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Effects of Vitamin D and Calcium on MSH2, TGF-α, and TGF-β Expression in Normal-Appearing Colorectal Mucosa of Sporadic Colorectal Adenoma Patients: A Randomized Clinical Trial

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Epidemiology

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Advisor

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Abstract

Effects of Vitamin D and Calcium on MSH2, TGF-α, and TGF-β Expression in Normal-Appearing Colorectal Mucosa of Sporadic Colorectal Adenoma Patients: A Randomized Clinical Trial

By Albert Kwan

Abnormal expression of the DNA mismatch repair protein MSH2 and autocrine/paracrine transforming growth factors TGF-α (growth promoter) and TGF-β_1 (growth inhibitor) is common during colorectal carcinogenesis. To estimate the effects of vitamin D and calcium on these biomarkers in the normal-appearing colorectal mucosa of sporadic colorectal adenoma patients, we conducted a randomized, double-blinded, placebo-controlled, modified 2×2 factorial chemoprevention clinical trial (N=104) of supplemental vitamin D_3 (1,000 IU daily) and calcium (1,200 mg daily), alone and in combination, versus placebo. The expression of the three biomarkers and Ki-67/MIB-1 in colorectal crypts in biopsies of normal-appearing rectal mucosa were detected using automated immunohistochemistry and quantified using image analysis. In the vitamin D_3 and vitamin D_3 plus calcium groups, relative to their reference groups, in the upper 40% (differentiation zone) of crypts, it was estimated that respectively, the MSH2/MIB-1 ratio increased by 47% (p=0.14) and 62% (p=0.08), TGF-β_1 expression increased by 41% (p=0.25) and 78% (p=0.14), and the TGF-α/TGF-β_1 ratio decreased by 25% (p=0.31) and 44% (p=0.13). Although not statistically significant, these results (i) suggest that supplemental vitamin D_3, alone or in combination with calcium may increase DNA mismatch repair relative to proliferation, increase TGF-β_1 expression, and decrease autocrine/paracrine growth promotion relative to growth inhibition in the colorectal epithelium, all hypothesized to reduce risk for colorectal carcinogenesis; and (ii) provide further support for the expression of MSH2 relative to MIB-1, TGF-β_1 alone, and TGF-α relative to TGF-β_1 in the normal-appearing rectal mucosa as modifiable, pre-neoplastic markers of risk for colorectal neoplasms.

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CHAPTER 1

INTRODUCTION

BACKGROUND AND SIGNIFICANCE

Overall Impact of Colorectal Cancer

In 2012, CRC remains among the top five cancers in the world in incidence and mortality, according to IARC GLOBOCAN estimates (1).

Incidence

In 2012, the International Agency for Research on Cancer (IARC), the cancer agency of the World Health Organization (WHO), listed colorectal cancer (CRC) as the third most common type of cancer worldwide (excluding non-melanoma skin cancer) among men and women (1). With an age-standardized incidence rate of 17.2 per 100,000 PY, CRC represents 9.7% (1.4 million cases) of all newly diagnosed cancer cases that year. In the United States alone, the National Cancer Institute's SEER program estimates CRC to be the fourth leading cause of cancer incidence (135,430 new cases) in 2017 (2).

Examining incidence among men and women separately, CRC is the third most common cancer among men (ASR=20.6 per 100,000 PY, 746,298 cases; 10.1% of all cancer incidence) and the second most common cancer among women (ASR=14.3 per 100,000; 614,304 cases; 9.2% of all cancer incidence) (1). The highest CRC incidence rates (ASR=44.8 per 100,000 PY among men and 32.2 per 100,000 PY among women) are observed in Australia/New Zealand, while the lowest incidence is seen in Western Africa (ASR=4.5 per 100,000 PY among men and 3.8 per 100,000 PY among women).

Mortality

Additionally, with an age-standardized mortality rate of 8.4 per 100,000 PY, CRC is the fourth leading cause of cancer mortality and is responsible for 8.5% of all cancer deaths annually (1). In the United States alone, the National Cancer Institute's SEER program estimates CRC to be the second leading causes of mortality (50,260 deaths) in 2017 (2).

Comparing CRC mortality between both sexes separately, CRC is the fourth leading cause of cancer-related deaths among men (ASR=10.0 per 100,000 PY; 373,698 cases; 8.0% of all cancers mortalities) and the third leading cause of death among women (ASR=6.9 per 100,000 PY; 320,294 cases; 9.0% of all cancer mortalities) (1). The highest estimated CRC mortality rates for both sexes are found in Central and Eastern Europe (ASR=20.3 per 100,000 PY among men and 11.7 per 100,000 PY among women), and the lowest mortality in Western Africa (ASR=3.5 per 100,000 PY among men and 3.0 per 100,000 PY among women).

Biologic and Molecular Basis for Colorectal Cancer (CRC)

CRC Subtypes

Colorectal cancer is a disease that is characterized by sporadic, familial, and genetic syndromes. The majority of CRC cases (65%) is attributed to environmental factors and thus classified as sporadic (3). About 35% of all CRC's are said to have a genetic component (4). About one-third of CRC cases are said to be inherited with

moderate penetrance (32%), in which associated genes have yet to be well-defined. These familial CRC cases may also include genetic-environmental interactions, which further complicates the picture (3). Consequentially, until moderately penetrant genes are more clearly identified and studied, genetic testing for such CRC cases is challenging and its value uncertain (5). The remaining 3% of cases are well-defined inherited syndromes that are highly penetrant (i.e. high risk of CRC). These last cases include hereditary nonpolyposis colorectal cancer (HNPCC) (i.e. Lynch syndrome) (1-3%), familial adenomatous polyposis (FAP) (<1%), and hamartomatous polyposis syndromes (<1%) (3).

Fearon and Vogelstein's Genetic Model for CRC Development (1990)

CRC is the result of a progressive accumulation of genetic and epigenetic changes that ultimately cause transformation and advancement of normal colonic epithelial cells to cancer. In the genetic model initially proposed by Fearon and Vogelstein in 1990, four key features define CRC development. First, there is mutational activation of oncogenes in tandem with mutational inactivation of tumor suppressor genes. Second, at least 4-5 gene mutations are required. Third, the total accumulation of genetic mutations is more critical for CRC development than the chronological order of the genetic mutations. Finally, mutant tumor suppressor genes exert a biologic effect even in their heterozygote states (6).

Fine-tuning of Fearon and Vogelstein's Genetic Model for CRC Development

There are two characterized genetic pathways to colorectal cancer, and they are likely to be linked — the "gatekeeper" and the "caretaker" pathways (7).

The gatekeeper pathway (i.e., the chromosomal instability pathway, or CIN) is involved in 85% of sporadic colorectal cancers and is also the pathway associated with FAP (8). It involves the disruption of genes that regulate cellular growth and promote apoptosis. For colorectal cancer, the key gene in the pathway is the tumor-suppressor gene APC. This pathway is responsible for direct CRC development.

The caretaker pathway (i.e., the microsatellite instability pathway, or MSI) is characterized by disruption to genes that maintain genetic stability, specifically genes involved in DNA MMR, which leads to microsatellite instability and ultimately CRC. It is responsible for 15% of all CRC's and is the pathway associated with Lynch Syndrome (formerly known as HNPCC) (8). Several tumor-suppressor genes are mutated in this pathway (9). This pathway is responsible for indirect CRC development, but also typically involve genes that directly affect CRC development (i.e., genes in the gatekeeper pathway).

The Adenoma-Carcinoma Sequence

This genetic model for CRC development is incorporated into the process of colorectal tumorigenesis known as the adenoma-carcinoma sequence (i.e., the chromosomal instability pathway) (10). During the adenoma-carcinoma sequence, aberrant crypt foci (ACF) form from normal mucosa, which progresses to early adenoma, late adenoma, and eventually to invasive cancer. Approximately 95% of all CRC cases are believed to progress from adenomatous polyps (11).

Microsatellite Instability, DNA Mismatch Repair System, and MSH2 in CRC

Microsatellite Instability (MSI)

In 1993, original articles reported the presence of microsatellite instability (MSI) as a frequent molecular phenomenon in CRC (12-14). Microsatellites are repetitive sequences found throughout the human genome and consist of mononucleotide, dinucleotide or higher-order nucleotide repeats such as (A)n or (CA)n. These sequence motifs are especially prone to accumulation of mutations, mainly due to DNA polymerase slippage in DNA synthesis. In DNA synthesis, DNA polymerase sometimes makes errors incorporating the correct number of bases during replication of these long repetitive DNA sequences like microsatellites. Slippage during replication of a repetitive sequence most frequently leads to temporary insertion-deletion loops (IDL) (i.e., extrahelical nucleotides that form DNA hairpins) and single base-pair mismatches, which escape the intrinsic proofreading activity of DNA polymerase. Usually, these errors can be recognized and repaired by the MMR system. However, if these errors are not repaired, during the second round of replication, the erroneously synthesized daughter strand (with the mismatch or IDL) contains a mutation even though the original parental strand is copied correctly. Single base-pair mismatches result in point mutations, whereas IDL's result in frame-shift mutations that usually lead to a downstream nonsense mutation and results in production of a truncated, nonfunctional protein. This is the basis of MSI (15,16).

General Function of the DNA Mismatch Repair System

The DNA mismatch repair (MMR) system consists of a complex set of proteins that identify and repair mismatch errors occurring during DNA replication and other areas involving DNA damage. It is responsible for the surveillance and correction of errors introduced in microsatellites and is highly conserved from bacteria to humans. The MMR proteins interact as heterodimers (15-20).

When a mismatch is detected, three steps take place: MSH2 associates with either MSH6 or MSH3 (forming MutSα and MutSβ complexes, respectively), and MLH1 couples with PMS2, PMS1, or MLH3 (forming MutLα, MutLβ, or MutLγ complexes, respectively). The recognition of mismatches and insertion–deletion loops is carried out by a sliding clamp formed by the combination of a MutS and a MutL complex, which interacts with replication factor C. Excision of hExo1 is performed by proteins such as exonuclease-1 and proliferating cell nuclear antigen. Resynthesis and religation is carried out by DNA polymerase δ and DNA ligase. Mutations in the genes responsible for the recognition step lead to an accumulation of errors in DNA, which results in MSI (15,16,21,22).

The Role of MSH2 in the DNA Mismatch Repair System

The human *mutS homologue 2* (*MSH2*) gene is located on chromosome 2p22, and is an integral component of the DNA mismatch repair (MMR) system (23). The MSH2 protein identifies DNA mismatches as part of two heterodimers: MSH2-MSH6 and MSH2-MSH3 (MutSα and MutSβ, respectively). These heterodimers have different relative abilities to bind to DNA mismatches and, as a result, yeast and other eukaryotes

have a broader ability to recognize and repair different types of DNA incorporation errors. MutSα recognizes single-base mismatches (MSH6 was initially called the "GT-binding protein") and short IDL's, while MutSβ identifies the larger IDL's. Both of these MSH2 heterodimers are also involved in recruiting the hMLH1α heterodimer (MLH1-PMS2) that facilitates mismatch correction (8,15-26).

DNA Mismatch Repair in CRC Development (the Microsatellite Instability Pathway)

The MMR system is one of the two primary molecular pathways in CRC development and is responsible for about 15% of all CRC's (15,16,27,28). As described previously, the caretaker pathway (i.e., the microsatellite instability pathway, or MSI) is characterized by disruption to genes that maintain genetic stability, specifically genes involved in DNA MMR, which leads to microsatellite instability and ultimately CRC. It is responsible for 15% of all CRC's and is the pathway associated with Lynch syndrome (formerly known as HNPCC) (8). Several tumor-suppressor genes are mutated in this pathway (9). This pathway is responsible for indirect CRC development, but also typically involve genes that directly affect CRC development (i.e., genes in the gatekeeper pathway).

In conclusion, measuring MSH2 protein expression in normal-appearing colorectal mucosa may serve as a valuable indicator in assessing the functionality of the DNA mismatch repair (MMR) system during CRC development.

Autocrine/Paracrine Growth Factors in CRC: TGF-α and TGF-β_1

TGF-α: Transforming growth factor alpha (TGF-α) and transforming growth factor beta (TGF-β_1) are autocrine/paracrine growth factors normally expressed in healthy colorectal crypts. However, protein expression for both biomarkers becomes abnormal in tumorigenesis (15,26,29-38). TGF-α is a member of the epidermal growth factor (EGF) family and is made in the colorectal crypt epithelium (30-34,39,40). Its only receptor, the epidermal growth factor receptor (EGFR) (34), is located on the basolateral surface of colorectal epithelial cells in the proliferation zone (40). Binding of TGF-α to EGFR produces a mitogenic stimulus that leads to cellular growth and proliferation (30-32). TGF-α is often overexpressed in human colorectal carcinogenesis (41-46), and separate studies found that the proliferation zone (lower 60% of the crypt) expands into the differentiation zone (upper 40% of the crypt) in CRC (47-49). Additionally, other investigators observed simultaneous overexpression of TGF-α and expansion of the proliferation zone into the differentiation zone (40,50), providing evidence that normal TGF-α expression helps maintain the balance between cellular proliferation and differentiation in healthy colorectal crypts.

TGF-β_1: TGF-β_1, the most abundantly and universally expressed TGF-β isoform in mammals (35), is classically considered a tumor suppressor but becomes a tumor promoter in the later stages of carcinogenesis (35,36,38,51-54). As a tumor suppressor, TGF-β_1 signaling occurs primarily through the TGF-β/Smad pathway (35-38). In the Smad-dependent pathway, TGF-β_1 either binds directly to the TGFβRII receptor or indirectly via the TGFβRIII receptor, which in turn presents TGF-β_1 to the TGFβRII receptor. Subsequently, the activated TGFβRII receptor phosphorylates TGFβRI, which

then recruits the Smad2-Smad3-Smad4 protein complex. The Smad complex translocates into the nucleus to ultimately regulate transcription of many TGF-β-responsive genes. TGF-β can also signal through Smad-independent pathways, but the exact molecular mechanisms are not as clear (35,36,55). Regardless of the pathway, TGF-β activation in the early stages of carcinogenesis results in the inhibition of cellular growth and proliferation; regulation of cellular adhesion, motility, and the extracellular matrix; and induction of apoptosis (35-38). Moreover, TGF-β and TGFβRII, its primary receptor, were found to play an important role in MSI-related CRCs (15,26,56,57).

