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Colorectal Adenomas, Calcium and Vitamin D

By

Veronika Fedirko
Doctor of Philosophy

Department of Epidemiology

[Roberd “Robin” M. Bostick, M.D., M.P.H.]
Advisor

[W. Dana Flanders, M.D., Sc.D.]
Committee Member

[Michael Goodman, M.D., M.P.H.]
Committee Member

[Stephanie L. Sherman, Ph.D.]
Committee Member

[Vin Tangpricha, M.D., Ph.D.]
Committee Member

Accepted:

Lisa A. Tedesco, Ph.D.
Dean of the Graduate School

Date

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By

Veronika Fedirko

B.Sc., National University “Kyiv Mohyla Academy”, 2002

M.P.H., Emory University, 2005

Advisor: Roberd “Robin” M. Bostick, M.D., M.P.H.

An Abstract of
a dissertation submitted to the Faculty of the Graduate School of Emory University
in partial fulfillment of the requirements for the degree of
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Abstract

Colorectal Adenomas, Calcium and Vitamin D

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Colorectal cancer is the second cause of cancer death in the US, and despite advances in treatment, screening, and prevention, its mortality has declined only modestly in recent years. Thus, there is need to develop new chemopreventive agents for colorectal neoplasms, and for new clinically accepted pre-neoplastic biomarkers of risk for colorectal neoplasms that may be used in colorectal cancer screening and in chemoprevention trials to assess the effectiveness of treatment. The goals for this dissertation are to investigate the roles of two evidentially well-supported chemopreventive agents, vitamin D_3 and calcium, in colorectal carcinogenesis, and to develop modifiable biomarkers of risk for colorectal neoplasia.

In a pooled analysis of three colonoscopy based case-control studies, a substantial, statistically significant, lower risk for incident, sporadic colorectal adenomas was found with higher levels of circulating 25-(OH)-vitamin D_3. This inverse association was stronger among those who took aspirin or other non-steroidal anti-inflammatory drugs. In a pilot randomized, double-blind, placebo-controlled clinical trial of calcium and vitamin D_3 supplementation, modulation of expression of cell cycle biomarkers in the normal-appearing colorectal mucosa of patients with previously resected colorectal adenomas was found in the calcium and vitamin D_3 groups. The strongest treatment effects were on the markers of cell differentiation and apoptosis, and vitamin D_3 related. Findings from this clinical trial also support a new hypothesis that vitamin D_3 and calcium reduce oxidative DNA damage in the colon, especially among those who have higher colorectal expression of the vitamin D receptor.

In conclusion, the results of my dissertation support hypotheses that higher intakes of vitamin D_3 and calcium favorably modulate biomarkers of risk for colorectal neoplasms, and higher circulating 25-(OH)-vitamin D_3 levels are associated with lower risk for incident, sporadic colorectal adenoma. The results of this dissertation warrant further investigation of the expression of cell cycle biomarkers in the normal-appearing colorectal mucosa as potential modifiable biomarkers of risk for colorectal neoplasms.

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CHAPTER 1. INTRODUCTION AND BACKGROUND

Introduction

Colorectal cancer is the third most common incident cancer and the second most common cause of cancer death in the U.S. in men and women combined (1, 2). It is a disease highly correlated with the Western-style diet, which is characterized by higher consumption of meat, and processed foods and lower consumption of fiber, calcium, and vitamin D (3, 4).

Vitamin D and calcium are promising dietary chemopreventive agents in colorectal cancer. Proposed mechanisms of calcium against colorectal cancer include protection of colonocytes against bile acids and fatty acids (5, 6), direct effects on cell cycle regulation (7), and modulation of E-cadherin and β-catenin expression via the calcium-sensing receptor (CaSR) (7-9). Beyond calcium homeostasis, for its non-classical, autocrine/paracrine functions, vitamin D regulates proliferation, differentiation, and apoptosis; promotes bile acid degradation and xenobiotic metabolism; and influences growth factor signaling, cell adhesion, DNA repair, angiogenesis, inflammation, and immune function (10-13). Moreover, vitamin D modulates more than 200 responsive genes (14, 15).

