Published in final edited form as:

J Steroid Biochem Mol Biol. 2015 April ; 148: 86–95. doi:10.1016/j.jsbmb.2015.01.010.

Effects of Supplemental Vitamin D and Calcium on Normal Colon Tissue and Circulating Biomarkers of Risk for Colorectal Neoplasms

Roberd M. Bostick, MD, MPH^{a,b}

^aDepartment of Epidemiology, Rollins School of Public Health, Emory University, 1518 Clifton Road NE, Atlanta, GA USA 30322

^bWinship Cancer Institute, Emory University, Atlanta, GA USA 30322

Abstract

This brief review, based on an invited presentation at the 17th Workshop on Vitamin D, is to summarize a line of the author's research that has been directed at the intertwined missions of clarifying and/or developing vitamin D and calcium and as preventive agents against colorectal cancer in humans, understanding the mechanisms by which these agents may reduce risk for the disease, and developing 'treatable' biomarkers of risk for colorectal cancer. The biological plausibility and observational and clinical trial evidence for vitamin D and calcium in reducing risk for colorectal neoplasms, the development of pre-neoplastic biomarkers of risk for colorectal neoplasms, and the clinical trial findings from the author's research group on the efficacy of vitamin D and calcium in modulating these biomarkers are summarized. Regarding the latter, we tested the efficacy of 800 IU (20 µg) of vitamin D₃ and 2.0g of calcium daily, alone and combined vs. placebo over 6 months on modulating normal colon tissue and circulating hypothesis-based biomarkers of risk for colorectal neoplasms in a randomized, double-blind, placebo-controlled, 2×2 factorial design clinical trial (n = 92). The tissue-based biomarkers were measured in biopsies of normal-appearing rectal mucosa using immunohistochemistry with quantitative image analysis, and a panel of circulating inflammation markers was measured using enzyme-linked immunoassays (ELISA). Statistically significant proportional tissue increases in the vitamin D group relative to the placebo group were found in bax (51%), p21 (141%), APC (48%), E-cadherin (78%), MSH2 (179%), the CaSR (39%), and CYP27B1 (159%). In blood, there was a 77% statistically significant decrease in a summary inflammation z-score. The findings for calcium were similar to those for vitamin D. These findings indicate that supplemental vitamin D₃ or calcium can favorably modulate multiple normal colon tissue and circulating hypothesis-based biomarkers of risk for colorectal neoplasms in sporadic colorectal adenoma patients.

© 2015 Elsevier Ltd. All rights reserved.

Telephone 011-404-727-2671; rmbosti@emory.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

vitamin D; calcium; colorectal neoplasms; biomarkers of risk

1.0 Introduction

The purpose of this paper, which is based on my invited presentation at the 17th Workshop on Vitamin D, is to summarize a line of my research over the past 25 years that has been directed at the intertwined missions of clarifying and/or developing calcium and vitamin D as chemopreventive agents against colorectal cancer in humans, understanding the mechanisms by which these agents may reduce risk for the disease, and developing ‘treatable’ biomarkers of risk for colorectal cancer. Accordingly, I briefly summarize the biological plausibility and the observational and clinical trial evidence for calcium and vitamin D in reducing risk for colorectal neoplasms, briefly describe the rationale and approach for developing pre-neoplastic biomarkers of risk for colorectal neoplasms as well as evidence for the validity of a set of such biomarkers, and then summarize the clinical trial findings from our research group on the efficacy of calcium and vitamin D in modulating these biomarkers. In the background sections (sections 2.0 – 6.0) I merely briefly summarize background with some updates from my previous more extensive review, and the reader is referred to that review [1] and other referenced articles below for a more extensive treatment of and references for the background. The studies and their results presented in sections 7.2 and 8.0 are summarized from our research group’s published reports over the years, and the reader is referred to those for more extensive detail.

2.0 Vitamin D and Calcium Insufficiency

My own interest in calcium and vitamin D and risk for colorectal cancer was initiated from literature that suggested that modern calcium intakes and exposure to vitamin D are lower than those of our ancestors during the late evolution of our species [1–6]. It has been hypothesized that such evolutionary discordances may increase risk for modern chronic diseases [2–6]. That skin pigmentation decreased with the prehistoric migration of humans from Africa into Northern Europe is thought to be evidence of an evolutionary adaptation to the lower light in northern latitudes, and thus the lower vitamin D exposure, that accompanied this migration. Modern lifestyles, via factors such as clothes, time spent indoors, and the use of sunscreens tend to result in less ultraviolet (UV) light exposure. Based on ecologic studies and studies of vitamin D in relation to parathyroid hormone (PTH) suppression, calcium absorption and bone mineral density, the Vmax of the vitamin D 25-hydroxylase enzyme (catalyzes the conversion of vitamin D to 25-OH-vitamin D, the circulating form of vitamin D and the best indicator of vitamin D exposure), and the serum 25-OH-vitamin D depletion rate in persons in northern latitudes during the winter, it has been hypothesized that 25-OH-vitamin D blood levels of 33 – 100 ng/ml (80 – 250 nmol/L) would be more consistent with pre-industrial exposures and more likely to be required for optimal health (i.e., to be “sufficient”) [1]. It has been estimated that approximately one-half of the United States (US) adult population maintains blood levels less than this range, which is achievable with vitamin D exposures of 1,000 – 4,000 IU (25 – 100 µg)/day. Note that

these amounts are greater than the current Recommended Daily Allowances (RDAs), which are primarily based on bone health, for adult men and women of 600 IU (15 µg)/day 70 years old and 800 IU (20 µg)/day for those > 70 years old [7].

Based on studies of fossils and extant animals and human hunter-gatherers, it has been estimated that human calcium intake during Paleolithic times was about 1,500 – 2,000 mg/day [5, 6]. The predominant source of calcium was from wild plant foods, which, in contrast to modern agriculturally produced vegetables and fruits, is high in calcium. Interestingly, the calcium intakes of all other mammalian species, adjusted for weight, are equivalent to human intakes of 1,500 – 2000 mg/day. This stands in contrast to the estimated average intake of adults in the US of 740 mg/day [8, 9]. Thus, it has been proposed that our modern intakes are low and may increase the risk of modern chronic diseases, especially colon cancer, as described below. Note that 1,500 – 2000 mg/day is greater than the current Recommended Daily Allowances (RDAs), which are primarily based on bone health, for adult men and women of 1,000 mg/day 50 years old and men 51 – 70 years old, and of 1,200 mg/day for women > 50 years old and men > 70 years old [7].

Research on calcium in relation to colorectal neoplasms preceded that for vitamin D in scope and volume, likely because the biological plausibility for calcium was established sooner and because until serum 25-OH-vitamin D was established as a valid marker of vitamin D exposure, observational assessment of vitamin D exposure was problematic. In keeping with this historic progression, for the remainder of this paper I preserve this order in presenting background and results.

3.0 Biological Plausibility for Calcium and Vitamin D in Reducing Risk for Colon Cancer

The two primary hypotheses as to how calcium may reduce risk for colon cancer are the bile acid binding hypothesis and the cell cycle regulation hypothesis [1]. If an amount of calcium is consumed such that there is an excess beyond that which is absorbed or becomes bound with phosphates in the gut, it can also bind toxic bile acids and fatty acids formed as a result of fat digestion, and thus prevent their mutagenic, mitogenic, and injury-induced inflammatory effects. It has been estimated that the amount of calcium required for this is 1,500 – 2,000 mg/day, corresponding to the estimated Paleolithic intakes discussed above. Calcium also has direct effects on the cell cycle, reducing proliferation and increasing differentiation. Some of the cell cycle effects may be mediated via calcium binding with the calcium sensing receptor (CaSR), which down-regulates pro-proliferative β -catenin and up-regulates the cell adhesion molecule E-cadherin. The reader is referred elsewhere for a more extensive review of the basic science of calcium in relation to colon cancer [10].

Until recently, vitamin D was primarily thought of in relation to its roles in maintaining bone health and calcium homeostasis. However, it is now known that the vitamin D receptor (VDR) and CYP24 (which catalyzes the first step in the catabolism of $1\alpha,25\text{-[OH]}_2\text{-vitamin D}$) are abundantly expressed in the colon and many other tissues, and that CYP27B1 (which catalyzes the conversion of 25-OH-vitamin D to the more active $1\alpha,25\text{-[OH]}_2\text{-vitamin D}$) has also been reported to be expressed in the colon and other tissues [1]. Furthermore,

vitamin D has autocrine/paracrine functions in these tissues where it modulates more than 200 genes involved in activities highly relevant to colorectal carcinogenesis, including cell cycle regulation, growth factor signaling, protection against oxidative stress, bile acid and xenobiotic metabolism, cell adhesion, DNA repair, angiogenesis, and inflammation and immune function [1]. The reader is referred elsewhere for a more extensive review of the basic science of vitamin D in relation to colon cancer [11].

