 (-1,108 - 727)	0.6764	0.95
At or above median								
Whole Crypts								
Calcium vs no calcium								
No calcium	13	2,955 (1,980 - 3,929)		3,224 (2,249 - 4,198)				
Calcium	17	2,387 (1,535 - 3,240)	0.377	2,775 (1,923 - 3,628)	0.484	119 (-724 - 962)	0.7748	1.07
Vitamin D vs no vitamin D								
No vitamin D	25	3,422 (2,673 - 4,171)		3,670 (2,921 - 4,418)				
Vitamin D	27	2,905 (2,185 - 3,625)	0.322	3,336 (2,616 - 4,056)	0.522	183 (-648 - 1,015)	0.6597	1.07
calcium + vitamin D vs Calcium								
Calcium	18	3,431 (2,541 - 4,322)		3,640 (2,749 - 4,530)				
Calcium + vitamin D	21	3,039 (2,214 - 3,863)	0.516	3,542 (2,718 - 4,367)	0.872	295 (-764 - 1,354)	0.5755	1.10

CI,Confidence intervel

[a] Sex-specific median

[b] *p* -value using a Mixed linear model

[c] Absolute effect= [(treatment group follow-up)-(treatment group baseline)]-[(placebo group follow-up)-(placebo group baseline)].

[d]Relative effect=[(treatment group follow-up)/(tratment group baseline)]/[(placebo group follow-up)/(placebo group baseline)]

[e] Non-steroidal anti-inflammatory drugs

[f] taken at least once in a week

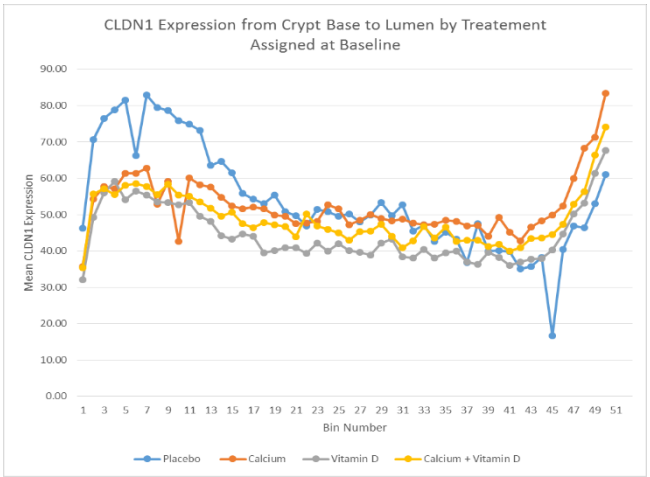
Table 7. (Continued)