Migration Studies

Although 35% of CRC cases are defined by a familial or genetic component, the majority of CRC cases are considered "sporadic" (65%) (3). Migration studies have demonstrated that CRC incidence rates among immigrants and their descendants become quite similar to those of their adopted country over a short period of time, sometimes within the migrating generation (26,58-66). Because the genetic pool of a population does not suddenly nor drastically change within one to two generations, these studies suggest that environmental factors play a key role in CRC development, and support further research on the prevention of CRC through modifiable risk factors like diet and lifestyle.

Dietary Risk Factors for Colorectal Cancer

In a landmark report, Doll and Peto (1981) stated that about 35% of U.S. cancers could be attributed to diet, excluding alcohol (60). Diet alone may actually reduce one's risk for colorectal cancer by up to 70% (67). Characterized by high intakes of animal protein and fat and low consumption of fiber, the Western diet is more prevalent in these regions and has been linked to increased risk of CRC (68). About 55% of all CRC cases occur in more developed regions, according to 2012 GLOBOCAN estimates (1).

Vitamin D and Calcium in CRC Development, Prevention, and Treatment

Biologic and Molecular Mechanisms

Vitamin D and calcium are two dietary factors that have been linked to protective effects against CRC development. Calcium protects the colorectal mucosa against free bile and fatty acids and also has direct effects on the cell cycle. Vitamin D, in addition to maintaining calcium homeostasis, modulates more than 200 genes highly relevant to colorectal carcinogenesis. Such modulation includes (but is not limited to) cell cycle regulation, growth factor signaling, bile acid and xenobiotic metabolism, and DNA repair. Ultimately, these biologic mechanisms of calcium and vitamin D result in the inhibition of cellular proliferation, induced differentiation, and promotion of apoptosis (47,69-85).

Animal Studies in CRC Prevention

Overall, vitamin D and calcium were both found, separately and combined, to reduce colonic epithelial cell hyperproliferation and hyperplasia, colorectal adenoma burden, and ultimately colorectal carcinogenesis overall, particularly in animals fed Western-style diets consisting of high fat content and low calcium and vitamin D (86-91).

Human Studies in CRC Prevention (Meta-Analyses)

Overall, conclusions from meta-analyses comprising of clinical trials, prospective cohort studies, case-control studies, and cross-sectional studies suggest protective effects for vitamin D and calcium intake, alone and combined, against CRC development in humans. Additionally, serum 25(OH)D levels were found to be inversely associated with CRC development as well. Also, protective effects of calcium intake may be mediated and masked by low vitamin D intake and serum 25(OH)D concentrations (92-99).

Human Studies in CRC Prevention (Clinical Trials)

Overall, results from the clinical trials on vitamin D and calcium's effects on colonic epithelial cell proliferation; polyp growth; and colorectal adenoma recurrence, an accepted neoplastic marker for CRC, were mixed. Although calcium supplementation may not decrease colonic epithelial cell proliferation, it may still normalize the distribution of the proliferating cells among sporadic adenoma patients. Some studies support calcium as a chemopreventive agent against adenoma recurrence, albeit the strength of the association was moderate at best. Another study found that calcium and vitamin D must work synergistically to reduce the risk of colorectal adenoma recurrence.

Another trial found that calcium and vitamin D had no impact on CRC incidence among post-menopausal women, although this trial has noticeable flaws. In the largest clinical trial to date, the authors did not find any meaningful reductions in risk of colorectal adenoma recurrence with daily vitamin D and calcium supplementation (100-109).

Reasoning, Purpose, and Goals for the MSH2, TGF-α, and TGF-β_1 Biomarkers

Identification and utilization of modifiable, phenotypic biomarkers of risk for ischemic heart disease, such as lipid profiles and blood pressures, has led to a 70% decline in heart disease deaths the United States since 1975 (110,111). Reflecting upon this medical model, our research group strives to develop similar biomarkers of risk for CRC. Currently, the adenomatous polyp, a benign neoplastic intestinal tumor from which most sporadic CRC's arise (10,11), is the only accepted biomarker of risk for CRC; there are no current accepted pre-neoplastic biomarkers of risk for the disease. The MSH2, TGF-α, and TGF-β_1 proteins may be potential pre-neoplastic biomarkers of risk for CRC for us to target.

Our goal is to investigate potential pre-neoplastic biomarkers to: 1) further understand relevant CRC mechanisms in humans and subsequently uncover potential preventive interventions in observational studies that may modulate these mechanisms; 2) utilize the biomarkers as endpoints in clinical trials to assess the potential efficacy, optimum dose, and potential long-term safety of interventions; 3) improve clinical risk assessment and management of CRC (e.g., use alongside colonoscopy to determine

appropriate screening intervals and manage risk in a primary care setting similarly to ischemic heart disease) (110).

Biomarker Studies in CRC Development and Prevention

Animal Studies in DNA MMR

There is no naturally occurring animal model of MMR deficiency (15). Moreover, although knockout strains of mice have been created for each of the Lynch syndrome genes: *MSH2* (112,113), *MLH1* (114,115), *PMS2* (115), and *MSH6* (116), none of the heterozygous knockout models produces a phenotype that is similar to the human disease. The principal tumors that develop in these mice are lymphomas. Furthermore, homozygous disruption of MMR genes leads to animals with a constitutional deficiency of MMR activity, a variety of genetic lesions, and some intestinal tumors; but are not useful in screens for agents that have therapeutic or preventive effects in humans. Another study found animals with compound knockouts of *Apc* and a DNA MMR gene have accelerated polyp progression, but these tumors predominantly form in the small intestine (117).

Additionally, the study of MMR in mouse models is limited because human genes associated with colorectal carcinogenesis include coding microsatellites in places where the microsatellites are absent in mouse genes. Even more, genes that are associated with colorectal tumorigenesis encode factors that are required for cell proliferation and

survival (e.g., TGF R2, BAX, and caspase-5), so it is a challenge to create mice with defects in these genes for cancer studies.

Human Biomarker Studies in Colorectal Carcinogenesis and Prevention

Overall, the studies suggest that certain biomarkers at the very least serve as good indicators of one's CRC status (110,118-124). Moreover, modulation of these biomarkers by dietary factors/treatment such as vitamin D and calcium may result in the favorable outcome of reducing one's risk of CRC by modifying DNA MMR activity (119, 122), enhancing apoptosis (120), and promoting colonic epithelial cell differentiation, and normalizing the proliferation zone (121).

MSH2

Support for MSH2 expression in the normal-appearing colorectal mucosa as a potential valid, modifiable, pre-neoplastic biomarker of risk for colorectal neoplasms in humans comes from a pilot, colonoscopy-based, case-control study of incident, sporadic, colorectal adenoma (N=37 cases, 41 controls) [Markers of Adenomatous Polyps II (MAP II) study] (119). In adenoma cases relative to controls, MSH2 expression was estimated to be 49% (p=0.01) lower in the ascending colon and 23% lower (p=0.06) in the rectum. Moreover, higher MSH2 expression in the rectum was estimated to be associated with approximately 13% lower risk for adenomas (OR=0.87 [95% CI: 0.44-1.71]). These findings support the validity of MSH2 as a biomarker of risk for colorectal neoplasms. Finally, MSH2 expression was 39% higher (p=0.04) in the rectum among those who

regularly took an NSAID than among those who did not, which suggests that MSH2 expression in the normal-appearing colorectal mucosa may be modifiable.

Support for the hypothesis that higher intakes of calcium and/or higher vitamin D exposure may increase MSH2 expression in the normal human colorectal mucosa comes from a pilot, randomized, double-blind, placebo-controlled, 2x2 factorial trial of calcium 2,000 mg/day, vitamin D_3 800 IU/day, both, or placebo over 6 months in sporadic colorectal adenoma patients (N=92) [Calcium and Vitamin D vs. Markers of Adenomatous Polyps trial (CaD v MAP)] (122). MSH2 expression along the full lengths of crypts increased by an estimated 61% (p=0.11) in the vitamin D group and 30% (p=0.36) in the calcium group, relative to the placebo group. The estimated calcium and vitamin D treatment effects were more pronounced in the upper 40% of crypts (differentiation zone), where MSH2 expression increased by 169% (p=0.04) in the vitamin D group and 107% (p=0.13) in the calcium group.

TGF-α

Support for TGF-α expression in the normal-appearing colorectal mucosa as a potential valid, modifiable, pre-neoplastic biomarker of risk for colorectal neoplasms in humans comes from a pilot, colonoscopy-based, case-control study (N=29 cases, 31 controls) of incident, sporadic, colorectal adenoma (45). In the cases relative to controls, TGF-α expression was 51% higher (p=0.05) in the whole crypts, 136% higher (p=0.02) in the upper 40% of crypts (the differentiation zone), and 110% higher (p=0.02) in the lower 60% of crypts (the proliferation zone). Furthermore, TGF-α expression was associated with more than a two-fold higher likelihood of adenoma (e.g., for TGF-α expression in

the whole crypt, OR=2.23 [95% CI: 0.98-5.07], and for the upper 40% of the crypt, OR=2.12 [95% CI: 1.03-4.38]). These findings support the validity of TGF-α as a biomarker of risk for colorectal neoplasms. Finally, TGF-α expression was 36% lower among regular NSAID users, and 49% lower among women taking hormone replacement therapy, which suggests that TGF-α expression in the normal-appearing colorectal mucosa may be modifiable.

Support for the hypothesis that higher intakes of calcium and/or higher vitamin D exposure may reduce TGF-α expression—at least in relation to $TGF\beta_1$ expression—in the normal human colorectal mucosa comes from the above-noted CaD v MAP chemoprevention trial (124). While there were minimal estimated changes in the active relative to the placebo group for TGF-α expression alone, the $TGF\alpha/TGF\beta_1$ ratio in the upper 40% of crypts in the calcium, vitamin D, and calcium plus vitamin D groups, was estimated to have decreased by 28% (p=0.09), 14% (p=0.41), and 22% (p=0.24), respectively.

TGF-β_1

Support for TGF-β_1 expression in the normal-appearing colorectal mucosa as a potential valid, modifiable, pre-neoplastic biomarker of risk for colorectal neoplasms in humans comes from a pilot, colonoscopy-based, case-control study (N=43 cases, 43 controls) of incident, sporadic, colorectal adenoma (46). Although TGF-β_1 expression alone in whole crypts was estimated to be only about 7% lower in the cases relative to the controls, the TGF-α/TGF-β_1 ratio in the whole crypts was approximately 110% greater (p=0.02), and it was associated with approximately two-fold higher likelihood for

adenomas (OR=2.42 [95% CI: 0.85–6.87]). These findings support the validity of TGF-β_1 and the TGF-α/TGF-β_1 ratio biomarkers of risk for colorectal neoplasms. Furthermore, the TGF-α/TGF-β_1 ratio was 105% higher (p=0.03) in smokers than in non-smokers, which suggest that TGF-α/TGF-β_1 expression ratio in the normal-appearing colorectal mucosa may be modifiable.

Support for the hypothesis that higher intakes of calcium and/or higher vitamin D exposure may modulate TGF-β_1 expression—including in relation to TGF-α expression—in the normal human colorectal mucosa comes from the above-described CaD v MAP chemoprevention trial (124). TGF-β_1 expression in the whole crypts in the calcium, vitamin D, and calcium plus vitamin D groups, was estimated to have increased by 14% (p=0.25), 19% (p=0.17), and 22% (p=0.09), respectively. As noted further above, the TGFα/TGFβ_1 ratio in the upper 40% of crypts in the calcium, vitamin D, and calcium plus vitamin D groups, was estimated to have decreased by 28% (p=0.09), 14% (p=0.41), and 22% (p=0.24), respectively.

Concluding Remarks

As previously mentioned, the adenomatous polyp is currently the only accepted biomarker of risk for CRC, and there are no currently accepted pre-neoplastic biomarkers of risk for the disease. The goal of my thesis aligns with the aforementioned goals of our research group. Specifically, my aim is to investigate the potential of MSH2, TGF-α, and TGF-β_1 expression in the normal-appearing rectal mucosa as modifiable, pre-neoplastic CRC biomarkers to better understand their roles in colorectal carcinogenesis.

Furthermore, I assess the potential potency of vitamin D_3 and calcium, alone and combined, as chemopreventive interventions to modify these biomarkers with the ultimate goal of preventing CRC development.

There is only one reported clinical trial that investigated the individual and combined effects of calcium and vitamin D_3 on MSH2, TGF-α, and TGF-β_1 expression, a pilot trial in 92 sporadic adenoma patients over 6 months (122,124). Based on the promising results of this pilot trial, we again tested the individual and combined effects of calcium and vitamin D_3 on the protein expression of these biomarkers in the crypts of the normal-appearing colorectal mucosa, this time in 104 sporadic colorectal adenoma patients over 12 months, using a lower dose of calcium (1,200 mg/d vs. 2,000 mg/d) and a higher dose of vitamin D_3 (1,000 IU/d vs. 800 IU/d).

