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Effects of Calcium and Vitamin D₃ on Transforming Growth Factors in Rectal Mucosa of Sporadic Colorectal Adenoma Patients: A Randomized Controlled Trial

Huakang Tu^{1,2}, W. Dana Flanders^{1,3,4}, Thomas U. Ahearn⁵, Carrie R. Daniel⁶, Amparo G. Gonzalez-Feliciano¹, Qi Long^{3,4}, Robin E. Rutherford⁷, and Roberd M. Bostick^{1,4,*}

¹Department of Epidemiology, Rollins School of Public Health, Emory University, Atlanta, Georgia

²Molecules to Mankind Program, Laney Graduate School, Emory University, Atlanta, Georgia

³Department of Biostatistics and Bioinformatics, Rollins School of Public Health, Emory University, Atlanta, Georgia

⁴Winship Cancer Institute, Emory University, Atlanta, Georgia

⁵Nutrition and Health Sciences, Graduate Division of Biological and Biomedical Sciences, Laney Graduate School, Emory University, Atlanta, Georgia

⁶Department of Epidemiology, Division of OVP, Cancer Prevention and Population Sciences, The University of Texas MD Anderson Cancer Center, Houston, Texas

⁷Division of Digestive Diseases, Department of Medicine, School of Medicine, Emory University, Atlanta, Georgia

Abstract

Transforming growth factor alpha (TGF α) and TGF β ₁ are growth-promoting and -inhibiting autocrine/paracrine growth factors, respectively, that may (1) affect risk for colorectal cancer and (2) be modifiable by anti-proliferative exposures. The effects of supplemental calcium and vitamin D₃ on these two markers in the normal-appearing colorectal mucosa in humans are unknown. We conducted a pilot, randomized, double-blind, placebo-controlled, 2 $\times$ 2 factorial clinical trial (n = 92; 23/treatment group) of calcium 2 g and/or vitamin D₃ 800 IU/d versus placebo over 6 mo. TGF α and TGF β ₁ expression was measured in biopsies of normal-appearing rectal mucosa using automated immunohistochemistry and quantitative image analysis at baseline and 6-mo follow-up. In the calcium, vitamin D₃, and calcium plus vitamin D₃ groups relative to the placebo group (1) the mean overall expression of TGF β ₁ increased by 14% (P = 0.25), 19% (P = 0.17), and 22% (P = 0.09); (2) the ratio of TGF α expression in the upper 40% (differentiation zone) to that in the lower 60% (proliferation zone) of the crypts decreased by 34% (P = 0.11), 31% (P = 0.22), and

*Correspondence to: Roberd M. Bostick, Department of Epidemiology, Rollins School of Public Health, Emory University, 1518 Clifton Road, NE, Atlanta, GA 30322.

Authors' contributions: R.M.B. designed and developed the research and conducted the study; R.M.B., W.D.F., and Q.L. developed the methodology; R.M.B. oversaw the study and provided administrative, technical, or material support; C.R.D., A.G.G.F., and R.E.R. collected the data; H.T., T.U.A., and R.M.B. performed data analyses; H.T., R.M.B., W.D.F., and T.U.A. drafted the manuscript; and all authors reviewed and approved the final content of the manuscript.

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26% ($P = 0.33$); and (3) the TGF α /TGF β_1 ratio in the upper 40% of the crypts decreased by 28% ($P = 0.09$), 14% ($P = 0.41$), and 22% ($P = 0.24$), respectively. These preliminary results, although not statistically significant, suggest that supplemental calcium and vitamin D₃ may increase TGF β_1 expression and shift TGF α expression downward from the differentiation to the proliferation zone in the crypts in the normal-appearing colorectal mucosa of sporadic colorectal adenoma patients, and support further investigation in a larger clinical trial.

Keywords

colorectal neoplasms; calcium; vitamin D₃; transforming growth factor alpha (TGF α); transforming growth factor beta 1 (TGF β_1)

INTRODUCTION

Colorectal cancer remains the second most common cause of cancer death in the United States [1], and it is widely accepted that most colorectal carcinomas develop from adenomas [2]. Modifications in diet and lifestyle have been proposed to reduce colorectal cancer incidence and mortality [3]. However, clinical trials of diet and lifestyle interventions with colorectal cancer incidence or mortality as the endpoints are limited due to the extended time to develop colorectal cancer and the large sample sizes and high costs involved. Therefore, modifiable pre-neoplastic biomarkers of risk for colorectal neoplasms that could be used as surrogate endpoints to investigate the potential efficacy of preventive interventions in short-term clinical trials are needed.