4.0 Human Observational and Clinical Trial Evidence for Calcium in Reducing Risk for Colorectal Neoplasms

Data from the numerous observational studies—especially from the prospective cohort studies—solidly supports the hypotheses that higher intakes of calcium reduce risk for colorectal cancer. Of 20 cohort studies, 18 (90%) found inverse associations of which eight were statistically significant, and two found direct associations of which none were statistically significant [1]. A pooled analysis of 10 cohort studies from five countries found statistically significant 22% lower risk for incident colorectal cancer among those consuming the highest vs. the lowest levels of calcium [12].

There have been seven clinical trials of calcium and adenoma recurrence, two of which had large sample sizes, and one major trial of colorectal cancer prevention [1]. In a US multi-center, randomized, double-blind, placebo-controlled clinical trial (n = 913) of calcium supplementation (1,200 mg of elemental calcium daily) and adenoma recurrence (the Calcium Polyp Prevention Study) [13], the relative risk (RR) for any recurrence of adenoma was 0.85 (95% confidence interval [95% CI] 0.74–0.98) and for advanced adenomas, 0.46 (95% CI 0.26–0.83). In a smaller trial (n = 665), the European Cancer Prevention Organization Intervention Study, there was a statistically non-significant reduction in adenoma recurrence (RR 0.66, 95% CI 0.38–1.17) among those randomized to 2,000 mg of elemental calcium daily relative to placebo [14]. When the results of all seven adenoma recurrence trials were combined in a meta-analysis, the summary RR was 0.80 (95% CI 0.68–0.93) [15]. Finally, in a Women's Health Initiative randomized, double-blind, placebo-controlled clinical trial, 36,282 postmenopausal women were randomized to 1,000 mg of elemental calcium plus 400 IU (10 µg) of vitamin D vs. placebo over an average of seven years. There was no evidence for a reduction in the incidence of invasive colorectal cancers (RR 1.08, 95% CI 0.86–1.34) [16]. However, because of the low pill-taking adherence in the active treatment group (only 60% took 80% or more of their pills) and the high rate of drop-in in the placebo group (69% took calcium and vitamin D supplements on their own resulting in intakes twice that of the national averages), the low doses administered, and the short length of follow-up for such a downstream endpoint, the interpretation of these results is problematic.

5.0 Human Observational and Clinical Trial Evidence for Vitamin D in Reducing Risk for Colorectal Neoplasms

An important advance in investigating an association between vitamin D exposure and risk for colorectal neoplasms was the recognition of 25-OH-vitamin D blood levels as the most

accurate indicator of total vitamin D exposure. Circulating 25-OH-vitamin D concentrations reflect vitamin D from sunlight exposure, which provides 90 – 95% of vitamin D in most people [1], plus dietary and supplemental intakes. The results from observational epidemiologic studies of colorectal cancer or adenomas that estimated vitamin D exposure from circulating 25-OH-vitamin D concentrations are quite consistent with there being an inverse association between vitamin D exposure and colorectal neoplasms. In a pooled analysis of three case-control studies (pooled $n = 616$ cases and 770 controls) of incident, sporadic colorectal adenoma we found that those in the highest quartile of circulating 25-OH-vitamin D₃ concentrations were at a statistically significant, approximately 40% lower risk of adenoma [17]. Of seven other observational studies of 25-OH-vitamin D and colorectal adenoma, six found inverse associations, among which three were statistically significant [18]. Of nine prospective cohort studies that investigated associations of circulating 25-OH-vitamin D and incident colorectal cancer, seven found inverse associations, of which two were statistically significant; when the data from all seven were combined in a meta-analysis the estimated relative risk for those in the upper relative to the lower quantiles of 25-OH-vitamin D was 0.66 (95% CI 0.54 – 0.81) [19]. This consistency for the 25-OH-vitamin D blood level studies may be remarkable given the low 25-OH-vitamin D blood levels in the studies (mean levels in controls were generally less than 32 ng/ml (80 nmol/L)—now considered by many to be the lower limit for vitamin D sufficiency [20–22]). However, given the relatively small number of studies that assessed 25-OH-vitamin D blood levels, the “consistency” among the studies should still be viewed as only suggestive. Currently, the only published peer-reviewed report of clinical trials of vitamin D and colon cancer is that of the Women’s Health Initiative trial discussed in the previous section.

6.0 Calcium Plus Vitamin D and Colorectal Cancer

Curiously, despite the long known intricate physiological relationship between calcium and vitamin D in relation to bone health and calcium homeostasis, there has been little study of a possible synergistic effect of the two against colon cancer. One experimental study in rodents found that calcium and vitamin D₃ supplementation together had a smaller protective effect than either supplement alone [23]. However, most studies have reported that vitamin D supplementation has a stronger anti-neoplastic effect in animals given relatively high-calcium diets [24–26]. Because of the measurement error associated with these two exposures, it is to be expected that human observational studies may have difficulty clarifying any interactive effects. Indeed, several studies seem to suggest that the effects of dietary calcium and vitamin D are independent of each other [27–29]. However, in two large cohort studies [30, 31] there was clear evidence of a positive interaction between the two nutrients. Also, in the Calcium Polyp Prevention Study, there were strong indications that vitamin D enhanced the chemopreventive effect of calcium [32]. Among subjects whose baseline 25-OH vitamin D levels were below the median, calcium had no effect on risk of one or more adenomas after randomization (RR 1.06; 95% CI 0.82–1.22); in contrast, among subjects with 25-OH vitamin D levels above the median, calcium substantially lowered the risk of adenoma occurrence (RR 0.71; 95% CI 0.57–0.88; p for interaction = 0.01). On the other hand, in the problematic Women’s Health Initiative trial

discussed above, there was no reduction in incident colorectal cancer among those randomized to 1,000 mg of calcium plus 400 IU (10 µg) of vitamin D daily. Thus, these data are somewhat mixed, but tend to suggest that vitamin D and calcium may work together to reduce risk for colorectal neoplasia.

7.0 Modifiable, Molecular Phenotypic Pre-neoplastic Biomarkers of Risk for Colorectal Neoplasms

7.1 Background

The development of modifiable pre-neoplastic biomarkers of risk for colorectal cancer, analogous to biomarkers of risk for ischemic heart disease, such as serum cholesterol, would be highly desirable for assessing and managing risk for colorectal cancer. Large (20-fold) international differences in colorectal cancer incidence coupled with the rapid (within 1 – 2 generations) increase in risk in migrants from low- to high-incidence countries [33], point to the strong environmental component to colorectal carcinogenesis, and thus to its preventability. Also in support of the predominant influence of environmental exposures on colorectal cancer risk are the findings from genome-wide association and other studies that are consistent with there being only minimal contributions of genotypes to risk for the sporadic disease, which comprises approximately 95% of all cases. Importantly, a major driver in the approximately 70% decline in heart disease deaths the US since 1975 [34] has been the identification and utilization of modifiable, phenotypic biomarkers of risk for ischemic heart disease, such as lipid profiles and blood pressures. Notably, risk prediction for ischemic heart disease has not been appreciably improved with genetic testing. Unlike ischemic heart disease, in which the mechanisms are pathophysiologic and thus the biomarkers of risk are pathophysiologic indicators, the mechanisms of colon carcinogenesis are molecular, and thus the biomarkers of risk likely will need to be molecular indicators. Finally, of the range of molecular indicators that are available, the most downstream indicators, those of phenotype, such as protein, may best reflect risk. The reasoning for this is that, as depicted in Fig. 1, phenotype is the result of complex interactions among genotypes, environmental exposures, epigenetics, and post-translational regulation by microRNAs. Such modifiable phenotypic biomarkers of risk, then, would help our understanding of relevant mechanisms in humans and could be used in observational studies to generate clues for potential modulators of these mechanisms (i.e., preventive interventions); as endpoints in clinical trials to assess the potential efficacy, optimum dose, and potential long-term safety of interventions; and for clinical risk assessment and management, such as using them as an adjunct to colonoscopy to assess the need for, and if indicated, interval for future colonoscopies, and for managing risk for colon cancer in the primary care setting in a manner similar to that for managing ischemic heart disease risk.