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CHAPTER 2

MANUSCRIPT

Effects of Vitamin D and Calcium on MSH2, TGF-α, and TGF-β Expression in Normal-Appearing Colorectal Mucosa of Sporadic Colorectal Adenoma Patients: A Randomized Clinical Trial

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ABSTRACT

Abnormal expression of the DNA mismatch repair protein MSH2 and autocrine/paracrine transforming growth factors TGF-α (growth promoter) and TGF-β_1 (growth inhibitor) is common during colorectal carcinogenesis. To estimate the effects of vitamin D and calcium on these biomarkers in the normal-appearing colorectal mucosa of sporadic colorectal adenoma patients, we conducted a randomized, double-blinded, placebo-controlled, modified 2×2 factorial chemoprevention clinical trial (N=104) of supplemental vitamin D_3 (1,000 IU daily) and calcium (1,200 mg daily), alone and in combination, versus placebo. The expression of the three biomarkers and Ki-67/MIB-1 in colorectal crypts in biopsies of normal-appearing rectal mucosa were detected using automated immunohistochemistry and quantified using image analysis. In the vitamin D_3 and vitamin D_3 plus calcium groups, relative to their reference groups, in the upper 40% (differentiation zone) of crypts, it was estimated that respectively, the MSH2/MIB-1 ratio increased by 47% (p=0.14) and 62% (p=0.08), TGF-β_1 expression increased by 41% (p=0.25) and 78% (p=0.14), and the TGF-α/TGF-β_1 ratio decreased by 25% (p=0.31) and 44% (p=0.13). Although not statistically significant, these results (i) suggest that supplemental vitamin D_3, alone or in combination with calcium may increase DNA mismatch repair relative to proliferation, increase TGF-β_1 expression, and decrease autocrine/paracrine growth promotion relative to growth inhibition in the colorectal epithelium, all hypothesized to reduce risk for colorectal carcinogenesis; and (ii) provide further support for the expression of MSH2 relative to MIB-1, TGF-β_1 alone, and TGF-α relative to TGF-β_1 in the normal-appearing rectal mucosa as modifiable, pre-neoplastic markers of risk for colorectal neoplasms.

INTRODUCTION

Colorectal cancer (CRC) is the second leading cause of cancer deaths among men and women combined in the U.S (1). Migration studies demonstrated that CRC incidence rates among immigrants and their descendants become quite similar to those of their adopted country over a short period of time, sometimes within the migrating generation (2-4). These studies indicate the importance of environmental factors—especially diet and lifestyle—in CRC development, and thus its preventability.

Calcium intakes and vitamin D exposure are thought to protect against CRC development (4-11). Calcium binds free bile and fatty acids in the gut lumen, preventing their mutagenic and mitogenic effects, and has direct and indirect cell cycle effects in the colorectal epithelium. Vitamin D, in addition to maintaining calcium homeostasis, modulates more than 200 genes highly relevant to colorectal carcinogenesis. Such modulation includes, but not limited to, cell cycle regulation, growth factor signaling, bile acid and xenobiotic metabolism, and DNA repair. Ultimately, these biologic mechanisms of calcium and vitamin D result in the inhibition of proliferation and promotion of differentiation and apoptosis in colon crypt epithelia.

A goal of our research group is to identify and utilize modifiable, pre-neoplastic, phenotypic biomarkers of risk for colorectal neoplasms, analogous to those for ischemic heart disease, such as lipid profiles and blood pressures that have played important roles in the 70% decline in heart disease deaths the United States since 1975 (11-12). Currently, the adenomatous polyp, a benign neoplastic intestinal tumor from which most sporadic CRCs arise (13-14), is the only accepted biomarker of risk for CRC; there are no currently accepted pre-neoplastic biomarkers of risk for the disease. Our goal is to

investigate potential pre-neoplastic biomarkers to: 1) further understand relevant CRC mechanisms in humans and subsequently uncover potential preventive interventions in observational studies that may modulate these mechanisms; 2) utilize the biomarkers as endpoints in clinical trials to assess the potential efficacy, optimum dose, and potential long-term safety of interventions; 3) improve clinical risk assessment and management of CRC (e.g., use in conjunction with a baseline colonoscopy to determine appropriate screening intervals and manage risk in a primary care setting similarly to that for ischemic heart disease) (11).

MutS-homologue 2 (MSH2), transforming growth factor alpha (TGF-α), and transforming growth factor beta 1 (TGF-β_1) are potential pre-neoplastic biomarkers of risk for CRC. MSH2 is an integral component of the DNA mismatch repair (MMR) system, which is involved in one of the two primary molecular pathways in CRC development (15-19). Impairment of MMR results in microsatellite instability (MSI), which is responsible for about 15% of all CRCs (20,21).

TGF-α and TGF-β_1 are autocrine/paracrine growth factors normally expressed in colorectal crypts. However, the expression of both biomarkers becomes altered in tumorigenesis (2,20,22-31). TGF-α binds to EGFR to produce a mitogenic stimulus that leads to normal cellular growth and proliferation (23-25), but often becomes overexpressed in human colorectal carcinogenesis (32-37). TGF-β_1 is classically considered a tumor suppressor but becomes a tumor promoter in the later stages of carcinogenesis (28,29,31,38-41). TGF-β_1 activation in the early stages of carcinogenesis results in inhibition of cellular growth and proliferation; regulation of cellular adhesion,

motility, and the extracellular matrix; and induction of apoptosis (28-31). Its primary receptor TGFβRII plays an important role in MSI-related CRCs (2,20,42,43).

To our knowledge, there is only one reported clinical trial that investigated the individual and combined effects of calcium and vitamin D_3 on MSH2, TGF-α, and TGF-β_1 expression, a pilot trial in 92 sporadic adenoma patients over 6 months (44,45). Based on the promising results of this pilot trial, we again tested the individual and combined effects of calcium and vitamin D_3 on the protein expression of these biomarkers in the crypts of the normal-appearing colorectal mucosa, this time in 104 sporadic colorectal adenoma patients over 12 months, using a lower dose of calcium (1,200 mg/d vs. 2,000 mg/d) and a higher dose of vitamin D_3 (1,000 IU/d vs. 800 IU/d).

METHODS

Participant Population

The participants in this study ("adjunct biomarker study") were recruited from those participating in a larger 11-center, randomized, double-blinded, placebo-controlled, 2×2 partial factorial chemoprevention clinical trial ("parent study") testing the efficacy of 1,200 mg of supplemental calcium and 1,000 IU of vitamin D_3, alone and in combination, over 3-5 years on adenoma recurrence in colorectal adenoma patients. Details of the parent study were previously reported (46). Briefly, eligible participants were 45-75 years of age, in general good health, had ≥1 histologically-verified neoplastic polyps (≥ 2 mm in diameter) removed from the large bowel within four months of study entry with no remaining polyps in the large bowel, and scheduled for a follow-up colonoscopy three or five years after their qualifying colonoscopy. Patients were excluded if they had

invasive colorectal carcinoma; familial adenomatous polyposis; inflammatory bowel disease; malabsorption syndromes; history of large bowel resection; diagnosed narcotic or alcohol dependence; serum calcium outside normal range; serum creatinine concentrations >20% above the upper limit of the normal range; serum 25-hydroxyvitamin D concentrations <12 ng/mL or >90 ng/mL; current use of a thiazide diuretic greater than the equivalent of 50 mg of hydrochlorothiazide; New York Heart Association Cardiovascular Diseases functional class 3 or 4; renal dialysis; history of kidney stones, unexplained hematuria, or sarcoidosis in the previous 20 years; history of hypo- or hyperparathyroidism; unwillingness to forgo individual calcium and vitamin D supplementation during the trial; unwillingness to forgo daily intake of more than a quart of milk (or other daily product equivalent) or daily dietary intake of vitamin D estimated to be greater than 400 IU; history of osteoporosis or other medical condition that may require supplemental calcium or vitamin D; current use of bisphonates; or current use of calcitonins. Additional exclusion criteria for the adjunct biomarker study were being unable to cease aspirin use for 7 days, history of a bleeding disorder, or current use of anticoagulant medication.

Study Design

For the parent study, between 2004 and 2008, 19,083 apparently eligible participants from 11 clinical centers were identified through initial screening of colonoscopy and pathology reports. From this initial study population, 2,259 participants ultimately underwent randomization as described below. Endpoints of the parent study included all adenomas diagnosed in any colorectal endoscopic or surgical procedure at

least 1 year after randomization and up to 6 months following the anticipated 3-year or 5-year colonoscopic examination.

At enrollment in the parent study, each study participant provided information on demographics, medical history, medications, nutritional supplements, and diet. Diet was assessed using the semi-quantitative Block Brief 2000 food frequency questionnaire (Nutritionquest, Berkeley, CA). Participants who did not adhere to the study protocol were excluded after a 3-month placebo run-in period (56-84 days). Randomization was then performed using computer-generated random numbers within permuted blocks and stratified by sex, clinical center, scheduled colonoscopic follow-up at 3 or 5 years, and full factorial or two-arm randomization. In the full factorial randomization group, participants were randomly assigned to one of the following four treatment groups: 1,200 mg/day calcium (1.5 g calcium carbonate twice/day), 1,000 IU/day vitamin D_3 (500 IU vitamin D_3 twice/day), both agents (1.5 g calcium carbonate + 500 IU vitamin D_3 twice/day), or placebo. In the two-arm randomization group, consisting of only women who declined to forego calcium supplementation, participants were randomly assigned calcium or calcium plus vitamin D_3. Each respective treatment was divided into two identical tablets taken twice daily with food, and bottles of study tablets were mailed to participants every four months during the treatment period. The study treatment period continued until the expected 3-year or 5-year colonoscopy. Neither clinical center staff nor study participants had knowledge of the treatment assignments.

Upon enrollment, participants agreed to avoid taking additional vitamin D or calcium supplements outside the trial. However, from April 2008 onward, daily supplement use of up to 1,000 IU of vitamin D and/or 400 mg of elemental calcium was

permitted, although discouraged. Participants who wanted to take a multivitamin were offered a special preparation that did not include calcium or vitamin D.

Participants were contacted by telephone every six months to gather information regarding their adherence to their treatment assignments, illnesses, medication and supplement use, dietary calcium intake, and colorectal procedures. During the first year of follow-up (the period relevant to the adjunct biomarker study), serum concentrations of 25-hydroxyvitamin D [25(OH)D and 1,25(OH)2D], calcium, and creatinine were measured at baseline and 1 year after randomization.

For the adjunct biomarker study, without knowledge of treatment group assignment, a total of 231 apparently eligible parent study participants at two parent study clinical centers in South Carolina (University of South Carolina) and Georgia (Emory University) were offered participation into the biomarker study. Of the 231 participants, 109 participants met final eligibility, provided informed consent, and were enrolled. Sufficient rectal biopsy tissue for biomarker measurements was obtained from 104 participants at the baseline and 1-year follow-up visits (discussed below). The Institutional Review Boards at the two clinical centers approved the research.

Participants in the adjunct biomarker study only underwent "non-prep" (i.e., no preceding bowel-cleansing preparation or procedure) biopsies of normal-appearing rectal mucosa at the baseline and 1-year follow-up visits. Six sextant ~1 mm thick biopsy specimens were taken from normal-appearing rectal mucosa 10 cm proximal to the external anal aperture through a short rigid proctoscope with a jumbo cup flexible biopsy forceps mounted on a semi-rigid rod. To avoid possible field effects, no biopsies were taken within 4.0 cm of a polypoid lesion. The biopsies were placed on a strip of bibulous

paper and immediately placed in phosphate buffered saline, oriented under a dissecting microscope so that they were not twisted or curled, transferred to 10% normal buffered formalin for 24 hours, and then transferred to 70% ethanol. Within a week, the biopsies were processed and embedded in paraffin blocks (two blocks of three biopsies per participant, per biopsy visit). From each block, for each biomarker, five slides with three levels of 3 μm-thick biopsy sections taken 40 μm apart were prepared, yielding a total of 15 levels. All biomarkers were measured in the biopsies using automated immunohistochemistry with image analysis.

Immunohistochemistry Protocol

Prior to immunohistochemical staining, the slides were deparaffinized using a Leica Automated H&E Stainer (Leica Biosystems, Buffalo Grove, IL). Then, the MSH2, TGF-α, and TGF-β_1, and MIB-1 (Ki-67 epitope) antigens were unmasked via heat-induced epitope retrieval by steaming them for 40 min. in a preheated PreTreatment Module (Lab Vision Corp., Fremont, CA) filled with 100x Citrate Buffer pH 6.0 (S1699, Dako Corp., Carpinteria, CA) (Dako Corp., henceforth referred to as Dako, is now a subsidiary of Agilent Technologies, Santa Clara, CA).

The slides were then immunohistochemically processed using the DakoCytomation Autostainer Plus automated immunostainer as follows. The slides were rinsed with Tris-Buffered Saline (TBS; 20X [1.0 M] with Tween 20 [Signet 2380, Signet Laboratories, Inc., Hayward, CA; Signet was subsequently acquired by BioLegend, San Diego, CA]), and then 3% H_2O_2 was applied for 5 min and cleared. Next, primary antibody was applied. For MSH2, we applied anti-MSH2 antibody (Calbiochem NA27)

at a 1:500 concentration in Background Reducing Antibody Diluent (Dako S3022) for 30 min. For TGF-α, we applied anti-TGF-α (Calbiochem GF10) at a concentration of 1:100 in Antibody Diluent (Dako S0809) for 45 min. For TGF-β_1, we applied anti-TGF-β_1 (Santa Cruz sc-146) at a concentration of 1:75 in Background Reducing Antibody Diluent (Dako S3022) for 45 min. For MIB-1, we applied anti-Ki-67 (Ki-67 (Dako M7240) at a concentration of 1:350 in Antibody Diluent (Dako S0809) for 30 min. Slides were then rinsed with TBS and cleared. Then, for MSH2, the EnVision+ Mouse System (Dako K4007) was applied for 30 min, and for the remaining antigens, the LSAB2 Detection System (Dako K0675) was applied (link antibody for 10 min, rinsed with TBS and cleared, and streptavidin peroxidase for 10 min). Next, the slides were rinsed with TBS and cleared, and then 3,3'-diaminobenzidine (DAB) was applied for 5 min (DAB+ [Dako K3438] was used for MSH2, and DAB [Dako K3466] was used for the remaining antigens). Then, the slides were rinsed with TBS, and cleared, and then rinsed with DH_2O and cleared. For MSH2 and MIB-1, Richard Allen Hematoxylin 7211 (Fisher 22-050-11) 1:6 in distilled water was applied for 1 min and then rinsed with TBS and then DH_2O. Slides for TGF-α and TGF-β_1 were not counterstained. For each participant, baseline and follow-up biopsy slides were stained in the same batch, and each staining batch included a balance of participants from each treatment group. The slides were coverslipped using a Leica CV5000 Coverslipper (Leica Microsystems, Inc., Buffalo Grove, IL) after the staining procedures.