Calcium and vitamin D are two plausible and evidentially well-supported dietary preventive agents against colorectal neoplasms. Observational epidemiological studies have consistently found calcium intake to be inversely associated with colorectal cancer risk [4], and calcium supplementation reduced sporadic colorectal adenoma recurrence [5]. Higher serum 25-OH-vitamin D concentrations, in a limited number of observational studies, have been consistently associated with lower risk for colorectal cancer [6] and adenomas [7]. The three most prominent mechanisms of calcium against colorectal cancer include protection of the colorectal mucosa against bile acids, direct effects on the cell cycle, and modulation of E-cadherin and β -catenin expression in the APC colon carcinogenesis pathway [8]. The four most prominent mechanisms for vitamin D include bile-acid catabolism, direct effects on the cell cycle, growth-factor signaling, and immunomodulation [8]. As with calcium, these potential mechanisms are probably complementary. Indeed, we previously reported that, in the same clinical trial reported herein, calcium and/or vitamin D₃ supplementation favorably modulated biomarkers of their metabolism [9], apoptosis [10], proliferation and differentiation [11], DNA damage [12], DNA mismatch repair [13], inflammation [14], and APC- β -catenin signaling [15].

Transforming growth factor alpha (TGF α) and transforming growth factor beta 1 (TGF β_1) are autocrine/paracrine growth factors that are classically thought of as potent promoters and inhibitors of cell growth, respectively, in normal tissues [16,17], and likely contribute to or at least affect colorectal carcinogenesis [18]. To our knowledge, no other human studies have assessed the effects of calcium and vitamin D₃ supplementation on the expression of

these two markers in the normal human colorectal mucosa. The goal of the present study was to estimate the effects of supplemental calcium and vitamin D₃ on TGF α and TGF β ₁ expression in the normal-appearing colorectal mucosa of sporadic colorectal adenoma patients.

MATERIALS AND METHODS

This study was approved by the Emory University Institutional Review Board. Written informed consent was obtained from each study participant.

Participant Population

The detailed protocol of this pilot, randomized, double-blind, placebo-controlled, 2 $\times$ 2 factorial clinical trial was published previously [10]. Briefly, all participants were recruited from patients attending the Digestive Diseases Clinic of The Emory Clinic, Emory University. Inclusion criteria included 30–75 yr of age, in general good health, capable of informed consent, and a history of at least one pathology-confirmed adenomatous colorectal polyp within the past 36 mo. Exclusion criteria included contraindications to calcium or vitamin D supplementation or rectal biopsy procedures, and medical conditions, habits, or medication usage that potentially could interfere with interpretation of the study results [10].

Clinical Trial Protocol

The detailed protocol for the clinical trial was published previously [10]. Briefly, between April 2005 and January 2006, 105 potential participants attended an eligibility visit during which they were interviewed; signed a consent form; their medication and nutritional supplement bottles reviewed; questionnaires (on sociodemographics, medical history, medication and nutrition supplement use, lifestyle, family history, and others) completed; and a blood sample procured. Diet was assessed with a semi-quantitative food frequency questionnaire [19]. Medical and pathology records were reviewed. Those still eligible and willing to participate entered a 30-d placebo run-in trial. Participants ($n = 92$) with no significant perceived side effects and who took $\geq 80\%$ of their assigned tablets were randomly assigned, stratified by sex and NSAID use, to the following four treatment groups ($n = 23/\text{group}$): placebo, 2.0 g elemental calcium supplementation (as calcium carbonate in equal doses twice daily), 800 IU vitamin D₃ supplementation (400 IU twice daily), and 2.0 g elemental calcium plus 800 IU vitamin D₃ supplementation. Study tablets were custom manufactured by Tishcon Corp. (Salisbury, MD). The corresponding supplement and placebo pills, which were taken with meals, were identical in size, appearance, and taste. The chosen calcium dose was at the upper range at which no side effects would be likely, and the chosen vitamin D dose was twice the Recommended Daily Allowance (RDA) for most adults at the time the study was conceived (2002). Additional details on the rationale for the doses and forms of calcium and vitamin D₃ supplements were previously published [10].

Participants were instructed to maintain their usual diet and not take any nutritional supplements that they were not taking at the time of entry into the study.

Over the 6-mo treatment period, participants attended two follow-up visits, which were 1 and 6 mo after randomization. At both follow-up visits, participants were asked about adherence and adverse events by questionnaire, interview, and pill count. At the final 6-mo follow-up, participants again underwent a rectal biopsy and provided a blood sample.

Six approximately 1.0 mm-thick biopsy specimens were taken from the normal appearing rectal mucosa 10 cm above the level of the external anal aperture through a rigid sigmoidoscope with a jumbo cup flexible biopsy forceps. No biopsy was taken within 4.0 cm of a polypoid lesion. Biopsies were placed onto a strip of bibulous paper and immediately placed in phosphate buffered saline, oriented, transferred to 10% normal buffered formalin for 24 h, and then transferred to 70% ethanol. Then, biopsies were processed and embedded in paraffin blocks within a week (2 blocks of 3 biopsies each per participant, per biopsy visit), cut and stained within another 4 wk, and analyzed within another 4 wk.