Treatment group	n	Baseline Mean (95% CI)	*p* -value[b]	1-yr follow-up Mean (95% CI)	*p* -value[b]	Absolute Treatment Effect[c] Mean (95% CI)	*p* -value[b]	Relative[d] Effect
Total Calcium[a]								
Below median								
Whole Crypts								
Calcium vs no calcium								
No calcium	20	2,820 (1,790 - 3,849)		2,975 (1,946 - 4,004)				
Calcium	18	2,672 (1,587 - 3,757)	0.843	3,075 (1,990 - 4,160)	0.893	247 (-502 - 996)	0.508	1.09
Vitamin D vs no vitamin D								
No vitamin D	29	3,524 (2,737 - 4,312)		3,639 (2,852 - 4,427)				
Vitamin D	29	2,866 (2,078 - 3,654)	0.242	2,947 (2,159 - 3,734)	0.218	-34 (-622 - 553)	0.907	1.00
calcium + vitamin D vs Calcium								
Calcium	20	3,639 (2,639 - 4,639)		3,558 (2,558 - 4,558)				
Calcium + vitamin D	18	3,120 (2,066 - 4,173)	0.473	3,352 (2,298 - 4,406)	0.775	313 (-461 - 1,087)	0.417	1.10
At or above median								
Whole Crypts								
Calcium vs no calcium								
No calcium	9	2,124 (1,044 - 3,204)		1,942 (862 - 3,022)				
Calcium	16	2,811 (2,001 - 3,621)	0.304	3,292 (2,482 - 4,102)	0.050	663 (-288 - 1,614)	0.163	1.28
Vitamin D vs no vitamin D								
No vitamin D	22	3,335 (2,581 - 4,090)		3,647 (2,892 - 4,401)				
Vitamin D	25	2,723 (2,015 - 3,431)	0.240	2,929 (2,221 - 3,636)	0.169	-106 (-1,072 - 860)	0.826	0.98
calcium + vitamin D vs Calcium								
Calcium	19	3,580 (2,761 - 4,399)		4,009 (3,190 - 4,828)				
Calcium + vitamin D	19	2,859 (2,040 - 3,678)	0.215	3,147 (2,328 - 3,966)	0.140	-141 (-1,310 - 1,028)	0.808	0.98
25(OH)D[a]								
Below median								
Whole Crypts								
Calcium vs no calcium								
No calcium	17	2,383 (1,621 - 3,145)		2,591 (1,830 - 3,353)				
Calcium	16	2,311 (1,525 - 3,096)	0.894	3,243 (2,458 - 4,028)	0.234	724 (55 - 1,392)	0.035	1.29
Vitamin D vs no vitamin D								
No vitamin D	27	3,535 (2,838 - 4,233)		3,809 (3,112 - 4,507)				
Vitamin D	25	2,519 (1,794 - 3,244)	0.048	2,939 (2,214 - 3,664)	0.089	147 (-590 - 883)	0.691	1.08
calcium + vitamin D vs Calcium								
Calcium	19	3,763 (2,864 - 4,662)		4,056 (3,157 - 4,955)				
Calcium + vitamin D	16	2,901 (1,922 - 3,881)	0.196	3,451 (2,472 - 4,431)	0.362	257 (-743 - 1,258)	0.604	1.10
At or above median								
Whole Crypts								
Calcium vs no calcium								
No calcium	12	2,917 (1,462 - 4,372)		3,119 (1,931 - 4,307)				
Calcium	18	3,117 (1,929 - 4,305)	0.829	3,119 (1,931 - 4,307)	0.686	174 (-732 - 1,081)	0.697	0.94
Vitamin D vs no vitamin D								
No vitamin D	24	3,339 (2,479 - 4,199)		3,455 (2,595 - 4,315)				
Vitamin D	29	3,042 (2,260 - 3,825)	0.611	2,937 (2,155 - 3,720)	0.376	-221 (-998 - 556)	0.571	0.93
calcium + vitamin D vs Calcium								
Calcium	20	3,465 (2,540 - 4,389)		3,513 (2,589 - 4,438)				
Calcium + vitamin D	21	3,050 (2,148 - 3,952)	0.520	3,090 (2,188 - 3,993)	0.512	-8 (-987 - 971)	0.987	1.00

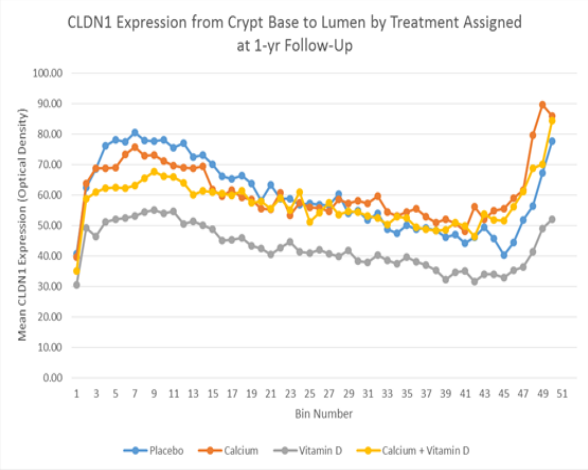
Figure

Figure 1.

A.



B.