In each slide staining batch, positive and negative control slides were included. For control tissues, we used a surgical specimen of normal human colon for MSH2, and for the remaining biomarkers we used a surgical specimen of human tonsil. The negative

and positive control slides were treated identically to the participant slides, except that antibody diluent was used instead of the primary antibody on the negative control slide.

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

A quantitative image analysis method ("scoring") was used to measure the immunohistochemically-detected biomarkers in colon crypts, as depicted in **Figure 1**. The imaging and analysis unit was the "hemicrypt," defined as one side of a colonic crypt bisected from base to colon lumen surface, or simply one half of the crypt. Intact hemicrypts extending from the muscularis mucosae to the colon lumen were considered eligible (i.e., "scorable") for quantitative image analysis.

The major equipment and software for the image analysis procedures were the Scanscope CS digital scanner (Aperio Technologies, Inc., Vista, CA), computer, digital drawing board, MatLab software (MathWorks, Inc., Natick, MA), CellularEyes Image Analysis Suite (DivEyes LLC, Atlanta, GA), and MySQL (Sun Microsystems, Inc., Redwood Shores, CA). Standardized settings were used on all equipment throughout the scoring procedures.

Whole slide images were acquired and digitized with the Aperio Scanscope CS digital scanner, and the resulting images were reviewed in the CellularEyes program to identify colon crypts acceptable for analysis. Images of negative and positive control slides were also checked for staining adequacy before analysis. Blinded to treatment assignment, the technician then selected two of three biopsies with 16-20 "scorable" hemicrypts per biopsy. After the borders of each selected hemicrypt were traced with the

digital drawing board, the CellularEyes program divided the traced outline into 50 equally spaced segments, roughly corresponding to the width of an average normal crypt epithelial cell (colonocyte). Finally, the program measured the background-corrected optical density of the biomarker labeling across the entire hemicrypt and within each segment. All resulting data were automatically transferred into a MySQL database. The quantitative image analysis procedure was repeated for each selected hemicrypt. Scoring reliability was assessed by rescoring blinded previously scored slides, and was confirmed before beginning scoring, at intervals, and at the completion of scoring. Scoring reliability, assessed using the intra-class correlation coefficient, was >0.90 throughout.

Statistical Analysis

The treatment groups were assessed for comparability of characteristics at baseline and at 1-year follow-up using the Fisher's exact test for categorical variables, and one-way ANOVA or the two-sample *t* test for continuous variables. All continuous variables were transformed by the natural logarithm to improve normality prior to hypothesis testing. Measurement reliability was assessed using the intra-class correlation coefficient.

Treatment effects were evaluated by comparing changes in mean biomarker labeling optical densities from baseline to 1-year follow-up between patients in each active treatment group relative to its respective reference group using marginal linear models. The absolute treatment effect was defined as: (treatment group follow-up - treatment group baseline) - (reference group follow-up - reference group baseline). For these analyses, as we previously reported (47), because of limited sample size, we

combined some treatment groups and compared participants who received 1) vitamin D relative to those who did not ("vitamin D vs. no vitamin D"), 2) calcium relative to those who did not ("calcium vs. no calcium"), and 3) calcium plus vitamin D relative to those who received only calcium ("calcium + vitamin D vs. calcium"). Based on previous literature (36,37,44,45,48), we assessed potential treatment effects on the expression of the biomarkers in whole crypts, upper 40% of crypts (differentiation zone), and lower 60% of the crypt (proliferation zone), as well as on the ratio of expression in the upper 40% of crypts relative to the whole crypts (distribution index, Φ_h). In addition, we created an MSH2/MIB-1 ratio to represent mismatch repair relative to cell proliferation and a TGF-α/TGFβ_1 ratio to represent the balance of autocrine/paracrine growth-promoting to growth-inhibiting factors. We assessed potential treatment effects on these ratios in the whole and upper 40% and lower 60% of crypts. To provide perspective on the magnitudes of the estimated treatment effects, since the biomarker measurements were in unit-less optical density, we also calculated relative treatment effects [(treatment group follow-up / treatment group baseline) / (reference group follow-up / reference group baseline)]. The interpretation of the relative treatment effect is similar to that for an odds ratio; for example, a relative treatment effect of 1.70 would indicate a 70% increase in mean biomarker expression in the treatment group of interest relative to that in the reference group. All analyses were based on treatment group assignment at the time of randomization, regardless of adherence status (intent-to-treat analysis).

To account for correlated outcomes, as noted above, the analyses were conducted using marginal linear models with compound symmetric R matrices specified in SAS Institute's PROC MIXED procedure. Prior to hypothesis testing, the TGF-β,

MSH2/MIB-1, and TGF-α/TGF-β biomarker values were transformed by the natural logarithm to improve normality. The model included the intercept, treatment group, visit, a treatment*visit interaction term, and selected potential confounding variables when indicated. We considered as potential confounding variables staining batch and any baseline participant characteristic that was imbalanced across the treatment groups (i.e., total energy intake, total calcium intake, dietary fiber intake, BMI, multivitamin use, and regular use of aspirin or non-aspirin NSAIDs once a week or more). The criterion for inclusion of a potential covariate in the final models was whether its inclusion/exclusion resulted in a change of ≥10% in the estimated relative treatment effect of interest. Total energy intake was included in the final models for MSH2 and the MSH2/MIB-1 ratio, total calcium intake was included in the final models for TGF-α and the TGF-α/TGF-β ratio, and total calcium intake and staining batch was included in the final model for TGF-β.

All statistical analyses were conducted using SAS software, Version 9.4 (SAS Institute, Cary, NC). Two-sided *P*-values ≤ 0.05 were considered statistically significant.

RESULTS

Baseline Participant Characteristics

Selected baseline characteristics of the adjunct biomarker study participants (N = 104) are summarized in **Table 1**. The mean age of the participants was 59 years, 46% were male, 79% were white, 50% were college graduates, 58% never smoked, and 79% were overweight (BMI ≥ 25 kg/m^2). Additionally, 9% had a family history of colorectal cancer in a first-degree relative, and 18% had at least one advanced adenoma at baseline.

The mean serum 25-hydroxyvitamin D level was 24.1 ng/mL (SD = 9.3). As noted above, there were differences across the treatment groups in physical activity, dietary fiber intake, total energy intake, total calcium intake, BMI, multivitamin use, and regular use of aspirin or non-aspirin NSAIDs once a week or more. During the first year after randomization, 76% of participants reported taking ≥ 80% of their study tablets, and at the 1-yr follow-up visit, there was a mean increase of 10.9 ng/mL (SD = 9.6) in serum 25-hydroxyvitamin D among participants randomized to vitamin D_3.

The estimated effects of the study interventions on most biomarker variables are summarized in **Tables 2-6** and described below. The results for the Φ_h distribution variable were all close to the null (Supplement Table 1). Graphical representations of the overall baseline distributions of each biomarker within the rectal crypts from base to apex are presented in Supplement Figures 1 and 2, and those for the distributions of each biomarker, by treatment group, at the baseline and follow-up visits are presented in Supplement Figures 3-6.

MSH2 and the MSH2/MIB-1 Ratio

All estimated treatment effects on MSH2 expression alone were close to the null (**Table 2**). However, as noted in **Table 3**, the expression of MSH2 relative to the cell proliferation marker MIB-1 (MSH2/MIB-1 ratio) was estimated to have increased by 10% and 14% in the whole crypts, by 47% and 62% in the upper 40% (differentiation zone) of crypts, and by 9% and 11% in the lower 60% (proliferation zone) of crypts in the vitamin D_3 and vitamin D_3 + calcium groups, respectively, relative to their reference groups, although these findings were not statistically significant. The estimated treatment

effects on the MSH2/MIB-1 ratio in the calcium relative to the no calcium group were close to the null, except for in the upper 40% of the crypt where it was estimated to have decreased by 29% (p=0.38).

TGF-α, TGF-β_1, and the TGF-α/TGF-β_1 Ratio

All estimated treatment effects on TGF-α expression alone were close to the null (**Table 4**). However, as noted in **Table 5**, TGF-β expression alone was estimated to have increased by 24% and 30% in the whole crypts, by 41% and 78% in the upper 40% of crypts, and by 30% and 27% in the lower 60% of crypts in the vitamin D_3 and vitamin D_3 + calcium groups, respectively, relative to their reference groups, although these findings were not statistically significant. The estimated calcium treatment effects on TGF-β in the calcium group relative to the no calcium group were for non-statistically significant decreases of 16%, 4%, and 22% in the whole, upper 40%, and lower 60% of the crypts.

As noted in **Table 6**, the expression of the growth-promoting TGF-α relative to growth-inhibiting TGF-β (TGF-α/TGF-β ratio) was estimated to have decreased by 20% and 24% in the whole crypts, by 25% and 44% in the upper 40% of crypts, and by 25% and 18% in the lower 60% of crypts in the vitamin D_3 and vitamin D_3 + calcium groups, respectively, relative to their reference groups, although these findings were not statistically significant. The estimated calcium treatment effects on the TGF-α/TGF-β ratio in the calcium group relative to the no calcium group were for non-statistically significant increases of 25%, 8%, and 39% in the whole, upper 40%, and lower 60% of the crypts.

DISCUSSION

Our results suggest that supplemental vitamin D_3, alone or in combination with calcium, may 1) increase colorectal crypt expression of MSH2 relative to proliferation, especially in the crypt differentiation zone; and 2) increase TGF-β_1 expression and decrease the expression of TGF-α relative to TGF-β_1, especially in the crypt differentiation zone, in the normal appearing colorectal mucosa of sporadic colorectal adenoma patients. On the other hand, our results are consistent with effects of calcium opposite to those for vitamin D, although of generally modest magnitudes. The estimated vitamin D treatment effects were in the directions hypothesized to reduce the risk for colorectal neoplasms, whereas those for calcium were not.

Located on chromosome 2p22, the *MSH2* gene produces a protein product (MSH2) crucial in DNA mismatch repair (MMR) (49), which consists of a complex set of proteins that identify and repair mismatch errors occurring during DNA replication (50,51). In MMR, the MSH2 protein identifies DNA mismatches as part of two heterodimers: MSH2-MSH6 (MutSα) and MSH2-MSH3 (MutSβ). MutSα recognizes single-base mismatches and short insertion-deletion loops, while MutSβ identifies the larger loops. These MSH2 heterodimers are also involved in recruiting the MLH1-PMS2 heterodimer (hMLH1α) that facilitates mismatch correction (20,21,52-53). The DNA MMR system is involved in one of the two primary molecular pathways in CRC development (15-19), and abnormalities in the pathway result in microsatellite instability (MSI), which is responsible for about 15% of all CRCs (20,21).

TGF-α, a member of the epidermal growth factor (EGF) family, is produced in the colorectal crypt epithelium (23-27,54,55). Its only receptor, the epidermal growth factor

receptor (EGFR) (27), is located on the basolateral surface of colorectal epithelial cells in the proliferation zone (55). TGF-α binding to EGFR produces a mitogenic stimulus that leads to cellular growth and proliferation (23-25). TGF-α is often overexpressed in human colorectal carcinogenesis (32-37), and separate studies found that the proliferation zone (lower 60% of the crypt) expands into the differentiation zone (upper 40% of the crypt) in colon carcinogenesis (7,56,57). Additionally, other investigators observed simultaneous overexpression of TGF-α and expansion of the proliferation zone into the differentiation zone (55,58), providing evidence that normal TGF-α expression helps maintain the balance between cellular proliferation and differentiation in healthy colorectal crypts, but may also facilitate colorectal carcinogenesis in unhealthy crypts.

TGF-β_1, the most abundantly and universally expressed TGF-β isoform in mammals (28), is classically considered a tumor suppressor, but also becomes a tumor promoter in the later stages of colorectal carcinogenesis (28,29,31,38-41). As a tumor suppressor, TGF-β_1 signaling occurs primarily through the TGF-β/Smad pathway to regulate transcription of many TGF-β-responsive genes (28-31). TGF-β_1 initially binds directly to the TGFβRII receptor (or indirectly via the TGFβRIII receptor, which in turn presents TGF-β_1 to the TGFβRII receptor). Now activated, the TGFβRII receptor phosphorylates TGFβRI, which subsequently recruits the Smad2-Smad3-Smad4 protein complex. The Smad complex then translocates into the nucleus to regulate transcription. TGF-β_1 can also signal through Smad-independent pathways, but the exact molecular mechanisms are less clear (28,29,59). Regardless of the pathway, TGF-β_1 activation in the early stages of carcinogenesis results in the inhibition of cellular growth and

proliferation; regulation of cellular adhesion, motility, and the extracellular matrix; and induction of apoptosis (28-31).

Although numerous animal and human studies investigated the chemopreventive effects of vitamin D and/or calcium in colorectal carcinogenesis, the exact biologic mechanisms of vitamin D and/or calcium on these aforementioned biomarkers in the prevention of colorectal carcinogenesis have not been elucidated. One reason is because there is no naturally occurring animal model of MMR deficiency (20). Knockout strains of mice were created for Lynch syndrome genes such as *MSH2* (60,61), but none of the heterozygous knockout models produced a phenotype that is similar to the human disease. Additionally, the study of MMR in mouse models is limited because human genes associated with colorectal carcinogenesis include coding microsatellites in places where the microsatellites are absent in mouse genes (20). However, in an animal study on TGF-α, vitamin D and high dietary calcium intake suppressed parathyroid hyperplasia in rats by inducing p21 expression, which functions in cell cycle arrest, and reducing TGF-α expression, which may suggest an interactive role between the two proteins (62). In another study on human colon cancer cells *in vitro,* 1,25-dihydroxyvitamin D_3, the active metabolite form of vitamin D_3, inhibited TGF-β_1/TGF-β_2-induced invasion and migration in epithelial-mesenchymal transition (EMT), which suggests that suppression of EMT may be one of the mechanisms underlying the anti-metastasis effect of 1,25-dihydroxyvitamin D_3 in colon cancer cells in which TGF-β_1 expression is modified (63).