Immunohistochemistry Protocol

From each block, five slides, with 4 levels of 3.0- μ m-thick biopsy sections (taken 40 μ m apart) on each slide, were prepared for each antigen, yielding a total of 20 levels for each antigen. Heat-mediated antigen retrieval was performed by steaming the slides in a preheated Pretreatment Module (Lab Vision Corp., Fremont, CA) with 100 $\times$ Citrate Buffer (pH 6.0; DAKO S1699; DAKO Corp.) for 40 min. Then, the slides were immunohistochemically processed in a DAKO Automated Immunostainer (DAKO Corp., Carpinteria, CA) using a labeled streptavidin–biotin method (TGF α antibody manufactured by Calbiochem (KGaA, Darmstadt, Germany), catalog No. GF10, dilution 1:100; TGF β ₁ antibody manufactured by Santa Cruz (Dallas, TX), catalog No. sc-146, dilution 1:75), but not counterstained. The processed slides were coverslipped with a Leica CV5000 Coverslipper (Leica Microsystems, Inc., Buffalo Grove, IL). Each staining batch contained approximately equal numbers of participants from each treatment group. Positive and negative control slides were included in each staining batch.

Protocol for Quantifying Labeling Optical Densities of Immunohistochemically Detected Biomarkers in Normal Rectum Crypts (“Scoring”)

A quantitative image analysis method to quantify the labeling optical densities (“expression”) of the immunohistochemically-detected biomarkers in normal rectal crypts was described in detail previously [10,13]. Briefly, the image analysis unit was a “hemicypt”, defined as one side of a rectal crypt bisected from base to rectal lumen surface. A “scorable” hemicypt was defined as an intact hemicypt extending from the muscularis mucosa to the colon lumen. Before image analysis, staining adequacy was checked by examining the batch’s negative and positive control slides.

Before scoring, the scorer, who was blinded to the intervention assignment, selected the two of the three biopsies with the greatest number of scorable hemicypts, captured background correction images for each slide, and captured 16-bit grayscale images of crypts at 200 $\times$ magnification. Then, the scorer traced the borders of the “hemicypt” in the image analysis program (Fig. 1). The program then segmented the traced hemicypt, and the background-

adjusted optical density of the labeling across the whole hemicrypt and within each segment was measured and exported to a Microsoft Access database. The goal was to score 16–20 hemicrypts per biopsy visit for each biomarker.

Reliability was assessed by selecting samples of previously analyzed slides (10%) to be re-analyzed by the same scorer. The scorer was blinded to the selection. Intra-reader reliability for TGF α and TGF β_1 was above 0.90 throughout.

Statistical Analysis

All statistical analyses were performed using SAS 9.3 statistical software (SAS Institute, Inc., Cary, NC). A P value ≤ 0.05 (two-sided) was considered statistically significant. Treatment groups were assessed for comparability of characteristics at baseline by the Fisher's exact test for categorical variables and analysis of variance for continuous variables. Slide scoring reliability was analyzed using intra-class correlation coefficients.

The mean biomarker expression in each study participant, at baseline and 6-mo follow-up, was calculated by averaging the biomarker expression on all the analyzed hemicrypts. To adjust for possible staining batch effects, batch-standardized mean biomarker expression was calculated by dividing an individual participant's biomarker expression by the mean biomarker expression on all the participants in the same batch [10]. To represent distinct functional zones of rectal crypts, the upper 40% of the crypts (differentiation zone) and the lower 60% of the crypts (proliferation zone) were selected *a priori* as measures of crypt biomarker distribution [20]. A TGF α /TGF β_1 ratio was calculated as an indicator of the balance between the growth-promoting and -inhibiting factors by dividing the mean batch standardized level of TGF α by that of TGF β_1 ; a higher ratio, thus, would indicate a more pro-growth balance.

The distributions of batch-standardized TGF α and TGF β_1 labeling optical densities along the full length of the hemicrypts were graphically plotted and modeled using the LOESS procedure. First, each hemicrypt was standardized to 50 sections. Then, the batch-standardized average of each section across all hemicrypts was calculated and predicted by the LOESS model separately for each treatment group, by visit. The results were graphically plotted along with smoothing lines. Although the plots illustrate the distribution of expression, they do not provide a complete analysis of treatment effects because they do not account for changes in the placebo group. Based on graphical assessments, an upper 40% to lower 60% ratio was created *a posteriori* for TGF α .