The greater challenge for developing modifiable biomarkers of risk for colorectal cancer relative to developing those for ischemic heart disease, is that, as noted above, colorectal cancer is a molecular disease, and it is unclear what might be detectable in blood or urine that may reflect risk in the tissue of the at-risk organ. Therefore, it was hypothesized that the most logical place to begin searching for modifiable biomarkers of risk for colorectal cancer is in the normal colorectal mucosa, especially the epithelium. Through basic science studies,

the molecular basis for colorectal cancer has become clearer. It is now understood as a disease in which there is an accumulation of genetic and epigenetic modifications in characteristic genes, [35] which also are important to the normal structure and function of colon crypts, which are epithelium lined invaginations in the colorectal mucosa that continuously replace the epithelial surface of the mucosa. It was also hypothesized that like virtually all other biological phenomena, the expression of the genes that can become altered during colon carcinogenesis may be normally distributed in the normal, at risk colon tissue, and, furthermore, that differences in expression along this normal distribution, particularly at the extremes, may reflect risk. It was also hypothesized that once biomarkers representing the most downstream expression of these genes (i.e., the protein, or molecular phenotype) are validated and confirmed to be modifiable, then correlates in surrogate fluids, such as blood or urine, could be identified and used as surrogate markers that are easier to sample and measure. Should such surrogates not be found, normal tissue markers could still be useful in research and for clinical application as adjuncts to colonoscopies. Use for managing risk in the primary care setting would likely be contingent on identifying surrogate markers.

So, just as one would diagram the pathophysiology of a disease such as ischemic heart disease to identify the various mechanistic steps and thus the indicators/biomarkers of them that may be normally distributed (e.g., serum cholesterol and blood pressure), for colon cancer we can now diagram the molecular basis of the disease (Fig. 2). Narrative to accompany this figure follows.

The molecular basis of colon carcinogenesis is a multi-step process involving genetic alteration of *APC*, *K-ras*, a tumor suppressor gene in chromosome 18q, and *p53* [35, 36]. There are at least two not necessarily entirely mutually exclusive major pathways driving this process. The first, the “APC Pathway”, accounts for familial adenomatous polyposis (FAP) and approximately 80% of sporadic cancers. In FAP, the affected person is born with an inactivating mutation in one allele of the “pathway gatekeeper” *APC* tumor suppressor gene, and has only to acquire an inactivation of the second allele to begin development of a colonic neoplasm; whereas, in the sporadic patient, inactivation of both alleles must be acquired, either through somatic mutation or epigenetic phenomena, predominantly the former. The second pathway, the “Mismatch Repair (MMR) Pathway” accounts for hereditary non-polyposis colon cancer (HNPCC) and approximately 15% of sporadic cancers. In HNPCC, the affected person is born with an inactivating mutation in one of the mismatch repair genes, predominantly *MSH2* and *MLH1*. The sporadic patient in this pathway must acquire an inactivation, either through mutation or epigenetic silencing, predominantly the latter. The protein product of the “gateway” gene, *APC*, functions to degrade β -catenin, which is both pro-proliferative and regulates **E-cadherin**, a calcium-dependent cell adhesion molecule necessary for colon crypt structure and function. Progressive alteration of cell proliferation and cell adhesion from the normal colon crypt to adenoma to carcinoma is a hallmark of colon carcinogenesis. When β -catenin is not degraded by *APC*, both c-myc and, further downstream, cyclin D1, are up-regulated, promoting entry of colonocytes into the proliferative phase of the cell cycle, with the *net*

effect being increased **proliferation** (direct effect) and, indirectly, decreased **differentiation** and **apoptosis**.

The DNA mismatch repair (MMR) genes serve to repair mismatches in paired DNA strands post replication [35, 36]. When MMR genes are impaired mismatches in other genes can be propagated and progressively expanded, eventually hampering the ability of the affected genes to function properly. Genes that characteristically become impaired as a result of deficient mismatch repair in MMR pathway colon carcinogenesis are *bax* and *TGFβRII*. Impairment of *bax*, a pro-apoptotic regulator, causes a decrease in apoptosis, while impairment of *TGFβRII* results in increased proliferation. The *net* result, as for the APC pathway, is increased **proliferation** and decreased **differentiation** and **apoptosis**.

Although not strictly considered part of the “pathway genes”, there are several indicators of net alterations in cell cycle characteristics, including cell proliferation, differentiation, and apoptosis. A marker of a cell in a proliferative phase is the **Mib-1** epitope of Ki-67 [37–39]; an informative long-term indicator of proliferation is **hTERT**, a catalytic subunit of telomerase [40]; and a marker of a cell that can no longer proliferate and is differentiated is **p21** [40, 41]. Detection of expression of inhibitors (**bcl-2**) and promoters (**bax**, *bak*) of apoptosis can be readily demonstrated in characteristic gradients in crypts of normal colon tissue [40, 42–46].

Autocrine or paracrine growth factors also contribute to colon carcinogenesis [35, 36]. **TGFα** and **TGFβ₁** are autocrine/paracrine growth factors classically thought of as potent promoters and inhibitors of growth, respectively [47]. However, many cancer tissues are resistant to *TGFβ₁* [47], *TGFβ₁* was found to have contextually-related tumor suppressor and pro-oncogenic activities [48, 49], and a variety of cancers [50–52], including colon cancer [50, 52], over express it. *TGFα* acts through the EGF receptor, *TGFβ₁* acts through the *TGFβ* receptor (*TGFβR*), and both receptors are abundantly expressed in the normal colon epithelium [47]. *TGFα* can synergize with c-myc to promote malignant transformation *in vitro* [47]; *TGFβ₁* inhibits c-myc [47, 53] and induces **p21** [53], and the growth suppressive activity of *TGFβ₁* is inhibited by β-catenin [54]. In immunohistochemical studies of the normal human colon crypt, *TGFα* expression is correlated with the distribution of **proliferating** cells [55, 56], and *TGFβ₁* expression tends to be located in the upper, non-proliferative zone of the crypt [47]. *TGFβ₁*, which not only inhibits **proliferation**, but promotes **apoptosis** and affects cell adhesion [57], is potently induced by **vitamin D₃** *in vitro* [47, 57].

Inflammation and inflammation regulation play important roles in colon carcinogenesis, and activity of genes for elements of this system, perhaps especially for **COX-2** in relation to **APC**, are important in colon carcinogenesis [58–66]. NSAIDs, which inhibit COX-2, reduced the occurrence and caused the regression or disappearance of adenomas in FAP patients [59–62, 66], and selective COX-2 inhibitors (celecoxib, rofecoxib) reduced sporadic adenoma recurrence in three large trials [67–69]. Oxidative stress, which is abundant in the colon, both causes and is caused by inflammation [70] and so is discussed here with inflammation.

Oxidative stress is commonly defined as “a disturbance in the prooxidant-antioxidant balance in favor of the former” [71]. According to a long-held view, oxidative stress occurs as a result of damage induced by free radicals or reactive oxygen and nitrogen species (RONS) [72–76]. (Recently, the theory of oxidative stress was refined to account for an alternative mechanism—a disruption of thiol-redox circuits, which leads to aberrant cell signaling and dysfunctional redox control without involving RONS-induced macromolecular damage [77, 78]). An available colon tissue biomarker of RONS-induced macromolecular damage is 8-hydroxydeoxyguanosine (**8-OH-dG**), which serves as a marker of DNA damage.

The candidate biomarkers drawn from this understanding and that were investigated in our subsequent studies are summarized according to category in Table 1.

In measuring these markers, given that they are involved in the normal structure and function of the colon crypt, it was hypothesized that both the total expression of a marker and its distribution within a crypt could be an important indicators of an at risk phenotype. Thus, the methodology chosen to measure the markers was automated immunohistochemistry with quantitative image analysis (Fig. 3). With these methods we can measure the relative quantities of biomarkers overall as well as their zones of expression to assess whether features such as total expression, shifting of an expression zone up or down, or expansion or contraction of an expression zone is most associated with risk and/or responsive to preventive interventions. With a strict protocol and appropriate training, the reliability of the image analysis procedures we developed is high, about 0.94 – 0.99, depending on the biomarker and image analysis operator [40, 56, 79–88].

7.2 Biomarker Validation: The Markers of Adenomatous Polyps II Study

To assess the potential validity of the hypothesis-based biomarkers of risk for colorectal neoplasms, we conducted a colonoscopy-based case-control study of incident, sporadic colorectal adenoma (n = 49 cases, 152 controls). The methods and findings from this study are reported in detail elsewhere [56, 81, 85, 87, 88]. Briefly, persons being scheduled for an elective, outpatient colonoscopy were recruited to fill out questionnaires prior to the day of colonoscopy, undergo venipuncture upon arriving for their colonoscopy visit (i.e., fasting but before receiving any colonoscopy-related medications or procedures), and undergo procurement of biopsies of normal-appearing colorectal mucosa from the rectum (10 cm above the anus), mid-sigmoid colon, and proximal ascending colon during colonoscopy. Only polyethylene glycol colon cleansing preparations were used because of known effects of other preparations on cell proliferation markers. The consent rate for the study was 87.5%.