In a pilot case-control study, the "Markers of Adenomatous Polyps II" (MAP II) study (N=49 cases, 154 controls), in incident, sporadic colorectal adenoma patients relative to normal controls, in the whole crypt the expression of MSH2 was estimated to

be 23% lower (p=0.06) (48), TGF-β_1 expression was estimated to be 6.7% lower (p=0.75) (37), and TGF-α relative to TGF-β_1 expression was estimated to be 109.9% higher (p=0.02) (37). Moreover, a larger TGF-α/TGF-β_1 ratio was found to be associated with more than a two-fold risk of primary sporadic colorectal adenoma (OR=2.42, [95% CI: 0.85-6.87]) (37). MSH2 expression relative to MIB-1 expression was not investigated in the MAP II case-control study.

Our findings for MSH2 and the transforming growth factors were fairly consistent with those from our pilot clinical trial, the "Calcium and Vitamin D vs. Markers of Adenomatous Polyps" (CaD v MAP) trial (44,45). In that trial, increases in MSH2 expression in the active treatment groups relative to the placebo group were consistently observed across all crypt parameters (44). The strongest estimated relative treatment effects were observed for vitamin D_3 alone, with relative increases of 61% (p=0.11) in the whole crypt, 169% (p=0.04) in the upper 40% of crypts, and 54% (p=0.16) in the lower 60% of crypts. MSH2 relative to MIB-1 expression was not investigated in that trial. Also in that trial, TGF-β_1 expression increased in all active treatment groups, relative to placebo, with the strongest estimated relative treatment effects observed for vitamin D_3 and calcium combined, with estimated increases of 22% (p=0.09) in whole crypts, 20% (p=0.12) in the upper 40% of crypts, and 25% (p= 0.06) in the lower 60% of crypts (45). Relative to placebo, the TGF-α/TGF-β_1 ratio in whole crypts was estimated to increase by 2% (p=0.93) in the vitamin D_3 only group, decrease by 14% (p=0.46) in the calcium only group, and decrease by 11% (p=0.55) in the vitamin D_3 plus calcium group (45). In the upper 40% of crypts, the TGF-α/TGF-β_1 ratio decreased by 14% (p=0.41) in the vitamin

D_3 only group, by 28% (p=0.09) in the calcium only group, and by 22% (p=0.24) in the vitamin D_3 plus calcium group.

There are several possible reasons for the discrepancies in the findings between the two trials. Although the present study had an overall larger sample size than did the previous trial, the study participants were not randomized to treatment within the adjunct biomarker study, resulting in imbalances in sample sizes (including a small sample size in the double-placebo group), necessitating combining treatment groups for analysis, and imbalances in various exposures at baseline, raising the possibility of confounding by unmeasured variables. Additionally, chance findings cannot be discounted due to the small sample sizes, which may explain the null results for MSH2 and TGF-α in the present trial. However, the MSH2 findings in our present study are consistent with those in other studies in which microsatellite instability was observed to be most prominent in the proximal colon (20). Nevertheless, the results of both trials suggest that vitamin D, alone and in combination with calcium, may favorably modulate the expression of mismatch repair proteins and autocrine/paracrine growth factors in the colorectal crypts of the normal-appearing mucosa of sporadic colorectal adenoma patients.

Our study had several limitations and strengths. As noted above, the limitations included the relatively small sample size, especially in the double-placebo group, which increased the possibility of chance findings. The small sample size also created an imbalance in the characteristics of the participants across the treatment groups, which increased the chance of uncontrolled confounding; however, in our models we tested controlling for any measured factor for which there was evidence for imbalance across the treatment groups. Another limitation is that we collected biopsies only from the

rectal mucosa, so possible treatment effects more proximal in the colon are unknown. Also, only protein expression of the selected biomarkers was measured, which does not necessarily represent actual protein activity. Finally, because the study was restricted to sporadic colorectal adenoma patients, our findings may not be generalizable to other populations. The strengths of our study included the high protocol adherence by the participants and the automated immunostaining and novel image analysis software, enabling quantification of the crypt biomarker distributions, and the high biomarker measurement reliability.

In conclusion, the results for this chemoprevention trial provide human *in vivo* evidence that supplemental vitamin D, alone or combined with calcium, may increase mismatch repair relative to proliferation, increase TGF-β_1 expression, and decrease autocrine/paracrine growth promotion relative to growth inhibition in the colorectal epithelium, all of which are hypothesized to reduce risk for colorectal carcinogenesis. However, our findings did not support beneficial effects of calcium on the investigated biomarkers. Our findings also provide further support for the expression of MSH2 relative to MIB-1, TGF-β_1 alone, and TGF-α relative to TGF-β_1 in the normal-appearing rectal mucosa as modifiable, pre-neoplastic markers of risk for colorectal neoplasms. Taken together with previous literature, our findings support further investigation of vitamin D and calcium and of our investigated biomarkers in larger observational studies and clinical trials.

AUTHORS' CONTRIBUTIONS

Conception and design was performed by RM Bostick. Development of methodology was performed by RM Bostick. Acquisition of data was performed by RM Bostick, CY Um, E Barry, and J Baron. Analysis and interpretation of data was done by AK Kwan, RM Bostick, and CY Um. Writing, review, and/or revision of the manuscript was done by AK Kwan, RM Bostick, CY Um, E Barry, and J Baron. Administrative, technical, or material support was performed by RM Bostick, CY Um, E Barry, and J Baron. Study supervision was done by RM Bostick.

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CHAPTER 2

TABLES AND FIGURES

Table 1. Baseline Characteristics* of the Adjunct Biomarker Study Participants (N = 104), According to Treatment Assignment†

	Treatment Assignment							
	Randomization to vitamin D_3 and to calcium (4-arm)					Randomization to vitamin D_3 only (2-arm)		
Characteristics	Placebo (n = 12)	Calcium (n = 16)	Vitamin D (n = 17)	Vitamin D + Calcium (n = 17)	*P*-value‡	Calcium + Placebo (n = 23)	Calcium + Vitamin D (n = 19)	*P*-value§
Male (%)	75.0	81.3	70.6	82.4	0.87	0	0	—
Age (years)	59.9 (7.2)	59.9 (6.5)	59.2 (7.8)	57.6 (7.1)	0.78	58.2 (5.3)	59.2 (7.3)	0.65
White (%)	83.3	75.0	70.6	94.1	0.42	69.6	84.2	0.57
≥ College graduate (%)	66.7	37.5	64.7	52.9	0.37	47.8	36.8	0.54
Family history of colorectal cancer (%)‖	0	12.5	20.0	5.9	0.41	4.4	11.1	0.57
At least one advanced adenoma (%)¶	36.4	6.3	23.5	29.4	0.25	8.7	15.8	0.64
Current smoker (%)	25.0	6.3	0	5.9	0.12	0	15.8	0.16
Drink alcohol (%)	75.0	68.8	88.2	82.4	0.51	43.5	52.6	0.76
Physical activity, MET-mins/wk#	1,620 (1,195)	2,128 (2,378)	2,782 (2,764)	3,875 (2,424)	0.03	1,458 (1,235)	3,021 (3,469)	0.07
Body mass index (kg/m^2)	29.4 (4.9)	32.3 (7.6)	28.7 (5.5)	30.0 (4.5)	0.31	29.7 (5.6)	27.5 (4.7)	0.18
Total energy intake (kcal/d)	1,341 (358)	1,731 (537)	1,437 (527)	1,613 (550)	0.16	1,254 (549)	1,429 (595)	0.27
Total fat intake (g/d)	58 (20)	69 (25)	60 (27)	63 (27)	0.66	50 (26)	61 (36)	0.31
Dietary fiber intake (g/d)	10 (4)	16 (5)	14 (6)	16 (6)	0.02	14 (5)	17 (5)	0.02
Total calcium intake (mg/d)**	696 (415)	891 (255)	663 (272)	667 (255)	0.05	938 (467)	1,213 (553)	0.06
Take multivitamin supplement (%)	41.7	81.3	47.1	64.7	0.11	69.6	89.5	0.15
Take aspirin ≥ once/week (%)	50.0	68.8	41.2	41.2	0.37	30.4	31.6	1.00
Take non-aspirin NSAID ≥ once/week (%)	33.3	43.8	23.5	29.4	0.68	26.1	31.6	0.74
Serum 25-hydroxyvitamin D level (ng/mL)	22.4 (8.2)	24.5 (13.4)	23.1 (8.7)	22.5 (6.5)	0.99	24.8 (8.9)	26.5 (9.6)	0.51

Abbreviations: MET, metabolic equivalents of task; NSAID, nonsteroidal anti-inflammatory drug

* Mean (SD) reported unless otherwise specified.

† Women who did not wish to cease prior calcium supplementation could elect to remain on calcium and be randomized to vitamin D only (2-arm randomization); all other patients were randomized to calcium, vitamin D, both agents, or placebo (4-arm randomization).

‡ From Fisher's exact test for categorical variables, and one-way ANOVA for continuous variables (transformed by the natural logarithm to improve normality when indicated).

§ From Fisher's exact test for categorical variables, and two-sample *t* test for continuous variables (transformed by the natural logarithm to improve normality when indicated).

‖ In a first-degree relative. Two missing values in "Vitamin D" (4-arm) and one missing value in "Calcium + Vitamin D" (2-arm).

¶ Multiple adenoma, adenoma ≥ 1.0 cm in diameter, and/or adenoma with a villous component or severe dysplasia. One missing value in "Placebo" (4-arm).

One missing value in "Calcium + Vitamin D" (2-arm).

** Dietary + supplemental sources.

Table 2. MSH2 Expression in the Normal-Appearing Colorectal Mucosa of the Adjunct Biomarker Study Participants*

	Baseline (n = 100)				1-Yr follow-up (n = 102)				Absolute Tx effect†			Relative Tx effect‡
MSH2 (OD)	n	Mean	95% CI	*P*	n	Mean	95% CI	*P*	Tx effect	95% CI	*P*§	
Whole crypt												
Vitamin D vs. No vitamin D												
No vitamin D	48	1905	(1705, 2106)	0.60	51	1956	(1747, 2164)	0.78	31	(-169, 230)	0.76	1.02
Vitamin D	52	1837	(1672, 2002)		51	1918	(1755, 2082)					
Calcium vs. No calcium												
No calcium	27	1925	(1703, 2146)	0.78	29	1999	(1775, 2224)	0.39	-95	(-358, 168)	0.47	0.95
Calcium	32	1877	(1619, 2135)		31	1857	(1617, 2096)					
Vitamin D + Calcium vs. Calcium												
Calcium	38	1837	(1615, 2059)	0.92	39	1858	(1625, 2092)	0.64	90	(-133, 313)	0.42	1.05
Vitamin D + Calcium	35	1820	(1600, 2040)		34	1932	(1718, 2146)					
Upper 40% of crypt												
Vitamin D vs. No vitamin D												
No vitamin D	48	349	(307, 392)	0.29	51	360	(309, 411)	0.49	9	(-45, 64)	0.73	1.03
Vitamin D	52	319	(280, 357)		51	339	(302, 376)					
Calcium vs. No calcium												
No calcium	27	318	(267, 369)	0.46	29	346	(291, 400)	0.76	-39	(-104, 25)	0.23	0.89
Calcium	32	346	(289, 403)		31	334	(280, 388)					
Vitamin D + Calcium vs. Calcium												
Calcium	38	344	(296, 392)	0.64	39	343	(288, 398)	0.75	28	(-35, 91)	0.38	1.08
Vitamin D + Calcium	35	328	(279, 377)		34	354	(305, 404)					
Lower 60% of crypt												
Vitamin D vs. No vitamin D												
No vitamin D	48	1498	(1339, 1658)	0.72	51	1536	(1376, 1696)	0.89	24	(-122, 170)	0.75	1.02
Vitamin D	52	1461	(1333, 1589)		51	1522	(1395, 1649)					
Calcium vs. No calcium												
No calcium	27	1548	(1368, 1728)	0.60	29	1593	(1420, 1766)	0.34	-47	(-244, 150)	0.64	0.97
Calcium	32	1472	(1273, 1671)		31	1470	(1285, 1654)					
Vitamin D + Calcium vs. Calcium												
Calcium	38	1434	(1260, 1609)	0.99	39	1460	(1279, 1642)	0.64	56	(-106, 218)	0.49	1.04
Vitamin D + Calcium	35	1436	(1265, 1606)		34	1518	(1355, 1681)					

Abbreviations: 95% CI, 95% confidence interval; OD, optical density; Tx, treatment.

* Geometric means, adjusted for baseline total energy intake shown. Four participants excluded at baseline and two at 1-yr follow-up because of unreliable MSH2 measurements.

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

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

§ From repeated-measures MIXED linear model.