Primary analyses were based on randomization treatment assignment, regardless of adherence status (intent-to-treat analysis). Treatment effects were evaluated by assessing the differences in the batch-adjusted biomarker expression from baseline to the 6-mo follow-up between participants in the active treatment groups and those in the placebo group by a repeated-measures linear MIXED effects model. The model included the intercept, treatment groups, visit (baseline and follow-up), and a treatment group by visit effect interaction term. Because optical density is measured in arbitrary units, to provide perspective on the magnitude of the treatment effects, we also calculated relative effects. The relative effect was calculated as the (treatment group at follow-up/treatment group at baseline)/(placebo

group at follow-up/placebo group at baseline). The relative effect provides a conservative estimate of the proportional change in the treatment group relative to that in the placebo group, and its interpretation is somewhat analogous to that of an odds ratio (e.g., a relative effect of two would mean that the proportional change in the treatment group was two times that in the placebo group) [10,21].

RESULTS

Characteristics of Study Participants

The mean age of study participants was 61 yr, 70% were men, 71% were white, and 20% had a family history of colorectal cancer in a first degree relative. The treatment groups were balanced on baseline characteristics except that there were higher proportions of regular aspirin use in the calcium and calcium plus vitamin D groups (Table 1). Average adherence to visit attendance was 92% and did not significantly differ among the four treatment groups. On average, at least 80% of pills were taken by 93% of participants at the first follow-up visit and by 84% of participants at the final follow-up visit. No adverse events were attributed to study procedures or treatments. Seven participants (8%) were lost to follow-up. Dropouts included one person from the vitamin D₃ supplementation group and two from each of the other three groups. Adequate biopsy specimens for image analysis for TGF α and TGF β ₁ were available for 84 and 86 participants at baseline and for 84 and 83 participants after a 6-mo follow-up, respectively.

Baseline serum 25-OH-vitamin D concentrations did not differ between the four treatment groups. At the 6-mo follow-up, serum 25-OH-vitamin D concentrations had increased 60% ($P < 0.0001$) and 56% ($P < 0.0001$) in the vitamin D₃ and calcium plus vitamin D₃ groups, respectively, relative to placebo [10]; however, mean post-treatment serum 25-OH-vitamin D concentrations remained below 30 ng/ml in all treatment groups (17.9, 23.2, 29.5, and 28.5 ng/ml in the placebo, calcium, vitamin D, and calcium plus vitamin D groups, respectively).

Effects on TGF α

At baseline, TGF α expression in the rectal mucosa did not differ significantly among the four treatment groups (Table 2). In the graphical assessment (Fig. 2), TGF α expression in all active treatment groups during the trial appeared to decrease in the approximate upper 40% (differentiation zone) of the crypts, but increase in the approximate lower 60% (proliferative zone) of the crypts. In the numerical assessment (Table 2), while TGF α expression in the whole crypts did not change substantially in the calcium, vitamin D, and calcium plus vitamin D groups, it decreased by 14% ($P = 0.35$), 2% ($P = 0.86$), and 1% ($P = 0.93$), respectively, in the upper 40% of the crypts, but increased by 29% ($P = 0.30$), 38% ($P = 0.17$), and 25% ($P = 0.32$) in the bottom 60% of the crypts. To quantify the apparent TGF α expression crypt zone shift seen in the graphical assessment, we created an upper 40% to lower 60% ratio and found that it decreased by 34% ($P = 0.11$), 31% ($P = 0.22$), and 26% ($P = 0.33$) in the calcium, vitamin D, and calcium plus vitamin D groups, respectively, relative to the placebo group.

Effects on TGF β ₁

At baseline, TGF β ₁ expression in the rectal mucosa did not differ significantly among the four treatment groups (Table 2). In the graphical assessment (Fig. 3), TGF β ₁ expression in all active treatment groups during the trial appeared to increase relatively uniformly throughout the lengths of the crypts. In the numerical assessment (Table 2), the mean overall expression of TGF β ₁ in the whole crypts increased by 14% ($P = 0.25$), 19% ($P = 0.17$), and 22% ($P = 0.09$) in the calcium, vitamin D, and calcium plus vitamin D groups, respectively, relative to the placebo group. Reflecting the graphical findings, the respective changes in the differentiation and proliferation zones of the crypts were similar to those for the whole crypts (Table 2).

Effects on TGF α /TGF β ₁ Ratios

After 6 mo of treatment, the TGF α /TGF β ₁ ratio in the whole crypts decreased by 14% ($P = 0.46$) and 11% ($P = 0.09$) in the calcium and calcium plus vitamin D groups, respectively, but increased by 2% ($P = 0.93$) in the vitamin D group, relative to the placebo group. In the upper 40% of the crypts, the TGF α /TGF β ₁ ratio decreased by 28% ($P = 0.09$), 14% ($P = 0.41$), and 22% ($P = 0.24$) in the calcium, vitamin D, and calcium plus vitamin D groups, respectively, relative to the placebo group; however, in the bottom 60% of the crypts, the TGF α /TGF β ₁ ratio increased by 16% ($P = 0.61$) and 30% ($P = 0.36$) in the calcium and vitamin D groups, but decreased by 2% ($P = 0.94$) in the calcium plus vitamin D group, relative to the placebo group (Table 2). A data-derived TGF α upper 40% to lower 60%/TGF β ₁ ratio that was created *a posteriori* as a best discriminator of the intervention effects decreased by 48% ($P = 0.08$), 44% ($P = 0.12$), and 35% ($P = 0.28$) in the calcium, vitamin D, and calcium plus vitamin D groups, respectively, relative to the placebo group.