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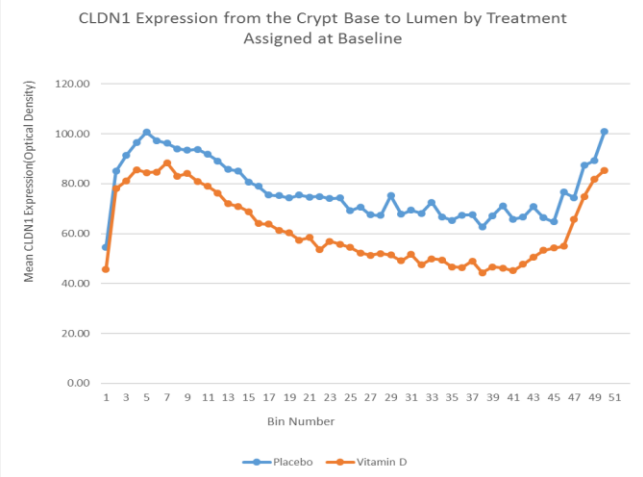

D.

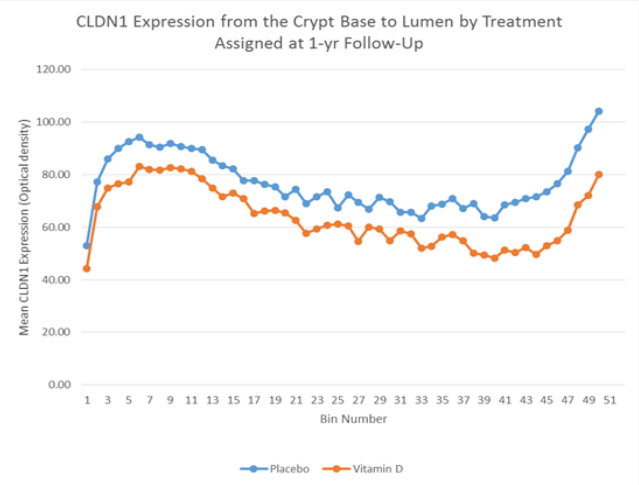

Figure 1. CLDN1 Expression from Crypt Base to Crypt Lumen by Treatment Assigned in Normal-Appearing Colorectal Mucosa. (A) at baseline in the 4-arm treatment group (B) at 1-yr follow-up in the 4-arm treatment group (C) at baseline in the 2-arm treatment group (D) at 1-yr follow-up in the 2-arm treatment group.

Appendix

Supplementary Table 1.

Table S1. Expression of CLDN1 in the Normal-Appearing Colorectal Mucosa of Study Participants (n=105) by Treatment Assigned[£]