Table 3. MSH2 Relative to MIB-1 Expression in the Normal-Appearing Colorectal Mucosa of the Adjunct Biomarker Study Participants*

	Baseline (n = 100)				1-Yr follow-up (n = 102)				Absolute Tx effect†	Relative Tx effect‡		
MSH2/MIB-1 Ratio (OD)	n	Mean§	95% CI§	*P*	n	Mean§	95% CI§	*P*		Tx effect§	95% CI§	*P*‖
Whole crypt												
Vitamin D vs. No vitamin D												
No vitamin D	48	1.58	(1.43, 1.75)	0.22	51	1.42	(1.28, 1.57)	0.94	0.14	1.10	(0.94, 1.29)	0.23
Vitamin D	52	1.44	(1.30, 1.60)		51	1.43	(1.27, 1.60)					
Calcium vs. No calcium												
No calcium	27	1.44	(1.25, 1.67)	0.73	29	1.53	(1.35, 1.74)	0.30	-0.10	0.93	(0.76, 1.15)	0.51
Calcium	32	1.40	(1.22, 1.60)		31	1.38	(1.19, 1.61)					
Vitamin D + Calcium vs. Calcium												
Calcium	38	1.62	(1.45, 1.82)	0.14	39	1.38	(1.22, 1.56)	0.99	0.19	1.14	(0.96, 1.35)	0.14
Vitamin D + Calcium	35	1.43	(1.26, 1.62)		34	1.38	(1.19, 1.60)					
Upper 40% of crypt												
Vitamin D vs. No vitamin D												
No vitamin D	48	6.66	(5.26, 8.43)	1.00	51	5.69	(4.35, 7.43)	0.08	2.66	1.47	(0.88, 2.46)	0.14
Vitamin D	52	6.66	(5.22, 8.48)		51	8.35	(5.96, 11.68)					
Calcium vs. No calcium												
No calcium	27	5.51	(3.93, 7.73)	0.23	29	7.87	(4.94, 12.54)	0.83	-2.21	0.71	(0.33, 1.53)	0.38
Calcium	32	7.22	(5.41, 9.64)		31	7.36	(4.89, 11.08)					
Vitamin D + Calcium vs. Calcium												
Calcium	38	7.27	(5.61, 9.41)	0.76	39	5.37	(4.01, 7.18)	0.09	3.25	1.62	(0.94, 2.80)	0.08
Vitamin D + Calcium	35	6.84	(5.08, 9.20)		34	8.19	(5.55, 12.08)					
Lower 60% of crypt												
Vitamin D vs. No vitamin D												
No vitamin D	48	1.31	(1.19, 1.45)	0.25	51	1.19	(1.08, 1.32)	0.97	0.10	1.09	(0.93, 1.26)	0.28
Vitamin D	52	1.21	(1.09, 1.34)		51	1.19	(1.06, 1.33)					
Calcium vs. No calcium												
No calcium	27	1.23	(1.06, 1.42)	0.48	29	1.29	(1.15, 1.46)	0.22	-0.06	0.96	(0.79, 1.16)	0.65
Calcium	32	1.14	(1.00, 1.31)		31	1.15	(1.00, 1.32)					
Vitamin D + Calcium vs. Calcium												
Calcium	38	1.34	(1.19, 1.51)	0.16	39	1.16	(1.03, 1.31)	0.83	0.13	1.11	(0.94, 1.31)	0.22
Vitamin D + Calcium	35	1.18	(1.04, 1.34)		34	1.14	(0.99, 1.31)					

Abbreviations: 95% CI, 95% confidence interval; OD, optical density; Tx, treatment.

* Geometric means, unadjusted shown. Four participants excluded at baseline and two at 1-yr follow-up because of unreliable MSH2 measurements.

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

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

§ Values for "Whole Crypt," "Upper 40% of Crypt," and "Lower 60% of Crypt" were transformed by the natural logarithm to improve normality the distribution for PROC MIXED, and then back-transformed by exponentiation.

‖ From repeated-measures MIXED linear model.

Table 4. TGF-α Expression in the Normal-Appearing Colorectal Mucosa of the Adjunct Biomarker Study Participants*

	Baseline (n = 94)				1-Yr follow-up (n = 97)				Absolute Tx effect†			Relative Tx effect‡
TGF-α (OD)	n	Mean	95% CI	*P*	n	Mean	95% CI	*P*	Tx effect	95% CI	*P*§	
Whole crypt												
Vitamin D vs. No vitamin D												
No vitamin D	45	6466	(5876, 7056)	0.82	49	6529	(5963, 7094)	0.94	-67	(-689, 504)	0.82	0.99
Vitamin D	49	6562	(5970, 7153)		48	6557	(6031, 7083)					
Calcium vs. No calcium												
No calcium	28	7114	(6386, 7843)	0.02	29	7205	(6402, 8008)	0.02	-68	(-813, 678)	0.86	0.99
Calcium	30	5959	(5274, 6644)		30	5982	(5362, 6602)					
Vitamin D + Calcium vs. Calcium												
Calcium	34	6280	(5647, 6913)	0.96	37	6241	(5650, 6831)	0.87	93	(-617, 803)	0.79	1.01
Vitamin D + Calcium	32	6256	(5463, 7048)		31	6309	(5712, 6907)					
Upper 40% of crypt												
Vitamin D vs. No vitamin D												
No vitamin D	45	3313	(3054, 3572)	0.53	49	3357	(3087, 3626)	0.41	33	(-251, 317)	0.82	1.01
Vitamin D	49	3429	(3175, 3684)		48	3506	(3274, 3737)					
Calcium vs. No calcium												
No calcium	28	3615	(3300, 3930)	0.04	29	3742	(3366, 4119)	0.03	-63	(-438, 313)	0.74	0.99
Calcium	30	3143	(2835, 3451)		30	3208	(2914, 3503)					
Vitamin D + Calcium vs. Calcium												
Calcium	34	3242	(2951, 3533)	0.78	37	3258	(2968, 3547)	0.61	34	(-315, 383)	0.85	1.01
Vitamin D + Calcium	32	3304	(2972, 3637)		31	3354	(3113, 3596)					
Lower 60% of crypt												
Vitamin D vs. No vitamin D												
No vitamin D	45	2713	(2382, 3045)	0.84	49	2711	(2424, 2997)	0.5	-93	(-410, 224)	0.56	0.97
Vitamin D	49	2664	(2322, 3006)		48	2569	(2271, 2866)					
Calcium vs. No calcium												
No calcium	28	3031	(2591, 3472)	0.03	29	2948	(2538, 3357)	0.02	24	(-385, 433)	0.91	1.00
Calcium	30	2387	(2015, 2759)		30	2327	(1997, 2658)					
Vitamin D + Calcium vs. Calcium												
Calcium	34	2602	(2265, 2939)	0.70	37	2525	(2228, 2821)	0.93	90	(-288, 469)	0.64	1.04
Vitamin D + Calcium	32	2491	(2037, 2945)		31	2504	(2132, 2875)					

Abbreviatoins: 95% CI, 95% confidence interval; OD, optical density; Tx, treatment.

* Geometric means, unadjusted shown. Ten participants excluded at baseline and seven at 1-yr follow-up because of unreliable TGF-α measurements.

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

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

§ From repeated-measures MIXED linear model.

Table 5. TGF-β_1 Expression in the Normal-Appearing Colorectal Mucosa of the Adjunct Biomarker Study Participants*

	Baseline (n = 103)				1-Yr follow-up (n = 103)				Absolute Tx effect†	Relative Tx effect‡		
TGF-β_1 (OD)	n	Mean§	95% CI§	*P*	n	Mean§	95% CI§	*P*		Tx effect§	95% CI	*P*‖
Whole crypt												
Vitamin D vs. No vitamin D												
No vitamin D	50	625	(372, 1051)	0.12	50	423	(239, 751)	0.31	150	1.24	(0.78, 1.98)	0.35
Vitamin D	53	329	(178, 609)		53	277	(155, 496)					
Calcium vs. No calcium												
No calcium	29	503	(245, 1033)	0.27	29	378	(184, 776)	0.15	19	0.84	(0.48, 1.47)	0.54
Calcium	33	290	(147, 572)		33	184	(93, 364)					
Vitamin D + Calcium vs. Calcium												
Calcium	38	617	(331, 1152)	0.14	38	413	(207, 823)	0.34	166	1.30	(0.71, 2.39)	0.39
Vitamin D + Calcium	36	293	(136, 632)		36	256	(125, 524)					
Upper 40% of crypt												
Vitamin D vs. No vitamin D												
No vitamin D	50	168	(90, 313)	0.14	50	118	(60, 231)	0.42	49	1.41	(0.79, 2.52)	0.25
Vitamin D	53	82	(39, 170)		53	81	(43, 152)					
Calcium vs. No calcium												
No calcium	29	143	(65, 312)	0.18	29	113	(52, 246)	0.15	14	0.96	(0.47, 1.99)	0.92
Calcium	33	66	(28, 154)		33	50	(23, 111)					
Vitamin D + Calcium vs. Calcium												
Calcium	38	173	(82, 366)	0.10	38	112	(49, 255)	0.47	71	1.78	(0.83, 3.84)	0.14
Vitamin D + Calcium	36	64	(25, 164)		36	73	(33, 162)					
Lower 60% of crypt												
Vitamin D vs. No vitamin D												
No vitamin D	50	405	(247, 662)	0.08	50	266	(155, 458)	0.27	109	1.30	(0.81, 2.08)	0.28
Vitamin D	53	201	(108, 374)		53	172	(97, 305)					
Calcium vs. No calcium												
No calcium	29	313	(152, 643)	0.33	29	236	(117, 475)	0.14	-4	0.78	(0.44, 1.36)	0.37
Calcium	33	195	(102, 373)		33	114	(58, 224)					
Vitamin D + Calcium vs. Calcium												
Calcium	38	391	(216, 707)	0.13	38	261	(137, 499)	0.30	102	1.27	(0.69, 2.35)	0.43
Vitamin D + Calcium	36	185	(86, 398)		36	157	(77, 322)					

Abbreviations: 95% CI, 95% confidence interval; OD, optical density; Tx, treatment.

* Geometric means, unadjusted shown. One participant excluded at baseline and one at 1-yr follow-up because of unreliable TGF-β_1 measurements.

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

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

§ Values for "Whole Crypt," "Upper 40% of Crypt," and "Lower 60% of Crypt" were transformed by the natural logarithm to improve normality the distribution for PROC MIXED, and then back-transformed by exponentiation.

‖ From repeated-measures MIXED linear model.

Table 6. TGF-α Relative to TGF-β_1 Expression in the Normal-Appearing Colorectal Mucosa of the Adjunct Biomarker Study Participants*

	Baseline (n = 94)				1-Yr follow-up (n = 97)				Absolute tx effect†	Relative tx effect‡		
TGF-α/TGF-β_1 Ratio (OD)	n	Mean§	95% CI§	*P*	n	Mean§	95% CI§	*P*		Tx effect§	95% CI§	*P*‖
Whole crypt												
Vitamin D vs. No vitamin D												
No vitamin D	45	9.5	(5.9, 15.5)	0.05	49	15.2	(8.8, 26.3)	0.18	-0.1	0.80	(0.52, 1.21)	0.29
Vitamin D	49	20.4	(11.4, 36.6)		48	26.0	(14.7, 46.0)					
Calcium vs. No calcium												
No calcium	28	14.4	(7.2, 28.6)	0.40	29	18.2	(9.4, 35.3)	0.19	8.8	1.25	(0.74, 2.12)	0.39
Calcium	30	21.5	(11.1, 41.5)		30	34.1	(17.1, 67.9)					
Vitamin D + Calcium vs. Calcium												
Calcium	34	8.9	(5.0, 15.9)	0.05	37	15.1	(7.8, 29.3)	0.18	0.5	0.76	(0.44, 1.34)	0.34
Vitamin D + Calcium	32	22.8	(10.9, 47.4)		31	29.5	(14.2, 61.2)					
Upper 40% of crypt												
Vitamin D vs. No vitamin D												
No vitamin D	45	18.9	(10.2, 35.0)	0.08	49	28.5	(14.7, 55.3)	0.23	-4.0	0.75	(0.42, 1.31)	0.31
Vitamin D	49	44.5	(21.3, 92.7)		48	50.0	(25.9, 96.7)					
Calcium vs. No calcium												
No calcium	28	26.3	(12.1, 57.3)	0.25	29	31.8	(15.3, 66.0)	0.18	10.1	1.08	(0.51, 2.26)	0.84
Calcium	30	52.0	(21.4, 126.4)		30	67.6	(29.6, 154.3)					
Vitamin D + Calcium vs. Calcium												
Calcium	34	16.8	(8.1, 34.7)	0.04	37	29.6	(13.1, 67.2)	0.26	-13.8	0.56	(0.26, 1.20)	0.13
Vitamin D + Calcium	32	58.7	(22.2, 155.4)		31	57.8	(24.6, 135.9)					
Lower 60% of crypt												
Vitamin D vs. No vitamin D												
No vitamin D	45	5.8	(3.8, 9.0)	0.03	49	9.6	(5.9, 15.9)	0.19	-0.9	0.75	(0.49, 1.13)	0.16
Vitamin D	49	12.6	(7.4, 21.4)		48	15.5	(9.2, 26.1)					
Calcium vs. No calcium												
No calcium	28	9.4	(4.9, 18.1)	0.57	29	11.5	(6.3, 21.2)	0.20	6.4	1.39	(0.83, 2.34)	0.2
Calcium	30	12.0	(6.8, 21.1)		30	20.5	(10.8, 39.0)					
Vitamin D + Calcium vs. Calcium												
Calcium	34	5.6	(3.3, 9.4)	0.05	37	9.3	(5.1, 16.8)	0.15	1.1	0.82	(0.48, 1.42)	0.48
Vitamin D + Calcium	32	13.1	(6.8, 25.0)		31	17.8	(9.1, 34.8)					

Abbreviations: 95% CI, 95% confidence interval; OD, optical density; Tx, treatment.

* Geometric means, unadjusted shown. Ten participants excluded at baseline and seven at 1-yr follow-up because of unreliabe TGF-α or TGF-β_1 measurements.

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

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

§ Values for "Whole Crypt," "Upper 40% of Crypt," and "Lower 60% of Crypt" were transformed by the natural logarithm to improve normality the distribution for PROC MIXED, and then back-transformed by exponentiation.

‖ From repeated-measures MIXED linear model.