Sensitivity Analyses

Neither multiple imputation to impute missing observations nor adjusting the analyses for baseline aspirin and/or NSAID use, serum 25-OH-vitamin D concentrations, and calcium intake appreciably changed our findings.

DISCUSSION

Our preliminary results, although not statistically significant, suggest that calcium and/or vitamin D₃ supplementation over 6 mo may increase the overall expression of TGF β ₁ and shift TGF α expression downward into the proliferation zone in the colorectal crypts in the normal-appearing colorectal mucosa of sporadic colorectal adenoma patients, and support a larger study to investigate this hypothesis further as well as other investigations of whether calcium and vitamin D₃ may reduce colorectal cancer risk, in part, by modulating growth factors.

Currently, there are no widely accepted pre-neoplastic biomarkers of risk for colorectal neoplasms. One potential biomarker is colorectal epithelial cell proliferation, which is regulated by growth factors [16]. Compared to patients at lower risk for colon cancer, patients with colon cancer and patients in every category known to be at higher risk for colon cancer on average, exhibit in their normal-appearing mucosa both an increased

colorectal epithelial cell proliferation rate and an extension of the colon crypt proliferative zone from the lower (basal) 60% of the crypt to include the upper (luminal) 40% of the crypt [22]. In patients with previous colon cancer or sporadic adenomas, these changes also predicted adenoma recurrence [23,24].

It was previously reported that, in the normal colorectal mucosa, immunohistochemically-detected TGF α was denser in the upper one-third to two thirds of colonic crypts [25–27] (differentiation zone), and our staining pattern was consistent with these findings. Consistent with its pro-growth and hyperproliferative role, TGF α expression was found to be greater in the normal-appearing rectal mucosa of sporadic colorectal adenoma patients than in adenoma-free patients [28], in colorectal adenomas and cancer [29,30], and in the blood of colorectal cancer patients [31,32]. In our study we found no substantial changes in the overall expression of TGF α in whole crypts in the normal-appearing colorectal mucosa after calcium and/or vitamin D₃ supplementation; however, our results suggest that calcium and/or vitamin D₃ supplementation may shift TGF α expression downward into the colorectal crypt proliferation zone. These findings are consistent with those we previously reported on the effects of calcium and vitamin D on cell proliferation markers. In the first trial ($n = 192$), we found that calcium supplementation, without changing the proliferation rate, substantially, statistically significantly decreased the proportion of proliferating cells in the upper 40% of the crypts relative to the whole crypts in the rectal mucosa of sporadic adenoma patients [21]. From the same trial reported herein, we found that calcium plus vitamin D₃ may shift downwards the expression of hTERT, a marker of long-term proliferation, in the crypt proliferation zone without affecting its overall expression [11]. Furthermore, reports from other groups also indicated that calcium could decrease cell proliferation in the upper 40% of the crypt, but not the overall cell proliferation rate [33,34]. The findings from the present study suggest that calcium and vitamin D may promote confinement of proliferating cells to the proliferation zone, at least in part, via modulating TGF α expression.

While the mechanism for the shift of TGF α expression downwards into the proliferation zone by calcium and vitamin D is unclear, this downward shift may reduce risk for colorectal neoplasms. First, a downward shift of TGF α may reduce the number of dividing cells in the luminal pole of the crypt. DNA is more susceptible to damage during cell division, and cells at the luminal pole of the crypt are more likely to be exposed to carcinogens in the colon lumen. Second, it has been proposed that colorectal adenomas originate from the upper crypt surface [35], and TGF α was found to be the main survival factor for early adenoma cells against apoptosis [36], so a downward shift of TGF α may decrease the likelihood of developing colorectal adenomas. Alternatively, it is possible that the downward shift in TGF α could stimulate initiated cells in the proliferation zone to grow faster and thus promote colorectal carcinogenesis; however, given that supplemental calcium statistically significantly reduced adenoma recurrence in a large, randomized controlled trial [37], this seems less likely.

No previous human studies tested the effects of supplemental calcium and/or vitamin D on TGF α expression in the normal colorectal mucosa; however, in two small ($n = 13$ and 10) clinical trials, cellulose [26] and 81 mg of aspirin [38] statistically significantly decreased

the percentage of TGF α expression-positive cells in the normal colorectal mucosa of adenoma patients.