The biomarkers were detected using automated immunohistochemistry, and for quantitative image analysis the aim was to analyze 16 full-length crypts, eight from each of two biopsies per colon site. In the data analysis procedures, the image analysis data were batch standardized and analyzed graphically using LOESS and quantitatively using analysis of covariance (ANACOVA) and logistic regression. *A priori*, the primary crypt expression parameters were specified as the expression of the biomarker in the entire crypt (overall expression), in the upper 40% of the crypt (the differentiation zone), and in the lower 60%

of the crypt (the proliferation zone), as well as the proportion of expression in the differentiation zone relative to that in the entire crypt (an indicator of the degree of expansion of a marker from the proliferative into the differentiation zone). In some cases, based on the graphical analysis, a variable for an *a posteriori* crypt parameter was created.

Example results of the analysis of covariance are summarized in Table 2 as the proportional differences in the batch-standardized means among the cases relative to the controls. Statistically significant findings are designated with an asterisk. Only the results for the crypt parameter with the greatest estimated case-control difference for each biomarker is shown; unless otherwise indicated in the footnotes, the finding summarized is the overall expression in the crypts of the rectum. So for example, in the table it can be seen that the overall expression of MSH2 in the proximal ascending colon was 49% lower in the cases than in the controls. In addition to the findings for the individual markers, those for four ratios are shown: APC/ β -catenin to reflect the balance of proliferation-inhibiting APC to proliferation-promoting β -catenin, bax/bcl-2 to reflect the balance of apoptosis-promoting bax to apoptosis-inhibiting bcl-2, MLH1/bax to reflect the balance of DNA mismatch repair to apoptosis promotion, and TGF α /TGF β_1 to reflect the balance of growth-promoting TGF α to growth-inhibiting TGF β_1 . Overall, as can be seen, the findings were in the hypothesized directions. In addition to what can be seen in the table, the case-control differences in the rectum persisted higher in the colon, except that the differences in the expression of MLH1 and MSH2 were small at the rectum and increased moving proximally, which would be consistent with hypotheses related to these DNA mismatch repair markers. Also in addition to what can be seen in the table, the various markers were associated with modifiable risk factors in hypothesized directions, which suggested that the biomarkers themselves may also be modifiable.

8.0 Modulation of Biomarkers of Risk for Colorectal Neoplasms with Calcium and/or Vitamin D

Interest in calcium as a chemopreventive agent against colorectal neoplasms preceded that for vitamin D, and pre-neoplastic tissue markers of risk were initially limited to those that were discretely expressed in nuclei or not and could be quantified by counting colon crypt epithelial cell nuclei with and without expression (i.e., cell proliferation markers, most of which were indicators of cells in S-phase). Following promising results from some small, uncontrolled trials of the effects of calcium and colorectal epithelial cell proliferation, we conducted the first full-scale, randomized, placebo-controlled clinical trial of the effects of supplemental calcium on colorectal epithelial cell proliferation [89]. Sporadic colorectal adenoma patients (n = 193) were randomized in this 3-arm parallel group trial to 1.0 or 2.0 g elemental calcium (as calcium carbonate) or placebo to be taken twice daily with food over six months. “Non-prep” (i.e., no preceding colon cleansing preparation) biopsies of normal appearing rectal tissue were taken 10 cm above the anus at baseline and at 1-, 2-, and 6-months follow up and proliferation was measured via immunohistochemical detection of proliferation cell nuclear antigen (PCNA) on biopsy specimens. The major, statistically significant finding of this study was that calcium substantially shifted the zone of

proliferation downward into the lower 60%, or normal proliferation zone, of the crypt, but did not affect the overall cell proliferation rate [89].

With increasing interest in vitamin D and the development of our image analysis software that allowed quantification of biomarkers that are expressed in density gradients along the colon crypt axis, we next conducted the Calcium and Vitamin D vs. Markers of Adenomatous Polyps trial (CaDvMAP) [40, 79, 80, 82–84, 90, 91]. In this pilot randomized, double-blind, placebo-controlled 2×2 factorial clinical trial we randomized 92 sporadic colorectal adenoma patients to vitamin D₃ (800 IU), calcium (2.0 g elemental calcium as calcium carbonate), both, or placebo given in two equally divided doses twice daily with meals over six months. “Non-prep” biopsies of normal appearing rectal tissue were taken 10 cm above the anus and blood was drawn at baseline and six months later. The endpoints were the same tissue biomarkers of risk as for the MAP II study plus the CaSR, the VDR, CYP27B1, CYP24, and 8-hydroxy-deoxyguanosine (8-OH-dG), all detected via automated immunohistochemistry and analyzed using our quantitative image analysis methods. We also measured serum 25-OH-D and 1 α ,25-(OH)₂-vitamin D via radioimmunoassay (CVs 2.3 and 6.2%, respectively) and a panel of inflammation markers using enzyme linked immunoassays (ELISA) (CVs < 12%). Visit attendance averaged 92%, and there were no differences by treatment group. Eighty percent or more of pills were taken by 93% of participants at the first follow-up and by 84% at final follow-up, and there were no differences by treatment group. The treatment groups were balanced on demographic, medical, dietary and lifestyle factors at baseline, and there were no adverse events attributable to pill taking or rectal biopsies during the trial. As in the MAPII study, for data analysis, we batch standardized and, to examine our results graphically, used the LOESS procedure. For quantitative analyses we used repeated measures linear MIXED effects models as implemented in SAS Institute’s Mixed Procedure (Proc Mixed). Because the results were in dimensionless optical density units, for easier interpretation, relative effects, which are similar to odds ratios, were calculated and interpreted as proportional change in an active treatment group relative to that in the placebo group. Proportional changes in serum 25-OH-vitamin D and 1 α ,25-(OH)₂-vitamin D are summarized in Table 3.

Changes in the tissue biomarkers are summarized in Table 4. The treatment effects are summarized as the proportional change in the active treatment group relative to that in the placebo group, and statistically significant findings are designated with an asterisk. Only the crypt parameter with the greatest estimated treatment effect on each biomarker is shown. In addition to the findings for the individual markers, those for three ratios are shown: APC/ β -catenin to reflect the balance of proliferation-inhibiting APC to proliferation-promoting β -catenin, bax/bcl-2 to reflect the balance of apoptosis-promoting bax to apoptosis-inhibiting bcl-2, and TGF α /TGF β ₁ to reflect the balance of growth-promoting TGF α to growth-inhibiting TGF β ₁. All findings were in the hypothesized directions, and more findings—especially in the vitamin D group—were statistically significant than would be expected by chance. Unexpectedly, for the most part, the findings for the calcium plus vitamin D groups tended to be weaker than those for the stronger of the two agents alone.

Changes in the serum inflammation markers are summarized in Table 5 in the same manner as that for the tissue markers (i.e., showing the proportional change in active treatment group

relative to that in the placebo group, with statistically significant results designated with an asterisk). Because no single marker can summarize the entire complexity of inflammation, we combined the inflammation markers into a summary inflammation score by converting the finding of each biomarker into a z-score and then summing the z-scores. In the vitamin D group, relative to the placebo group, the summary inflammation z-score decreased by a statistically significant 77%; in the calcium group it decreased by an estimated 48% and in the calcium plus vitamin D group by 33%; however, these latter two findings were not statistically significant in this pilot trial.

9.0 Summary, Conclusions, Future Directions

In summary, there is strong biological rationale for vitamin D₃ and calcium in reducing risk for colorectal cancer. There is a high prevalence of low exposure to vitamin D and low intakes of calcium in the US. The observational literature for calcium in reducing risk for colorectal neoplasms is consistent, especially from cohort studies, and calcium reduced adenoma recurrence in large randomized controlled trials. The observational literature for circulating 25-OH-vitamin D concentrations and colorectal neoplasms is consistent but still relatively sparse. We developed a panel of hypothesis-based, modifiable, phenotypic biomarkers of risk for colorectal neoplasms that is analogous to a lipid panel for risk for cardiovascular disease that can be used as endpoints for prevention trials to bridge the gap between basic science and human observational studies and tumor endpoint trials. The effects of vitamin D₃ and calcium on markers in this panel were tested in a pilot randomized, controlled trial in sporadic colorectal adenoma patients. The findings were consistent with both agents favorably modulating the markers in the normal-appearing rectal mucosa and with favorably modulating a panel of circulating inflammation markers. Unexpectedly, the results for vitamin D₃ and calcium combined were generally weaker than those for the stronger of the two agents alone; the explanation for this is unknown. Taken altogether, these findings provide support for further investigation of vitamin D and calcium as potential chemopreventive agents against colorectal neoplasms, as well as the development and application of modifiable phenotypic biomarkers of risk for colorectal neoplasms in research and for eventual clinical application. Proposed future work includes a vitamin D-biomarker dose-response trial (e.g., administering 1,000, 2,000, and 4,000 IU [25, 50, and 100 µg] daily); a calcium and vitamin D trial in which biopsies of normal-appearing colorectal mucosa are taken from multiple levels of the colon, preferably in the context of an adenoma recurrence trial; a larger, preferably prospective study of an expanded version of our biomarker panel and risk for colorectal neoplasms; and investigations to identify easier to measure correlates of our tissue biomarkers in easier to acquire surrogate fluids, such as blood or urine.