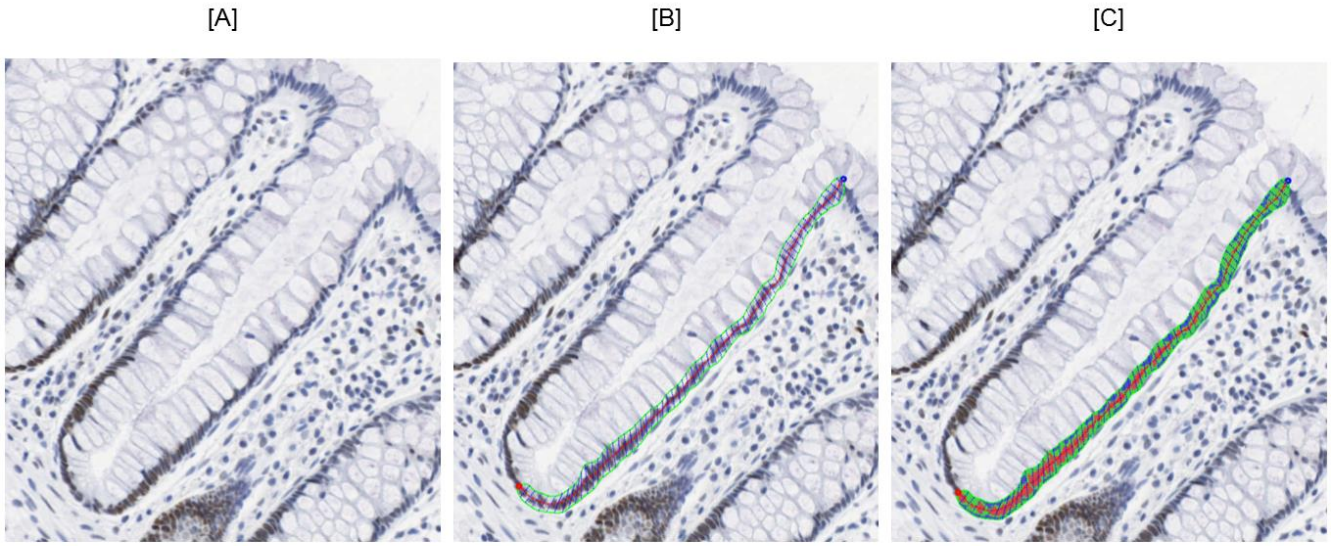


Figure 1. Depicts measurement of biomarker (MSH2) expression in crypts of normal appearing rectal mucosa using custom-designed quantitative image analysis software. (A) Identifying a full length hemicrypt. (B) Outlining the hemicrypt and sections are automatically generated. (C) Quantification of MSH2 labeling optical density, overall and within each section of the hemicrypt.

CHAPTER 2

SUPPLEMENTARY MATERIALS

Supplement Table 1. Distribution Index (Φ_h) for MSH2, TGF-α, and TGF-β_1 Expression in the Normal-Appearing Colorectal Mucosa of the Adjunct Biomarker Study Participants*

	Baseline				1-Yr follow-up				Absolute Tx effect†			Relative Tx effect‡
Φ_h (OD)	n	Mean	95% CI	*P*	n	Mean	95% CI	*P*	Tx effect	95% CI	*P*§	
MSH2‖												
Vitamin D vs. No vitamin D												
No vitamin D	48	0.18	(0.17, 0.19)	0.13	51	0.18	(0.17, 0.19)	0.42	0.01	(-0.01, 0.02)	0.44	1.06
Vitamin D	52	0.17	(0.16, 0.18)		51	0.18	(0.17, 0.18)					
Calcium vs. No calcium												
No calcium	27	0.17	(0.15, 0.18)	0.10	29	0.17	(0.16, 0.18)	0.68	-0.01	(-0.03, 0.00)	0.14	1.00
Calcium	32	0.18	(0.17, 0.19)		31	0.18	(0.16, 0.19)					
Vitamin D + Calcium vs. Calcium												
Calcium	38	0.19	(0.18, 0.20)	0.37	39	0.18	(0.17, 0.20)	0.88	0.01	(-0.01, 0.02)	0.45	1.06
Vitamin D + Calcium	35	0.18	(0.17, 0.19)		34	0.18	(0.17, 0.19)					
TGF-α¶												
Vitamin D vs. No vitamin D												
No vitamin D	45	0.52	(0.51, 0.54)	0.19	49	0.52	(0.51, 0.54)	0.03	0.01	(-0.01, 0.03)	0.42	1.02
Vitamin D	49	0.54	(0.52, 0.56)		48	0.55	(0.53, 0.56)					
Calcium vs. No calcium												
No calcium	28	0.52	(0.50, 0.54)	0.14	29	0.53	(0.51, 0.55)	0.19	0.00	(-0.03, 0.02)	0.72	1.00
Calcium	30	0.54	(0.52, 0.56)		30	0.55	(0.52, 0.57)					
Vitamin D + Calcium vs. Calcium												
Calcium	34	0.52	(0.51, 0.54)	0.11	37	0.53	(0.51, 0.55)	0.30	-0.01	(-0.03, 0.01)	0.41	0.96
Vitamin D + Calcium	32	0.55	(0.52, 0.57)		31	0.54	(0.52, 0.57)					
TGF-β_1#												
Vitamin D vs. No vitamin D												
No vitamin D	50	0.29	(0.27, 0.32)	0.91	50	0.30	(0.28, 0.33)	0.71	0.01	(-0.03, 0.05)	0.63	1.03
Vitamin D	53	0.29	(0.26, 0.32)		53	0.31	(0.28, 0.34)					
Calcium vs. No calcium												
No calcium	29	0.31	(0.27, 0.35)	0.16	29	0.31	(0.28, 0.35)	0.70	0.03	(-0.03, 0.09)	0.34	1.11
Calcium	33	0.27	(0.23, 0.30)		33	0.30	(0.27, 0.34)					
Vitamin D + Calcium vs. Calcium												
Calcium	38	0.31	(0.28, 0.34)	0.12	38	0.30	(0.27, 0.34)	0.70	0.05	(0.00, 0.10)	0.04	1.19
Vitamin D + Calcium	36	0.27	(0.23, 0.31)		36	0.31	(0.27, 0.35)					

Abbrevations: 95% CI, 95% confidence interval; OD, optical density; Tx, treatment; Φ_h (distribution index), ratio of expression in the upper 40% of the crypt (i.e., differentiation zone) to that in the whole crypt.

* Geometric means, adjusted for baseline total energy intake (MSH2), unadjusted (TGF-α and TGF-β_1) shown.

† Absolute Tx Effect = [(treatment group follow-up) - (treatment group baseline) - [(placebo group follow-up) - (placebo group baseline)].

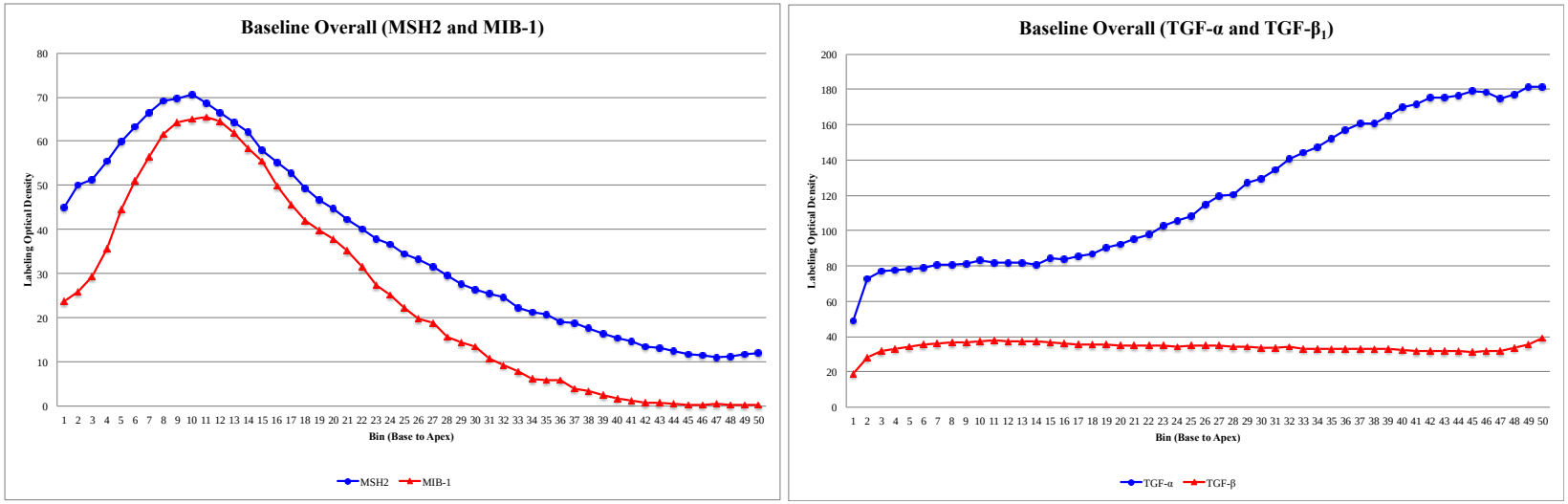
‡ Relative Tx Effect = [(treatment group follow-up)/(treatment group baseline)]/[(placebo group follow-up)/(placebo group baseline)]; interpretation similar to that for an odds ratio.

§ From repeated-measures MIXED linear model.

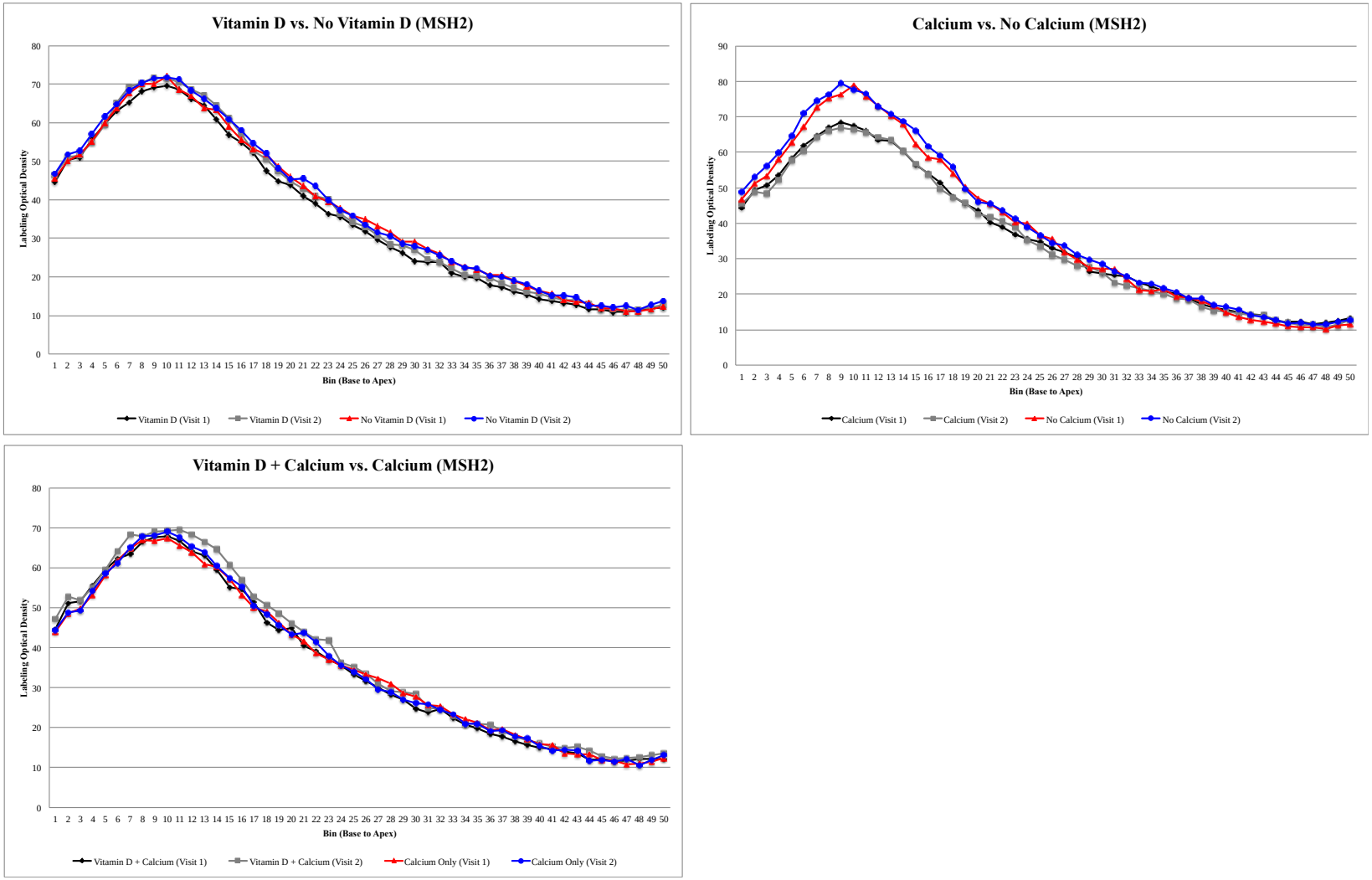
‖ Four participants excluded at baseline and two at 1-yr follow-up because of unreliable MSH2 measurements.

¶ Ten participants excluded at baseline and seven at 1-yr follow-up because of unreliable TGF-α measurements.

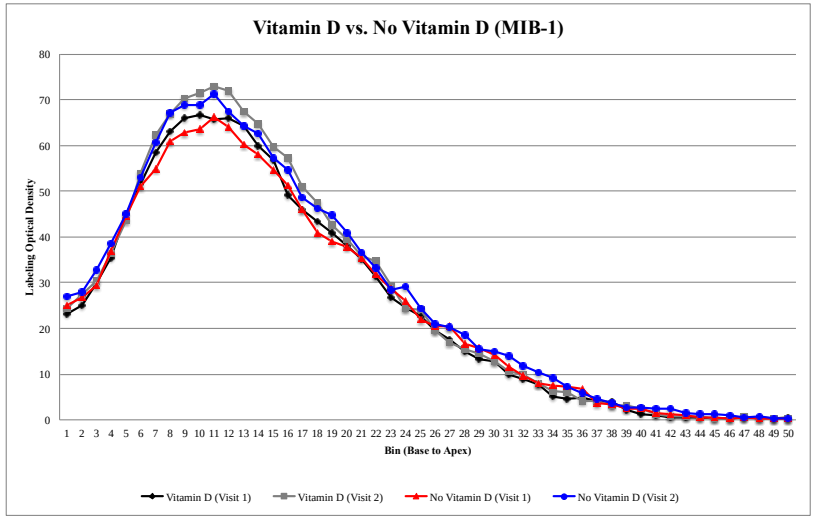
One participant excluded at baseline and one at 1-yr follow-up because of unreliable TGF-β_1 measurements.