TGF β_1 immunoreactivity was previously reported to localize mainly in the upper third of the crypts of the normal colorectal mucosa [39]. TGF β_1 signaling, which is complex in cancer progression, regulates cell proliferation, apoptosis, autophagy, inflammation, tumor angiogenesis, and metastasis [40]. A dual role of TGF β_1 has been proposed, because TGF β_1 suppresses the growth of normal epithelial cells but promotes tumor metastasis in later stages of cancer [40]. In a mouse model, the absence of TGF β_1 expression promoted the progression from hyperplasia to adenoma and allowed the development of carcinoma [41]. Our study suggests that calcium and/or vitamin D₃ supplementation may increase TGF β_1 expression in the normal-appearing colorectal mucosa where TGF β_1 should function as a cell growth suppressor. Findings from studies in cell lines and animals suggested that calcium [42] and vitamin D [43–46] could induce TGF β_1 expression, and our results are consistent with these findings.

No previous human studies tested the effect of supplemental calcium and/or vitamin D on TGF β_1 expression in the normal colorectal mucosa, but one small ($n = 39$) randomized, controlled trial in multiple sclerosis patients found that 1,000 IU of supplemental vitamin D daily over 6 mo statistically significantly increased serum TGF β_1 levels [47]. The molecular mechanism by which vitamin D induces TGF β_1 expression is unclear. While one study identified two vitamin D response elements (VDREs) in the *TGF β_2* gene promoter region [48], it is unclear whether the *TGF β_1* gene promoter region contains a VDRE. Also, vitamin D stimulates activator protein-1 (AP-1) expression and enhances its binding to DNA [49], and AP-1 is a mediator of TGF β_1 autoinduction by binding to specific promoter elements in the *TGF β_1* gene [50].

Our study had several limitations. First, it was a pilot study with limited statistical power, especially for stratified analyses. Second, we collected biopsies only from the rectum; however, previous studies found that levels of cell proliferation markers in the rectum reflected those in other areas of the colon [51,52]. Third, we measured protein expression rather than protein activity, although measuring either is likely to represent protein function in normal tissue. Fourth, the dose of vitamin D supplementation used in the present study may have been insufficient; however, when the study was designed in 2002, 800 IU/d at twice the RDA was considered a bold choice. Finally, our participants were limited to sporadic colorectal adenoma patients and caution should be taken when generalizing our results to other populations.

The strengths of our study are: (i) to our knowledge, it is the first randomized, double-blind, placebo-controlled clinical trial to test the effects of calcium and/or vitamin D₃ supplementation on TGF α and TGF β_1 expression in the normal colorectal epithelium in sporadic adenoma patients; (ii) the high protocol adherence; and (iii) the automated immunostaining and newly designed image analysis software that allowed quantification of TGF α and TGF β_1 expression overall as well as their distributions within the colorectal crypts.

In summary, the results of this pilot clinical trial, although not statistically significant, suggest that calcium and/or vitamin D₃ supplementation over 6 mo may increase overall TGFβ₁ expression, and shift downwards (“normalize”) the expression of TGFα from the differentiation zone to the proliferation zone in the normal colorectal mucosa of sporadic colorectal adenoma patients, and support further investigation in a larger clinical trial. Taken together with previous literature that suggests that anti-carcinogenic effects of supplemental calcium and vitamin D₃ may, in part, depend on the ability of these agents to favorably modulate the expression of the TGFα and TGFβ₁ expression, our results also support further investigation of (1) calcium and vitamin D₃ as chemopreventive agents against colorectal neoplasms and (2) whether TGFα and TGFβ₁ expression could be used as modifiable biomarkers and surrogate endpoints to investigate the potential efficacy of preventive interventions against colorectal neoplasms.

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Abbreviations

TGFα	transforming growth factor alpha
TGFβ₁	transforming growth factor beta 1

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Figure 1.

Process of quantitative image analysis. (A) Identifying scorable crypts; (B) tracing the hemicrypt; (C) automated sectioning of the trace; and (D) automated quantification of TGF α labeling optical density in the whole hemicrypt and each section.

Figure 2. Standardized transforming growth factor alpha (TGFα) expression (labeling optical density) along normal colorectal crypts by treatment group (A - Placebo group, B - Calcium group, C - Vitamin D₃ group, D - Calcium plus Vitamin D₃ group) at baseline and 6-mo follow-up. The distributions were modeled and graphically plotted using the LOESS procedure.

Figure 3.

Standardized transforming growth factor beta 1 (TGF β ₁) expression (labeling optical density) along normal colorectal crypts by treatment group (A - Placebo group, B - Calcium group, C - Vitamin D₃ group, D -Calcium plus Vitamin D₃ group) at baseline and 6-mo follow-up. The distributions were modeled and graphically plotted using the LOESS procedure.