		Baseline		1-Yr Follow-Up		Treatment Effect		
						Absolute Effect[b]		Relative Efffect[d]
Treatment Group	n	Mean (95% CI)	*p* -value[b]	Mean (95% CI)	*p* -value[b]	Mean (95%CI)	*p* -value[b]	
Whole crypts								
4-Arm								
Placebo	12	2,698 (1,554 - 3,843)		3,003 (1,858 - 4,147)				
Calcium	16	2,540 (1,504 - 3,575)	0.829	2,972 (1,936 - 4,007)	0.966	128 (-728 , 983)	0.766	1.05
Vitamin D	17	2,118 (1,150 - 3,086)	0.424	1,990 (1,022 - 2,957)	0.165	-433 (-1,277 , 412)	0.310	0.84
Calcium+Vitamin D	18	2,333 (1,356 - 3,309)	0.612	2,779 (1,802 - 3,755)	0.756	142 (-693 , 977)	0.735	1.07
2-Arm								
Placebo	23	4,261 (3,521 - 5,002)		4,245 (3,505 - 4,985)				
Vitamin D	19	3,275 (2,476 - 4,075)	0.076	3,361 (2,561 - 4,160)	0.110	102 (-950 , 1,153)	0.846	1.03
Upper 40% of crypts								
4-Arm								
Placebo	12	826 (393 - 1,258)		967 (534 - 1,399)				
Calcium	16	952 (561 - 1,343)	0.650	1,100 (709 - 1,492)	0.630	-7 (-331 , 346)	0.965	0.99
Vitamin D	17	772 (406 - 1,138)	0.844	671 (305 - 1,037)	0.282	-242 (-576 , 92)	0.153	0.74
Calcium+Vitamin D	18	836 (467 - 1,205)	0.969	1,003 (634 - 1,372)	0.893	26 (-304 , 356)	0.875	1.02
2-Arm				0 (0 - 0)				
Placebo	23	1,513 (1,233 - 1,793)		1,544 (1,264 - 1,824)				
Vitamin D	19	1,101 (798 - 1,404)	0.051	1,142 (838 - 1,445)	0.056	9 (-429 , 447)	0.966	1.02
Lower 60% of crypts								
4-Arm								
Placebo	12	1,717 (1,063 - 2,370)		1,847 (1,194 - 2,501)				
Calcium	16	1,399 (808 - 1,990)	0.450	1,673 (1,082 - 2,263)	0.677	143 (-368 , 653)	0.579	1.11
Vitamin D	17	1,196 (644 - 1,749)	0.211	1,212 (659 - 1,765)	0.128	-115 (-619 , 389)	0.650	0.94
Calcium+Vitamin D	18	1,322 (765 - 1,880)	0.340	1,585 (1,028 - 2,143)	0.525	133 (-366 , 631)	0.597	1.11
2-Arm								
Placebo	23	2,473 (2,039 - 2,908)		2,413 (1,979 - 2,848)				
Vitamin D	19	1,960 (1,491 - 2,429)	0.114	2,008 (1,538 - 2,477)	0.209	107 (-485 , 700)	0.716	1.05
ϕh [e]								
4-Arm								
Placebo	12	30 (27 - 34)		32 (29 - 36)				
Calcium	16	38 (35 - 41)	0.001	38 (35 - 41)	0.018	-2 (-7 , 3)	0.40	0.93
Vitamin D	17	35 (32 - 38)	0.021	34 (31 - 37)	0.505	-4 (-9 , 1)	0.13	0.89
Calcium+Vitamin D	18	35 (32 - 38)	0.028	37 (34 - 40)	0.033	0 (-5 , 5)	0.95	0.99
2-Arm								
Placebo	23	36 (33 - 38)		36 (34 - 38)				
Vitamin D	19	32 (30 - 35)	0.0485	33 (31 - 36)	0.1308	0 (-2 , 3)	0.9153	1.02

CI, confidence interval.

[b] *p* -value for the difference between each treatment group and placebo group from repeated-measures MIXED model.

[c] Absolute effect= [(treatment group follow-up)-(treatment group baseline)]-[(placebo group follow-up)-(placebo group baseline)].

[d] Relative effect=[(treatment group follow-up)/(treatment group baseline)]/[(placebo group follow-up)/(placebo group baseline)]; interpretation similar to that for an odds ratio.

[e] ϕh , proportion (%) of CLDN1 density in the differentiating zone (upper 40%)

[£] Adjusted for age, sex, and study center.

Supplementary Table 2.

Table S2. Expression of CLDN1 in the Normal-Appearing Colorectal Mucosa of Study Participants (n=105)* by Treatment Assigned[£]