Acknowledgements

I thank all my co-authors in references 1, 17, 40, 56, and 79 – 91, all of whom materially contributed to the work summarized on their behalf by me in this manuscript. I also acknowledge the following funding sources for my efforts over the years that contributed to this work: National Cancer Institute, National Institutes of Health grants R01 CA51932, P01 CA50305, R01 CA66539, R01 CA104637, R03 CA115230, R03 CA121873, R01 CA116795, R03 CA136113, and P30 CA138292; Fullerton Foundation; Emory Winship Cancer Institute; Georgia Cancer Coalition Distinguished Scholar award; and the Franklin Foundation. The National Cancer Institute, the Georgia Cancer Coalition, the Fullerton Foundation, the Emory Winship Cancer Institute, and the Franklin Foundation had

no influence on the design of the study; the collection, analysis, and interpretation of the data; the decision to submit the manuscript for publication; or the writing of the manuscript.

Abbreviations

CaSR	calcium sensing receptor
CRP	C-reactive protein
ELISA	enzyme-linked immunoassay
8-OH-dG	8-hydroxy-deoxyguanosine
IL	interleukin
MMR	mismatch repair
RONS	reactive oxygen and nitrogen species
TGF	transforming growth factor
VDR	vitamin D receptor

REFERENCES

1. Bostick, RM.; Goodman, M.; Sidelnikov, E. Calcium and vitamin D. In: Potter, JD.; Lindor, NM., editors. Genetics of Colorectal Cancer. New York, NY: Springer Science + Business Media, LLC; 2009. p. 273-294.
2. Cordain L, Eaton SB, Miller JB, Mann N, Hill K. The paradoxical nature of hunter-gatherer diets: meat-based, yet non-atherogenic. *Eur J Clin Nutr.* 2002; 56(Suppl 1):S42–S52. [PubMed: 11965522]
3. Eaton SB, Eaton SB 3rd. Paleolithic vs. modern diets--selected pathophysiological implications. *Eur J Clin Nutr.* 2000; 39:67–70.
4. Eaton SB, Eaton SB 3rd, Konner MJ. Paleolithic nutrition revisited: a twelve-year retrospective on its nature and implications. *Eur J Clin Nutr.* 1997; 51:207–216. [PubMed: 9104571]
5. Eaton SB, Konner M. Paleolithic nutrition. A consideration of its nature and current implications. *N Engl J Med.* 1985; 312:283–289. [PubMed: 2981409]
6. Eaton, SB.; Shostak, M.; Konner, M. The Paleolithic Prescription. New York: Harper and Row; 1988.
7. Ross, AC.; Taylor, CL.; Yaktine, AL.; Del Valle, HB., editors. Dietary Reference Intakes for Calcium and Vitamin D. Washington (DC): 2011.
8. Dietary Goals for the United States. Washington, DC: 1977. Select Committee on Nutrition and Human Needs, United States Senate.
9. Dietary Reference Intakes for Calcium, Phosphorus, Magnesium, Vitamin D and Fluoride, Standing Committee on the Scientific Evaluation of Dietary Reference Intakes. Washington, DC: Institute of Medicine. Food and Nutrition Board; 1997. p. 432
10. Peterlik M, Kallay E, Cross HS. Calcium nutrition and extracellular calcium sensing: relevance for the pathogenesis of osteoporosis, cancer and cardiovascular diseases. *Nutrients.* 2013; 5:302–327. [PubMed: 23340319]
11. Cross HS, Nittke T, Kallay E. Colonic vitamin D metabolism: implications for the pathogenesis of inflammatory bowel disease and colorectal cancer. *Mol Cell Endocrinol.* 2011; 347:70–79. [PubMed: 21801808]
12. Cho E, Smith-Warner S, Spiegelman D, Beeson W, van den Brandt P, Colditz G, Folsom A, Fraser G, Freudenheim J, Giovannucci E, Goldbohm R, Graham S, Miller A, Pietinen P, Potter J, Rohan T, Terry P, Toniolo P, Virtanen M, Willett W, Wolk A, Wu K, Yaun S, Zeleniuch-Jacquotte A,

- Hunter D. Dairy foods, calcium, and colorectal cancer: a pooled analysis of 10 cohort studies. *J Natl Cancer Inst.* 2004; 96:1015–1022. [PubMed: 15240785]
13. Baron JA, Beach M, Mandel JS, van Stolk RU, Haile RW, Sandler RS, Rothstein R, Summers RW, Snover DC, Beck GJ, Bond JH, Greenberg ER. Calcium supplements for the prevention of colorectal adenomas, Calcium Polyp Prevention Study Group. *N Engl J Med.* 1999; 340:101–107. [PubMed: 9887161]
14. Bonithon-Kopp C, Kronborg O, Giacosa A, Rath U, Faivre J. Calcium and fibre supplementation in prevention of colorectal adenoma recurrence: a randomised intervention trial. European Cancer Prevention Organisation Study Group. *Lancet.* 2000; 356:1300–1306. [PubMed: 11073017]
15. Shaukat A, Scouras N, Schunemann HJ. Role of supplemental calcium in the recurrence of colorectal adenomas: a meta analysis of randomized controlled trials. *Am J Gastroenterol.* 2005; 100:390–394. [PubMed: 15667497]
16. Wactawski-Wende J, Kotchen JM, Anderson GL, Assaf AR, Brunner RL, O'Sullivan MJ, Margolis KL, Ockene JK, Phillips L, Potters L, Prentice RL, Robbins J, Rohan TE, Sarto GE, Sharma S, Stefanick ML, Van Horn L, Wallace RB, Whitlock E, Bassford T, Beresford SA, Black HR, Bonds DE, Brzyski RG, Caan B, Chlebowski RT, Cochrane B, Garland C, Gass M, Hays J, Heiss G, Hendrix SL, Howard BV, Hsia J, Hubbell FA, Jackson RD, Johnson KC, Judd H, Kooperberg CL, Kuller LH, LaCroix AZ, Lane DS, Langer RD, Lasser NL, Lewis CE, Limacher MC, Manson JE. Calcium plus vitamin D supplementation and the risk of colorectal cancer. *N Engl J Med.* 2006; 354:684–696. [PubMed: 16481636]
17. Fedirko V, Bostick RM, Goodman M, Flanders WD, Gross MD. Blood 25-Hydroxy vitamin D3 concentrations and incident sporadic colorectal adenoma risk: A pooled case-control study. *Am J Epidemiol.* 2010; 172:489–500. [PubMed: 20650953]
18. Wei MY, Garland CF, Gorham ED, Mohr SB, Giovannucci E. Vitamin D and prevention of colorectal adenoma: a meta-analysis. *Cancer Epidemiol Biomarkers Prev.* 2008; 17:2958–2969. [PubMed: 18990737]
19. Lee JE, Li H, Chan AT, Hollis BW, Lee IM, Stampfer MJ, Wu K, Giovannucci E, Ma J. Circulating levels of vitamin D and colon and rectal cancer: the Physicians' Health Study and a meta-analysis of prospective studies. *Cancer Prev Res.* 2011; 4:735–743.
20. Heaney RP. The vitamin D requirement in health and disease. *J Steroid Biochem Mol Biol.* 2005; 97:13–19. [PubMed: 16026981]
21. Holick MF. Vitamin D deficiency. *N Engl J Med.* 2007; 357:266–281. [PubMed: 17634462]
22. Hollis BW. Circulating 25-hydroxy vitamin D levels indicative of vitamin D sufficiency: implications for establishing a new effective dietary intake recommendation for vitamin D. *J Nutr.* 2005; 135:317–322. [PubMed: 15671234]
23. Pence BC, Buddingh F. Inhibition of dietary fat-promoted colon carcinogenesis in rats by supplemental calcium or vitamin D3. *Carcinogenesis.* 1988; 9:187–190. [PubMed: 2826033]
24. Beaty MM, Lee EY, Glauert HP. Influence of dietary calcium and vitamin D on colon epithelial cell proliferation and 1,2-dimethylhydrazine-induced colon carcinogenesis in rats fed high fat diets. *J Nutr.* 1993; 123:144–152. [PubMed: 8421225]
25. Sitrin MD, Halline AG, Abrahams C, Brasitus TA. Dietary calcium and vitamin D modulate 1,2-dimethylhydrazine-induced colonic carcinogenesis in the rat. *J Nutr.* 1991; 123:144–152.
26. Kawaura A, Tanida N, Sawada K, Oda M, Shimoyama T. Supplemental administration of 1 alpha-hydroxy vitamin D3 inhibits promotion by intrarectal instillation of lithocholic acid in N-methyl-N-nitrosourea-induced colonic tumorigenesis in rats. *Carcinogenesis.* 1989; 10:647–649. [PubMed: 2702712]
27. Bostick R, Potter J, Sellers T, McKenzie D, Kushi L, Folsom A. Relation of calcium, vitamin D, and dairy food intake to incidence of colon cancer among older women. The Iowa Women's Health Study. *Am J Epidemiol.* 1993; 137:1302–1317. [PubMed: 8333412]
28. Boutron MC, Faivre J, Marteau P, Couillaud C, Senesse P, Quipourt V. Calcium, phosphorus, vitamin D, dairy products and colorectal carcinogenesis: a French case-control study. *Br J Cancer.* 1996; 74:145–151. [PubMed: 8679449]
29. Kampman E, Slattery ML, Caan B, Potter JD. Calcium, vitamin D, sunshine exposure, dairy products and colon cancer risk (United States). *Cancer Cause Control.* 2000; 11:459–466.