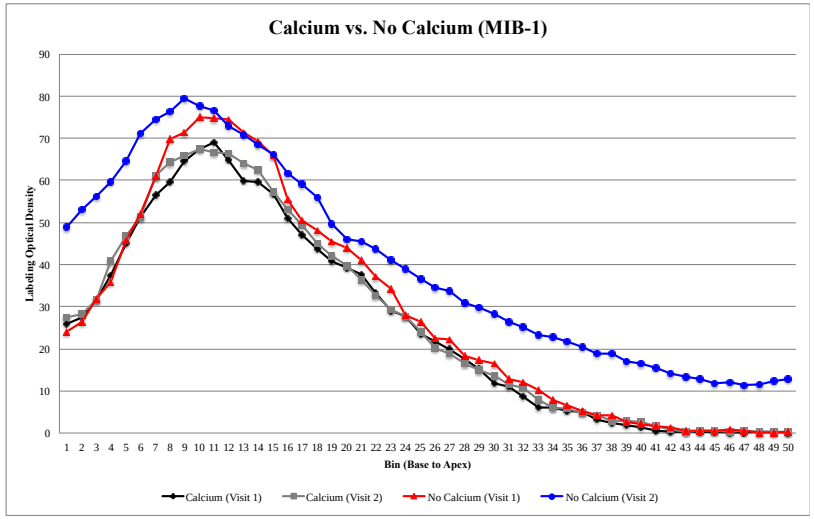


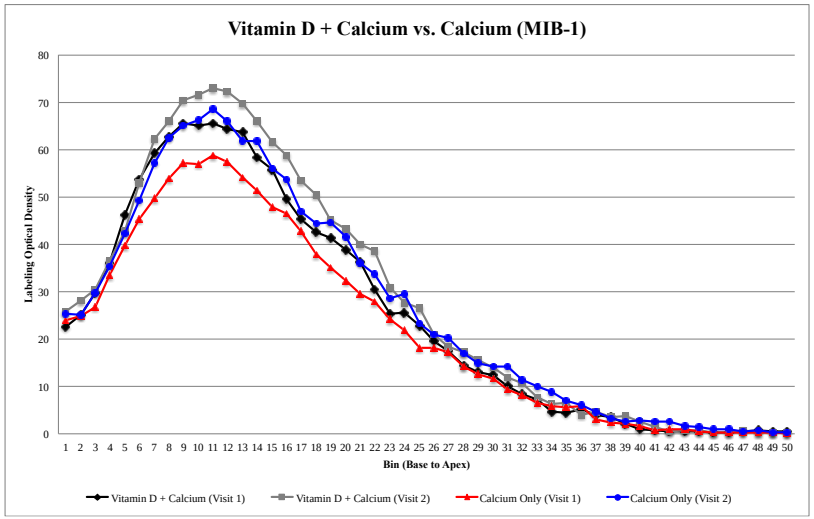
Supplement Figure 1. Comparisons of the overall mean distributions of MSH2 versus MIB-1 expression and TGF-α versus TGF-β_1 expression, respectively, at baseline from the base to apex of colorectal crypts, divided into 50 segments ("bins").



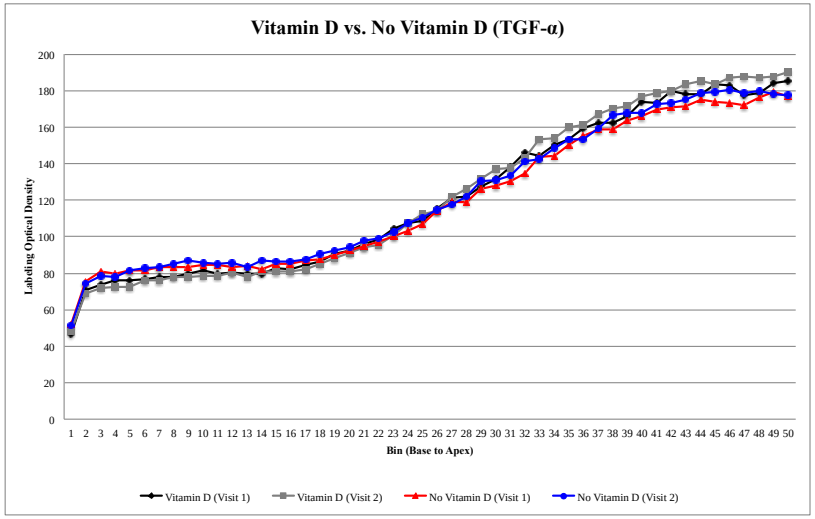
Supplement Figure 2. Distribution of MSH2 expression from base to apex of colorectal crypts, divided into 50 segments ("bins"), by treatment group and visit (baseline and 1-yr follow-up).

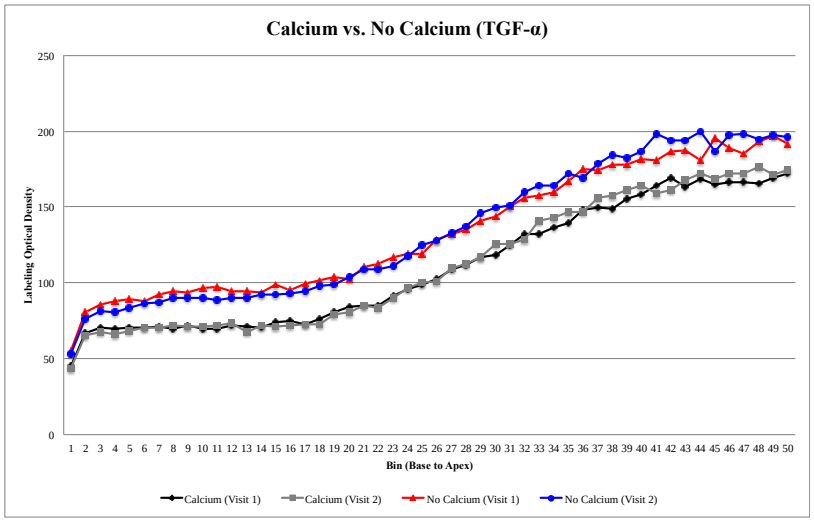


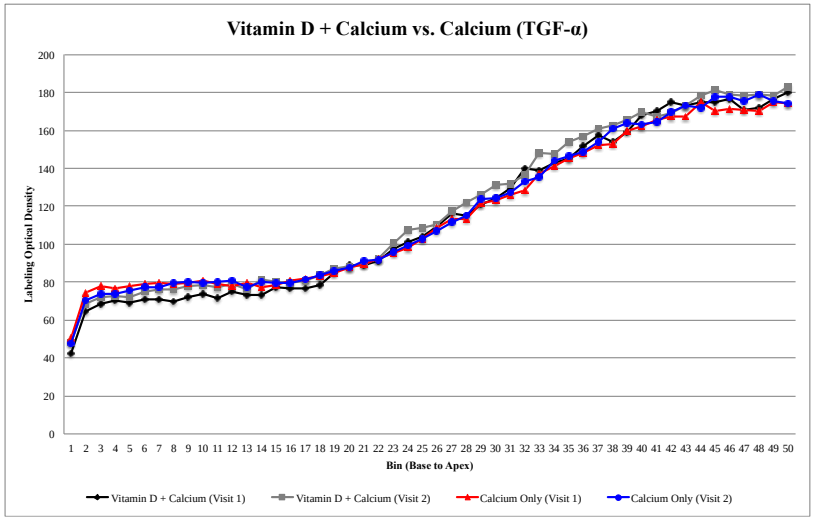




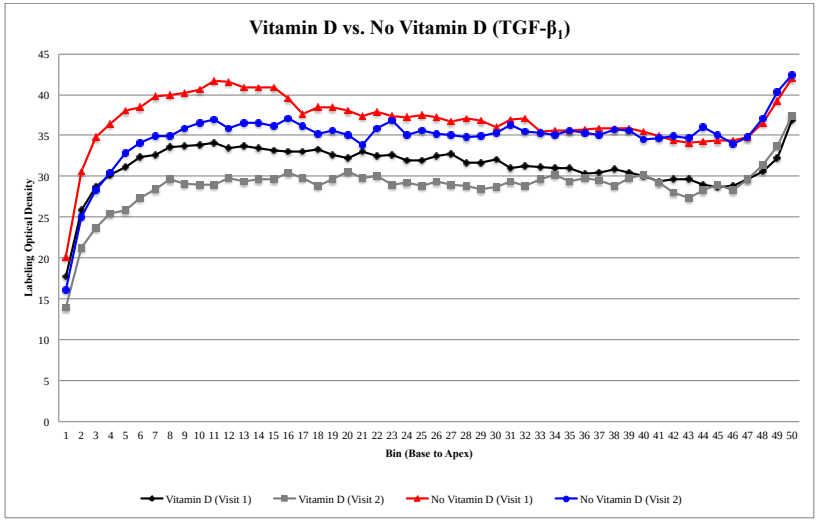
Supplement Figure 3. Distribution of MIB-1 expression from base to apex of colorectal crypts, divided into 50 segments ("bins"), by treatment group and visit (baseline and 1-yr follow-up).

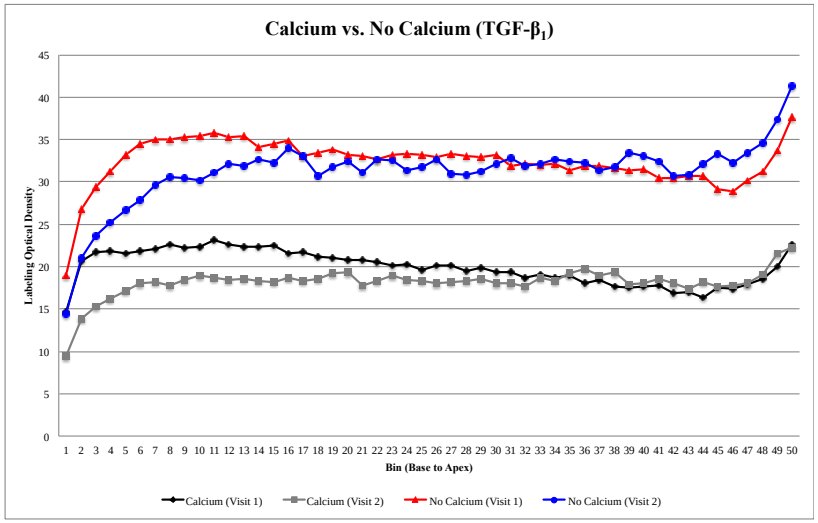


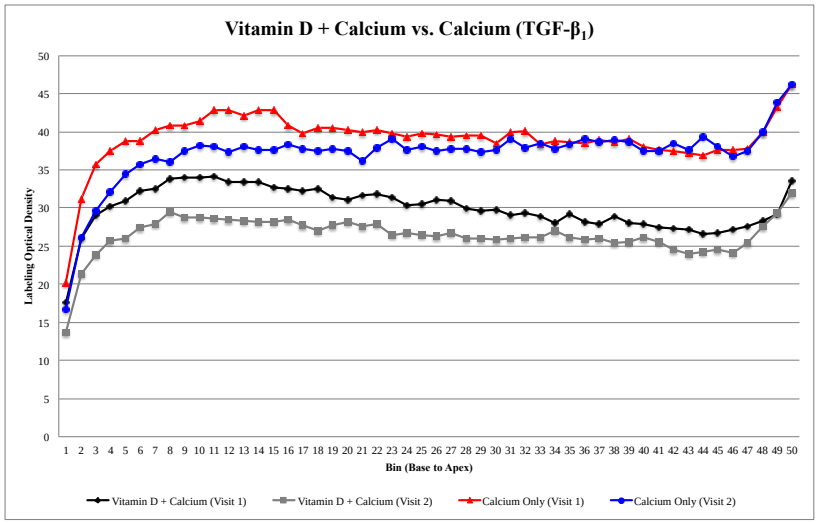




Supplement Figure 4. Distribution of TGF-α expression from base to apex of colorectal crypts, divided into 50 segments ("bins"), by treatment group and visit (baseline and 1-yr follow-up).







Supplement Figure 5. Distribution of TGF-β_1 expression from base to apex of colorectal crypts, divided into 50 segments ("bins"), by treatment group and visit (baseline and 1-yr follow-up).

CHAPTER 3

CONCLUSIONS AND FUTURE DIRECTIONS

CONCLUSIONS AND FUTURE DIRECTIONS

In conclusion, the results for this chemoprevention trial provide human *in vivo* evidence that supplemental vitamin D, alone or in combination with calcium, may increase mismatch repair relative to proliferation, increase TGF-β expression, and decrease autocrine/paracrine growth promotion relative to growth inhibition in the colorectal epithelium, all of which are hypothesized to reduce risk for colorectal carcinogenesis. However, our findings did not support beneficial effects of calcium on the investigated biomarkers. Our findings also provide further support for the expression of MSH2 relative to MIB-1, TGF-β_1 alone, and TGF-α relative to TGF-β_1 in the normal-appearing rectal mucosa as modifiable, pre-neoplastic markers of risk for colorectal neoplasms. Taken together with previous literature, our findings support further investigation of vitamin D and calcium and of our investigated biomarkers in larger observational studies and clinical trials.

Further proposed research includes a similar but larger-scale clinical trial to investigate the effects of supplemental vitamin D and/or calcium on MSH2, TGF-α, and TGF-β_1 expression in multiple levels of colon (proximal, distal, and rectum) from normal-appearing colorectal mucosa and to see if this modulation is associated with decreased recurrence of sporadic colorectal adenomatous polyps; a trial to investigate whether biomarker responses to treatments vary according to vitamin D receptor genotype, genes involved in vitamin D metabolism (CYP24A1 and CYP27B1), and colorectal adenoma status; a trial to investigate other biomarkers including mismatch repair proteins vital in colorectal carcinogenesis (e.g., MLH1); and a dose-response trial

to investigate a possible dose-response relationship of vitamin D and calcium supplementation with MSH2, TGF-α, and TGF-β_1 expression.