		Baseline		1-Yr Follow-Up		Treatment Effect		
						Absolute Effect[c]		Relative Efffect[d]
Treatment Group	n*	Mean (95% CI)	*p*-value[b]	Mean (95% CI)	*p*-value[b]	Mean (95% CI)	*p*-value[b]	
Whole crypts								
4-Arm								
Placebo	12	2,393 (975 - 3,811)		2,697(1,279 - 4,115)				
Calcium	16	2,329 (938 - 3,188)	0.943	2,761(1,370 - 4,152)	0.943	128(-728 , 983)	0.766	1.05
Vitamin D	17	1,810 (432 - 3,720)	0.489	1,682(304 - 3,060)	0.230	-433(-1,277 , 412)	0.310	0.82
Calcium+Vitamin D	18	2,149 (773 - 3,526)	0.787	2,595(1,219 - 3,972)	0.910	142(-693 , 977)	0.735	1.07
2-Arm								
Placebo	23	4,540 (3,256 - 5,824)		4,524(3,240 - 5,807)				
Vitamin D	19	3,911 (2,714 - 5,108)	0.321	3,996(2,800 - 5,193)	0.405	102(-950 , 1,154)	0.846	1.03
Upper 40% of crypts								
4-Arm								
Placebo	12	704 (170 - 1,239)		845(310 - 1,379)				
Calcium	16	856 (332 - 1,380)	0.651	1,004(480 - 1,528)	0.635	-7(-331 , 346)	0.965	0.98
Vitamin D	17	636 (117 - 1,155)	0.831	535(16 - 1,054)	0.331	-242(-576 , 92)	0.153	0.70
Calcium+Vitamin D	18	749 (230 - 1,267)	0.896	915(397 - 1,434)	0.836	26(-304 , 356)	0.875	1.02
2-Arm								
Placebo	23	1,599 (1,123 - 2,075)		1,630(1,154 - 2,106)				
Vitamin D	19	1,299 (853 - 1,744)	0.209	1,339(894 - 1,785)	0.224	9(-429 , 448)	0.966	1.01
Lower 60% of crypts								
4-Arm								
Placebo	12	1,553 (744 - 2,362)		1,684(875 - 2,493)				
Calcium	16	1,320 (527 - 2,114)	0.647	1,593(800 - 2,387)	0.859	143(368 , 653)	0.579	1.11
Vitamin D	17	1,064 (279 - 1,850)	0.311	1,080(294 - 1,866)	0.212	-115(-619 , 389)	0.650	0.94
Calcium+Vitamin D	18	1,260 (475 - 2,045)	0.569	1,523(738 - 2,308)	0.755	133(-366 , 631)	0.597	1.12
2-Arm								
Placebo	23	2,626 (1,870 - 3,383)		2,566(1,810 - 3,323)				
Vitamin D	19	2,359 (1,655 - 3,063)	0.471	2,406(1,702 - 3,111)	0.666	107(-486 , 700)	0.716	1.04
ϕh[e]								
4-Arm								
Placebo	12	30 (25 - 34)		32(28 - 36)				
Calcium	16	37 (33 - 40)	0.008	37(33 - 41)	0.064	-2.16(-7.20 , 2.88)	0.3954	0.93
Vitamin D	17	34 (30 - 38)	0.088	32(29 - 36)	0.858	-3.81(-8.79 , 1.17)	0.1311	0.89
Calcium+Vitamin D	18	33 (29 - 37)	0.148	36(32 - 39)	0.165	-0.16(-5.08 , 4.76)	0.9497	0.99
2-Arm								
Placebo	23	36 (32 - 39)		36(33 - 40)				
Vitamin D	19	33 (29 - 36)	0.068	33(30 - 37)	0.155	0.76(-2.98 , 4.51)	0.6819	1.02

* 3 patient has missing information on total energy intake and total dietary fiber intake (2 from placebo group 4-arm and 1 from calcium group 4-arm) and 1 patient has missing information in vitamin d group 2-arm. Determined the median of these missing value for the treatment group by gender and visitnumber to include into anlysis.

[b] *p*-value for the difference between each treatment group and placebo group from repeated-measures MIXED model.

[c] Absolute effect= [(treatment group follow-up)-(treatment group baseline)]-[(placebo group follow-up)-(placebo group baseline)].

[d] Relative effect=[(treatment group follow-up)/(treatment group baseline)]/[(placebo group follow-up)/(placebo group baseline)]; interpretation similar to that for an odds ratio.

[e] ϕh, proportion (%) of CLDN1 density in the differentiation zone (upper 40%)

[£] Adjusted for age, sex, study center, smoking, multivitamin use, physical activity, total energy intake and total dietary fiber intake.