30. Zheng W, Anderson KE, Kushi LH, Sellers TA, Greenstein J, Hong CP, Cerhan JR, Bostick RM, Folsom AR. A prospective cohort study of intake of calcium, vitamin D, and other micronutrients in relation to incidence of rectal cancer among postmenopausal women. *Cancer Epidemiol Biomarkers Prev.* 1998; 7:221–225. [PubMed: 9521437]
31. Wu K, Willett WC, Fuchs CS, Colditz GA, Giovannucci EL. Calcium intake and risk of colon cancer in women and men. *J Natl Cancer Inst.* 2002; 94:437–446. [PubMed: 11904316]
32. Grau MV, Baron JA, Sandler RS, Haile RW, Beach ML, Church TR, Heber D. Vitamin D, calcium supplementation, and colorectal adenomas: results of a randomized trial. *J Natl Cancer Inst.* 2003; 95:1765–1771. [PubMed: 14652238]
33. Potter JD, Slattery ML, Bostick RM, Gapstur SM. Colon cancer: a review of the epidemiology. *Epidemiol Rev.* 1993; 15:499–545. [PubMed: 8174669]
34. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. *CA Cancer J Clin.* 2012; 62:10–29. [PubMed: 22237781]
35. Potter JD. Colorectal cancer: molecules and populations. *J Natl Cancer Inst.* 1999; 91:916–932. [PubMed: 10359544]
36. Kinsler, K.; Vogelstein, B. Colorectal tumors. In: Kinsler, K.; Vogelstein, B., editors. *The Genetic Basis of Human Cancer*. New York: McGraw-Hill; 2002.
37. Ellis PA, Makris A, Burton SA, Titley J, Ormerod MG, Salter J, Powles TJ, Smith IE, Dowsett M. Comparison of MIB-1 proliferation index with S-phase fraction in human breast carcinomas. *Br J Cancer.* 1996; 73:640–643. [PubMed: 8605100]
38. Holt PR, Moss SF, Kapetanakis AM, Petrotos A, Wang S. Is Ki-67 a better proliferative marker in the colon than proliferating cell nuclear antigen? *Cancer Epidemiol Biomarkers Prev.* 1997; 6:131–135. [PubMed: 9037564]
39. Langford LA, Cooksley CS, DeMonte F. Comparison of MIB-1 (Ki-67) antigen and bromodeoxyuridine proliferation indices in meningiomas. *Hum Pathol.* 1996; 27:350–354. [PubMed: 8617477]
40. Fedirko V, Bostick RM, Flanders WD, Long Q, Sidelnikov E, Shaikat A, Daniel CR, Rutherford RE, Woodard JJ. Effects of vitamin D and calcium on proliferation and differentiation in normal colon mucosa: a randomized clinical trial. *Cancer Epidemiol Biomarkers Prev.* 2009; 18:2933–2941. [PubMed: 19861511]
41. Fenoglio-Preiser CM, Noffsinger A. Aberrant crypt foci: A review. *Toxicol Pathol.* 1999; 27:632–642. [PubMed: 10588543]
42. Bedi A, Pasricha PJ, Akhtar AJ, Barber JP, Bedi GC, Giardiello FM, Zehnbauser BA, Hamilton SR, Jones RJ. Inhibition of apoptosis during development of colorectal cancer. *Cancer Res.* 1995; 55:1811–1816. [PubMed: 7728743]
43. Bosari S, Moneghini L, Graziani D, Lee AK, Murray JJ, Coggi G, Viale G. bcl-2 oncoprotein in colorectal hyperplastic polyps, adenomas, and adenocarcinomas. *Hum Pathol.* 1995; 26:534–540. [PubMed: 7750937]
44. Bronner MP, Culin C, Reed JC, Furth EE. The bcl-2 proto-oncogene and the gastrointestinal epithelial tumor progression model. *Am J Pathol.* 1995; 146:20–26. [PubMed: 7856728]
45. Hague A, Moorghen M, Hicks D, Chapman M, Paraskeva C. BCL-2 expression in human colorectal adenomas and carcinomas. *Oncogene.* 1994; 9:3367–3370. [PubMed: 7936663]
46. Merritt AJ, Potten CS, Watson AJ, Loh DY, Nakayama K, Hickman JA. Differential expression of bcl-2 in intestinal epithelia. Correlation with attenuation of apoptosis in colonic crypts and the incidence of colonic neoplasia. *J Cell Sci.* 1995; 108(Pt 6):2261–2271. [PubMed: 7673346]
47. Mendelsohn, J.; Baird, A.; Fan, Z.; Markowitz, S. Growth factors and their receptors in epithelial malignancies. In: Mendelsohn, J.; Israel, M.; Liotta, L., editors. *The Molecular Basis of Cancer*. Philadelphia, PA: WB Saunders Co; 2001. p. 137–161.
48. Tian F, Byfield SD, Parks WT, Stuelten CH, Nemani D, Zhang YE, Roberts AB. Smad-binding defective mutant of transforming growth factor beta type I receptor enhances tumorigenesis but suppresses metastasis of breast cancer cell lines. *Cancer Res.* 2004; 64:4523–4530. [PubMed: 15231662]