Table 1

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

Characteristics	Treatment group				P-value ^d
	Placebo (n = 23)	Calcium (n = 23)	Vitamin D ₃ (n = 23)	Calcium +Vit. D ₃ (n = 23)	
Demographics, medical history, habits, anthropometrics					
Age, yr	58.5 (8.2)	61.9 (8.2)	60.2 (8.1)	62.1 (7.5)	0.39
Men (%)	70	70	70	70	1.00
White (%)	74	83	65	61	0.40
College graduate (%)	65	64	57	44	0.53
History of colorectal cancer in 1° relative (%)	17	30	17	13	0.60
Take NSAID ^b regularly ^c (%)	22	13	4	13	0.43
Take aspirin regularly (%)	22	52	30	57	0.05
If woman (n = 28), taking estrogens (%)	4 (14)	4 (14)	4 (14)	9 (29)	1.00
Current smoker (%)	9	4	0	0	0.61
Take multivitamin (%)	30	30	26	39	0.86
Body mass index (BMI), kg/m ²	30.6 (7.2)	29.4 (5.5)	28.9 (5.6)	31.6 (6.0)	0.44
Mean dietary intakes ^d					
Total energy intake, kcal/d	1596 (528)	1788 (691)	1848 (821)	1845 (752)	0.59
Total ^e calcium, mg/d	619 (308)	746 (335)	843 (526)	824 (714)	0.41
Total ^e vitamin D, IU/d	277 (230)	336 (202)	360 (317)	415 (315)	0.40
Total fat, g/d	67 (32)	72 (35)	70 (32)	74 (28)	0.89
Dietary fiber, g/d	15 (7)	17 (9)	18 (9)	17 (11)	0.70
Alcohol intake, g/d	9 (14)	11 (15)	14 (18)	10 (20)	0.76
Serum vitamin D					
25-OH-vitamin D, ng/ml	20.4 (7.6)	25.7 (7.6)	21.0 (8.3)	20.9 (9.6)	0.12

^a By Fisher's exact test for categorical variables, and ANOVA for continuous variables.

^b Nonsteroidal anti-inflammatory drug.

^c At least once a week.

^d All nutrients energy adjusted using residual method.

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^c Diet plus supplements.
^{*} Data are given as means (SD) unless otherwise specified.

Table 2

Standardized Expression of Transforming Growth Factor Alpha (TGF α) and Transforming Growth Factor Beta 1 (TGF β ₁) in the Normal-Appearing Colorectal Mucosa During the Clinical Trial

Treatment Group	Baseline				6-mo follow-up				Absolute Rx effect				Relative effect ^c	
	n	Mean	Std Err	P	n	Mean	Std Err	P	n	Rx effect ^a	Std Err	P ^b		
(A) TGF α														
Whole crypts														
Placebo	20	1.06	0.13		21	1.00	0.04		18	0	—	—	1.00	
Calcium	23	1.00	0.08	0.71	21	0.97	0.08	0.74	21	0.03	0.16	0.87	1.03	
Vitamin D ₃	20	0.96	0.09	0.53	21	0.98	0.06	0.85	19	0.09	0.16	0.61	1.09	
Ca +Vit. D ₃	21	0.98	0.14	0.62	21	1.01	0.07	0.88	19	0.09	0.16	0.59	1.10	
Upper 40% of crypts														
Placebo	20	0.59	0.07		21	0.53	0.02		18	0	—	—	1.00	
Calcium	23	0.67	0.06	0.42	21	0.52	0.04	0.79	21	-0.09	0.10	0.35	0.86	
Vitamin D ₃	20	0.61	0.06	0.84	21	0.54	0.03	0.89	19	-0.01	0.10	0.86	0.98	
Ca +Vit. D ₃	21	0.59	0.08	0.95	21	0.53	0.03	0.97	19	-0.01	0.10	0.93	0.99	
Lower 60% of crypts														
Placebo	20	0.47	0.08		21	0.47	0.02		18	0	—	—	1.00	
Calcium	23	0.34	0.04	0.13	21	0.44	0.04	0.53	21	0.10	0.10	0.30	1.29	
Vitamin D ₃	20	0.35	0.06	0.18	21	0.48	0.04	0.82	19	0.13	0.10	0.17	1.38	
Ca +Vit. D ₃	21	0.39	0.07	0.34	21	0.48	0.04	0.83	19	0.10	0.10	0.32	1.25	
Top 40% to bottom 60% of the crypts														
Placebo	20	1.69	0.28		21	1.17	0.06		18	0.00	—	—	1.00	
Calcium	23	2.79	0.45	0.07	21	1.27	0.07	0.29	21	-0.99	0.63	0.11	0.66	
Vitamin D ₃	20	2.53	0.43	0.18	21	1.20	0.07	0.74	19	-0.80	0.64	0.22	0.69	
Ca +Vit. D ₃	20	2.28	0.51	0.34	20	1.17	0.08	0.99	19	-0.59	0.64	0.33	0.74	
(B) TGF β ₁														
Whole crypts														
Placebo	21	1.04	0.06		21	1.00	0.02		19	0	—	—	1.00	
Calcium	22	1.03	0.06	0.95	21	1.13	0.07	0.12	20	0.14	0.13	0.25	1.14	