49. Ye SC, Foster JM, Li W, Liang J, Zborowska E, Venkateswarlu S, Gong J, Brattain MG, Willson JK. Contextual effects of transforming growth factor beta on the tumorigenicity of human colon carcinoma cells. *Cancer Res.* 1999; 59:4725–4731. [PubMed: 10493532]
50. Guda K, Claffey KP, Dong M, Nambiar PR, Rosenberg DW. Defective processing of the transforming growth factor-beta1 in azoxymethane-induced mouse colon tumors. *Mol Carcinogen.* 2003; 37:51–59.
51. Lu SL, Reh D, Li AG, Woods J, Corless CL, Kulesz-Martin M, Wang XJ. Overexpression of transforming growth factor beta1 in head and neck epithelia results in inflammation, angiogenesis, and epithelial hyperproliferation. *Cancer Res.* 2004; 64:4405–4410. [PubMed: 15231647]
52. Tsamandas AC, Kardamakis D, Ravazoula P, Zolota V, Salakou S, Tepetes K, Kalogeropoulou C, Tsota I, Kourelis T, Makatsoris T, Karavias D, Scopa CD, Bonikos DS, Kalofonos HP, Petsas T. The potential role of TGFbeta1, TGFbeta2 and TGFbeta3 protein expression in colorectal carcinomas. Correlation with classic histopathologic factors and patient survival. *Strahlenther Onkol.* 2004; 180:201–208. [PubMed: 15057430]
53. Vijayachandra K, Lee J, Glick AB. Smad3 regulates senescence and malignant conversion in a mouse multistage skin carcinogenesis model. *Cancer Res.* 2003; 63:3447–3452. [PubMed: 12839923]
54. Sekiya T, Adachi S, Kohu K, Yamada T, Higuchi O, Furukawa Y, Nakamura Y, Nakamura T, Tashiro K, Kuhara S, Ohwada S, Akiyama T. Identification of BMP and activin membrane-bound inhibitor (BAMBI), an inhibitor of transforming growth factor-beta signaling, as a target of the beta-catenin pathway in colorectal tumor cells. *J Biol Chem.* 2004; 279:6840–6846. [PubMed: 14660579]
55. Cameron IL, Hardman WE. Paracrine action of transforming growth factor-alpha in rectal crypt epithelium of humans. *Cell Biol Int.* 2002; 26:1029–1034. [PubMed: 12468378]
56. Daniel CR, Bostick RM, Flanders WD, Long Q, Fedirko V, Sidelnikov E, Seabrook ME. TGF- α expression as a potential biomarker of risk within the normal-appearing colorectal mucosa of patients with and without incident sporadic adenoma. *Cancer Epidemiol Biomarkers Prev.* 2009; 18:65–73. [PubMed: 19124482]
57. D'Amico A, Whittington R, Malkowicz S, Schultz D, Blank K, Broderick G, Tomaszewski J, Renshaw A, Kaplan I, Beard C, Wein A. Biochemical outcome after radical prostatectomy, external beam radiation therapy, or interstitial radiation therapy for clinically localized prostate cancer. *J Am Med Assoc.* 1998; 280:969–974.
58. Baron J, Cole B, Sandler R, Haile R, Ahnen D, Bresalier R, McKeown-Eyssen G, Summers R, Rothstein R, Burke C, Snover D, Church T, Allen J, Beach M, Beck G, Bond J, Byers T, Greenberg E, Mandel J, Marcon N, Mott L, Pearson L, Saibil F, van Stolk R. A randomized trial of aspirin to prevent colorectal adenomas. *N Engl J Med.* 2003; 348:891–899. [PubMed: 12621133]
59. Giardiello FM, Hamilton SR, Krush AJ, Piantadosi S, Hyland LM, Celano P, Booker SV, Robinson CR, Offerhaus GJ. Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. *N Engl J Med.* 1993; 328:1313–1316. [PubMed: 8385741]
60. Labayle D, Fischer D, Vielh P, Drouhin F, Pariente A, Bories C, Duhamel O, Troussset M, Attali P. Sulindac causes regression of rectal polyps in familial adenomatous polyposis. *Gastroenterol.* 1991; 101:635–639.
61. Ladenheim J, Garcia G, Titzer D, Herzenberg H, Lavori P, Edson R, Omary MB. Effect of sulindac on sporadic colonic polyps. *Gastroenterol.* 1995; 108:1083–1087.
62. Steinbach G, Lynch P, Phillips R, Wallace M, Hawk E, Gordon G, Wakabayashi N, Saunders B, Shen Y, Fujimura T, Su L, Levin B. The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. *N Engl J Med.* 2000; 342:1946–1952. [PubMed: 10874062]
63. Smalley WE, DuBois RN. Colorectal cancer and nonsteroidal anti-inflammatory drugs. *Adv Pharmacol.* 1997; 39:1–20. [PubMed: 9160111]
64. Taketo MM. Cyclooxygenase-2 inhibitors in tumorigenesis (part I). *J Natl Cancer Inst.* 1998; 90:1529–1536. [PubMed: 9790545]
65. Taketo MM. Cyclooxygenase-2 inhibitors in tumorigenesis (Part II). *J Natl Cancer Inst.* 1998; 90:1609–1620. [PubMed: 9811310]

66. Winde G, Gumbinger HG, Osswald H, Kemper F, Bunte H. The NSAID sulindac reverses rectal adenomas in colectomized patients with familial adenomatous polyposis: clinical results of a dose-finding study on rectal sulindac administration. *Int J Colorectal Dis.* 1993; 8:13–17. [PubMed: 8388011]
67. Arber N, Eagle CJ, Spicak J, Racz I, Dite P, Hajer J, Zavoral M, Lechuga MJ, Gerletti P, Tang J, Rosenstein RB, Macdonald K, Bhadra P, Fowler R, Wittes J, Zauber AG, Solomon SD, Levin B. Celecoxib for the prevention of colorectal adenomatous polyps. *N Engl J Med.* 2006; 355:885–895. [PubMed: 16943401]
68. Baron J, Sandler R, Bresalier R, Quan H, Riddell R, Lanos A, Bolognese J, Oxenius B, Horgan K, Loftus S, Morton D. A randomized trial of rofecoxib for the chemoprevention of colorectal adenomas. *Gastroenterol.* 2006; 131:1674–1682.
69. Bertagnolli MM, Eagle CJ, Zauber AG, Redston M, Solomon SD, Kim K, Tang J, Rosenstein RB, Wittes J, Corle D, Hess TM, Woloj GM, Boissarie F, Anderson WF, Viner JL, Bagheri D, Burn J, Chung DC, Dewar T, Foley TR, Hoffman N, Macrae F, Pruitt RE, Saltzman JR, Salzberg B, Sylwestrowicz T, Gordon GB, Hawk ET. Celecoxib for the prevention of sporadic colorectal adenomas. *N Engl J Med.* 2006; 355:873–884. [PubMed: 16943400]
70. Bruce W, Giacca A, Medline A. Possible mechanisms relating diet and risk of colon cancer. *Cancer Epidemiol Biomarkers Prev.* 2000; 9:1271–1279. [PubMed: 11142411]
71. Sies, H. *Oxidative stress.* London, UK: Academic Press; 1985.
72. Ames B. Mutagenesis and carcinogenesis: endogenous and exogenous factors. *Environ Mol Mutagen.* 1989; 14:66–77. [PubMed: 2659334]
73. Ames B. Endogenous DNA damage as related to cancer and aging. *Mutat Res.* 1989; 214:41–46. [PubMed: 2671700]
74. Ames B. Endogenous oxidative DNA damage, aging, and cancer. *Free Radic Res Commun.* 1989; 7:121–128. [PubMed: 2684796]
75. Cross C, Halliwell B, Borish E, Pryor W, Ames B, Saul R, McCord J, Harman D. Oxygen radicals and human disease. *Ann Intern Med.* 1987; 107:526–545. [PubMed: 3307585]
76. Frenkel K. Carcinogen-mediated oxidant formation and oxidative DNA damage. *Pharmacol Ther.* 1992; 53:127–166. [PubMed: 1641400]
77. Jones DP. Redefining oxidative stress. *Antioxid Redox Signal.* 2006; 8:1865–1879. [PubMed: 16987039]
78. Jones DP. Radical-free biology of oxidative stress. *Am J Physiol Cell Physiol.* 2008; 295:C849–C868. [PubMed: 18684987]
79. Ahearn TU, McCullough ML, Flanders WD, Long Q, Sidelnikov E, Fedirko V, Daniel CR, Rutherford RE, Shaikat A, Bostick RM. A randomized clinical trial of the effects of supplemental calcium and vitamin D3 on markers of their metabolism in normal mucosa of colorectal adenoma patients. *Cancer Res.* 2011; 71:413–423. [PubMed: 21084270]
80. Ahearn TU, Shaikat A, Flanders WD, Rutherford RE, Bostick RM. A randomized clinical trial of the effects of supplemental calcium and vitamin D3 on APC, β -catenin, and E-cadherin expression in the normal mucosa of colorectal adenoma patients. *Cancer Prev Res.* 2012; 5:1247–1256.
81. Ahearn TU, Shaikat A, Flanders WD, Seabrook ME, Bostick RM. Markers of the APC/ β -catenin signaling pathway as potential treatable, preneoplastic biomarkers of risk for colorectal neoplasms. *Cancer Epidemiol Biomarkers Prev.* 2012; 21:969–979. [PubMed: 22539608]
82. Fedirko V, Bostick RM, Flanders WD, Long Q, Shaikat A, Rutherford RE, Daniel CR, Cohen V, Dash C. Effects of vitamin D and calcium supplementation on markers of apoptosis in normal colon mucosa: a randomized, double-blind, placebo-controlled clinical trial. *Cancer Prev Res.* 2009; 2:213–223.
83. Fedirko V, Bostick RM, Long Q, Flanders WD, McCullough ML, Sidelnikov E, Daniel CR, Rutherford RE, Shaikat A. Effects of supplemental vitamin D and calcium on oxidative DNA damage marker in normal colorectal mucosa: a randomized clinical trial. *Cancer Epidemiol Biomarkers Prev.* 2010; 19:280–291. [PubMed: 20056649]
84. Sidelnikov E, Bostick RM, Flanders WD, Long Q, Fedirko V, Shaikat A, Daniel CR, Rutherford RE. Effects of calcium and vitamin D on MLH1 and MSH2 expression in rectal mucosa of

- sporadic colorectal adenoma patients. *Cancer Epidemiol Biomarkers Prev.* 2010; 19:1022–1032. [PubMed: 20332274]
85. Tu H, Ahearn TU, Daniel CR, Gonzalez-Feliciano AG, Seabrook ME, Bostick RM. Transforming growth factors and receptor as potential modifiable pre-neoplastic biomarkers of risk for colorectal neoplasms. *Mol Carcinogen.* 2014 (in press).
86. Tu H, Flanders WD, Ahearn TU, Daniel CR, Gonzalez-Feliciano AG, Long Q, Rutherford RE, Bostick RM. Effects of calcium and vitamin D3 on transforming growth factors in rectal mucosa of sporadic colorectal adenoma patients: A randomized controlled trial. *Mol Carcinogen.* 2014 (in press).
87. Sidelnikov E, Bostick RM, Flanders WD, Long Q, Cohen VL, Dash C, Seabrook ME, Fedirko V. MutL-homolog 1 (MLH1) expression and risk of incident, sporadic colorectal adenoma: search for prospective biomarkers of risk for colorectal cancer. *Cancer Epidemiol Biomarkers Prev.* 2009; 18:1599–1609. [PubMed: 19423536]
88. Sidelnikov E, Bostick RM, Flanders WD, Long Q, Seabrook ME. Colorectal mucosal expression of MSH2 as a potential biomarker of risk for colorectal neoplasms. *Cancer Epidemiol Biomarkers Prev.* 2009; 18:2965–2973. [PubMed: 19861524]
89. Bostick RM, Fosdick L, Wood JR, Grambsch P, Grandits GA, Lillemoe TJ, Louis TA, Potter JD. Calcium and colorectal epithelial cell proliferation in sporadic adenoma patients: a randomized, double-blinded, placebo-controlled clinical trial. *J Natl Cancer Inst.* 1995; 87:1307–1315. [PubMed: 7658483]
90. Hopkins MH, Owen J, Ahearn TU, Fedirko V, Flanders WD, Jones DP, Bostick RM. Effects of supplemental vitamin D and calcium on biomarkers of inflammation in colorectal adenoma patients: A randomized, controlled clinical trial. *Cancer Prev Res.* 2011; 4:1645–1654.
91. McCullough ML, Bostick RM, Daniel CR, Flanders WD, Shaikat A, Davison J, Rangaswamy U, Hollis BW. Vitamin D status and impact of vitamin D3 and/or calcium supplementation in a randomized pilot study in the southeastern U.S. *Am J Coll Nutr.* 2009; 28:678–686.

Highlights

- Strong biological rationale for vitamin D and calcium in preventing colon cancer
- Biomarkers developed to help screen potential preventive interventions
- Vitamin D and calcium favorably modulated the biomarkers in a chemoprevention trial

Fig. 1.
Factors involved in generating a specific amount of protein

Molecular Basis of Colon Carcinogenesis

Fig. 2.

Fig. 3.
Quantitative image analysis of immunohistochemically-detected biomarker in colon crypts of normal-appearing colorectal mucosa

Table 1

Hypothesis-based molecular phenotypic biomarkers of risk for colorectal neoplasms

1. Colon Carcinogenesis Pathway Gene Expression	
a. APC Pathway:	APC, E-cadherin (also see Cell Adhesion)
b. MMR Pathway:	MSH2, MLH1, TGF β RII (also see Growth Factor Signaling), bax (also see Apoptosis)
2. Cell Cycle	
a. Proliferation	
i. Short-term:	Mib-1 (Ki-67)
ii. Long-term:	hTERT
b. Differentiation:	p21
c. Apoptosis	
i. Inhibition:	bcl-2
ii. Promotion:	bax (also see MMR Pathway)
3. Cell Adhesion:	E-cadherin (also see APC Pathway)
4. Inflammation:	COX-2, CRP, IL-6, IL-1 β , IL-8, IL-10, TNF α
5. Oxidative Balance:	8-OH-dG
6. Vitamin D/Calcium Metabolism:	CaSR, VDR, CYP27B1, CYP24
7. Growth Factor Signaling:	TGF α , TGF β ₁ , IGF-I, IGFBP3

Table 2

Example MAPII study tissue biomarker results

Biomarkers	Biomarker Expression in Cases Relative to Controls ^a
APC/ β -catenin ^b	↓ 14% *
MSH2 ^c	↓ 49% *
MLH1 ^c	↓ 49% *
hTERT ^d	↑ 7%
Bax / bcl-2	↓ 26% *
MLH1 / bax	↓ 42% *
TGF α ^e	↑ 49% *
TGF β ₁	↓ 7%
TGF β RII	↓ 7%
TGF α /TGF β ₁	↑ 110% *

*
p < 0.05^a Proportional differences between cases and controls; results are for overall crypt expression for all biomarkers except where indicated^b Φ h APC/ β -catenin in the whole crypt, where Φ h APC = ratio of APC in upper 40% of crypts to the whole crypts^c Results for proximal ascending colon^d Results shown are for proportion of overall crypt biomarker expression extending into upper 40% of crypts^e Results shown are for upper 40% (differentiation zone) of crypt

Table 3

Changes in serum vitamin D metabolites in the CaD v MAP trial

	BL	6-mon. F/U	Abs. Diff.	Rel. (%)	P
25-OH-vitamin D (ng/ml) ^a					
Placebo	20.4	17.9	-2.6		
Vitamin D ₃	21.0	29.5	8.44	↑ 60	< 0.0001
Calcium	25.7	23.2	-2.5	↑ 3	0.88
Vit. D + Cal.	20.9	28.5	7.6	↑ 56	< 0.0001
1α,25-(OH) ₂ -vitamin D (pg/ml) ^b					
Placebo	39.2	37.1	-2.0		
Vitamin D ₃	44.5	47.4	2.9	↑ 12	0.57
Calcium	45.4	31.7	-13.7	↓ 26	0.21
Vit. D + Cal.	37.9	36.7	-1.2	↑ 2	0.97

Abbreviations: BL, baseline; F/U, follow up; Abs. Diff., absolute difference; Rel. , relative change (in the active treatment group relative to placebo)

^a nmol/L = ng/L × 2.5^b pmol/L = pg/L × 0.42

Table 4

Changes in tissue biomarkers in the CaD v MAP trial

Biomarkers	Crypt Parameter	Treatment		
		Calcium (% Rel.)	Vitamin D ₃ (% Rel.)	Ca ⁺⁺ &Vit. D ₃ (% Rel.)
APC	Upper 40%	↑ 7	↑ 48*	↑ 10
β-catenin	Whole	↓ 15	↓ 12	↓ 11
APC/β-catenin	Mixed	↑ 41*	↑ 31*	↑ 16
E-cadherin	Upper 40%	↑ 1	↑ 78*	↑ 56*
MSH2	Upper 40%	↑ 107	↑ 169*	↑ 90
MLH1	Upper 40%	↑ 14	↑ 26	↑ 10
Mib-1	Upper 40% / Whole	↓ 6	↓ 3	↓ 3
hTERT	Upper 40% / Whole	↓ 10	↓ 3	↓ 15*
p21	Whole	↑ 101*	↑ 142*	↑ 25
Bax	Whole	↑ 33	↑ 56*	↑ 33
Bax/bcl-2	Whole	↑ 62	↑ 47	↑ 71
TGFα	Upper 40% / Lower 60%	↓ 34	↓ 31	↓ 26
TGFβ ₁	Whole	↑ 14	↑ 19	↑ 22
TGFα/TGFβ ₁	Mixed	↓ 48	↓ 44	↓ 35
8-OH-dG	Whole	↓ 22	↓ 25	↑ 6
CaR	Whole	↑ 27*	↑ 39*	↑ 24*
VDR	Whole	↑ 5	↑ 10	↑ 19
CYP27B1	Whole	↑ 34	↑ 159*	↑ 34
CYP24A1	Whole	↓ 21	↑ 65	↑ 51

Abbreviations: Ca⁺⁺, calcium; Vit., vitamin; % Rel. , proportional change in the active treatment group relative to placebo*
p< 0.05

Table 5

Changes in serum inflammation markers in the CaD v MAP trial

Biomarkers	Treatment		
	Calcium (% Change)	Vitamin D ₃ (% Change)	Ca ⁺⁺ &Vit. D ₃ (% Change)
CRP	↓ 8	↓ 32	↑ 9
TNF α	↑ 6	↓ 13	↑ 3
IL-6	↓ 37	↓ 32	↓ 8
IL-8	↓ 11	↓ 15	↓ 13
IL-1 β	↓ 23	↓ 50	↓ 35
IL-10	↓ 7	↓ 5	↓ 4
Inflammation z-score	↓ 48	↓ 77*	↓ 33

Abbreviations: Ca⁺⁺, calcium; Vit., vitamin; % Change, proportional change in the active treatment group relative to the placebo group; CRP, C-reactive protein; TNF, tumor necrosis factor; IL, interleukin

*
p< 0.05