Treatment Group	Baseline			6-mo follow-up				Absolute Rx effect				Relative effect ^c	
	n	Mean	Std Err	P	n	Mean	Std Err	P	n	Rx effect ^a	Std Err		P ^b
Vitamin D ₃	22	0.93	0.07	0.23	20	1.07	0.06	0.42	19	0.17	0.13	0.17	1.19
Ca + Vit. D ₃	21	1.00	0.05	0.64	21	1.18	0.07	0.04	19	0.22	0.13	0.09	1.22
Upper 40% of crypts													
Placebo	21	0.39	0.02		21	0.38	0.01		19	0	—	—	1.00
Calcium	22	0.40	0.02	0.83	21	0.43	0.03	0.11	20	0.04	0.05	0.35	1.11
Vitamin D ₃	22	0.35	0.03	0.25	20	0.41	0.03	0.34	19	0.07	0.05	0.15	1.20
Ca + Vit. D ₃	21	0.39	0.02	0.86	21	0.45	0.02	0.03	19	0.08	0.05	0.12	1.20
Lower 60% of crypts													
Placebo	21	0.65	0.04		21	0.62	0.02		19	0	—	—	1.00
Calcium	22	0.64	0.04	0.80	21	0.70	0.03	0.10	20	0.10	0.07	0.18	1.16
Vitamin D ₃	22	0.58	0.04	0.18	20	0.67	0.04	0.37	19	0.11	0.07	0.12	1.20
Ca + Vit. D ₃	21	0.59	0.03	0.23	21	0.70	0.04	0.11	19	0.14	0.07	0.06	1.25
(C) TGFα/TGFβ ₁													
Whole crypts													
Placebo	19	1.03	0.12		21	1.00	0.03		17	0.00	—	—	1.00
Calcium	22	1.06	0.10	0.90	21	0.88	0.06	0.13	20	−0.14	0.19	0.46	0.86
Vitamin D ₃	20	0.99	0.08	0.80	19	0.97	0.07	0.73	17	0.02	0.20	0.93	1.02
Ca + Vit. D ₃	21	1.05	0.18	0.94	21	0.90	0.06	0.21	19	−0.12	0.19	0.55	0.89
Upper 40% of crypts													
Placebo	19	1.48	0.16		21	1.41	0.06		17	0.00	—	—	1.00
Calcium	22	1.85	0.20	0.19	21	1.26	0.09	0.21	20	−0.52	0.29	0.09	0.72
Vitamin D ₃	20	1.71	0.16	0.43	19	1.39	0.10	0.88	17	−0.24	0.30	0.41	0.86
Ca + Vit. D ₃	21	1.62	0.23	0.61	21	1.21	0.08	0.08	19	−0.35	0.30	0.24	0.78
Lower 60% of crypts													
Placebo	19	0.77	0.12		21	0.76	0.04		17	0.00	—	—	1.00
Calcium	22	0.55	0.07	0.19	21	0.63	0.06	0.08	20	0.09	0.18	0.61	1.16
Vitamin D ₃	20	0.56	0.08	0.22	19	0.72	0.06	0.61	17	0.17	0.18	0.36	1.30
Ca + Vit. D ₃	21	0.71	0.17	0.75	21	0.69	0.05	0.36	19	−0.01	0.18	0.94	0.98
(D) TGFα upper 40% to lower 60% TGFβ ₁													

Treatment Group	Baseline			6-mo follow-up			Absolute Rx effect			Relative effect ^c
	n	Mean	Std Err	P	n	Mean	Std Err	P	Rx effect ^d	
Placebo	19	1.62	0.30		21	1.19	0.07		0.00	—
Calcium	22	3.10	0.53	0.07	21	1.18	0.07	0.95	-1.49	0.85
Vitamin D ₃	20	3.11	0.65	0.07	19	1.27	0.10	0.62	-1.40	0.87
Ca + Vit. D ₃	20	2.46	0.66	0.31	20	1.16	0.21	0.90	-0.85	0.86

TGFα, transforming growth factor alpha; TGFβ1, transforming growth factor beta 1; Std Err, standard error.

^a Rx effect = [(treatment group follow-up) – (treatment group baseline)] – [(placebo group follow-up) – (placebo group baseline)].

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

^c Relative effect = [(treatment group follow-up)/(treatment group baseline)]/[(placebo follow-up)/(placebo baseline)]; interpretation similar to that for an odds ratio (e.g., a relative effect of 1.7 indicates a 70% proportional increase in the treatment group relative to that in the placebo group).