Vitamin D was shown in some epidemiologic studies to reduce risk for colorectal cancer (16-20) and adenoma (21-24). In studies that investigated dietary vitamin D intake without considering exposure to UVB light, the association between vitamin D intake and colorectal adenoma/cancer was not consistent. This inconsistency between these studies can be explained by misclassification of actual vitamin D exposure that leads to an underestimation of the main effect. In those few studies that assessed the main form of circulating vitamin D, 25-(OH)-vitamin D (collective term for 25-(OH)-vitamin D_2 and D_3), an inverse association was observed between 25-(OH)-vitamin D levels and colorectal cancer (25-27) or adenomas (21, 22). The results of these studies suggest that circulating vitamin D level is a better marker of vitamin D exposure than indirect estimates of vitamin D exposure based solely on a diet due to its long half-life in the circulation and lack of tight homeostatic regulation of its concentration.

In numerous epidemiologic studies, higher intakes of calcium were consistently shown to be inversely associated with risk for colorectal cancer (16, 19, 28-37). The same inverse association was observed in colorectal adenoma studies (21, 24, 38-40). Moreover, several clinical trials found reduced colorectal adenoma recurrence with calcium supplementation(41). A large number of studies investigated the effect of vitamin D and calcium separately from each other, and the few studies that addressed the interaction of these two agents found inconclusive results, likely due to using dietary assessment of vitamin D intake as a sole indicator of vitamin D exposure.

It is also biologically plausible that there are multiple agents or conditions that can modify the vitamin D and calcium association with colorectal adenoma risk that were not fully considered in the previous epidemiologic studies (e.g., retinol, inflammation status, HRT in women, folate, obesity).

The few human studies that investigated the local effect of vitamin D and calcium on cell cycle markers in the normal colon mucosa yielded inconsistent results (42-45). None of the published human studies reported on effects of vitamin D and calcium on oxidative stress markers in the normal colon mucosa. Thus, possible effects of calcium and vitamin D on tissue markers of apoptosis, differentiation, proliferation, and oxidative DNA damage require further investigation.

Overall, calcium and vitamin D are promising, safe chemopreventive agents against colorectal neoplasms that require further investigation. The objective of this dissertation is to clarify the role of vitamin D and calcium in colorectal carcinogenesis. The specific research questions are: 1) do high circulating 25-(OH)-vitamin D levels alone or in combination with high calcium intake reduce risk for colorectal adenomas; 2) is this association modified by inflammation status, obesity, HRT use in women, or dietary intakes of retinol, soy products, and folate; and 3) do calcium and vitamin D alter levels of oxidative DNA damage and expression of biomarkers of cell proliferation, differentiation, and apoptosis in the normal rectal mucosa? The three studies included in this dissertation examined these questions by using data from a pooled analysis of three case-control studies of incident, sporadic colorectal adenomas, and a randomized, double-blind, placebo-controlled 2x2 factorial clinical trial of calcium and/or vitamin D in sporadic adenoma patients. This dissertation will lead to a better understanding of the mechanisms of colorectal cancer prevention by vitamin D and calcium.

Background

Overview of Colorectal Cancer Epidemiology

Colorectal cancer (CRC) is the second leading cause of cancer deaths in the United States. Approximately 218,350 new cases and 49,920 deaths were anticipated in 2009 (2). Colon cancer affects men and women approximately equally, but rectal cancer frequency can be up to twice as high in men as in women (46). The colorectal cancer incidence rate stayed relatively unchanged during the past 30 years, while the mortality rate decreased, particularly in females (47). Colorectal cancer incidence rates increase sharply with age (46). Risk of developing colorectal cancer is influenced by both genetic and environmental factors. International ecologic studies and studies of immigrants demonstrated the importance of lifestyle and nutritional factors, such as physical activity and consumption of red meat, in the etiology of colon cancer (4, 48-50). Incidence rates vary 20-fold between countries, with the highest rates in Japan, North America and Europe, and the lowest rates in Africa (46, 51). It is estimated that diet-related factors could contribute up to 80% of the differences between countries (51). However, whether the associations observed in epidemiologic studies between colorectal cancer and diet are causal are subject to debate.

Colon cancer is usually observed in one of three specific patterns: sporadic, inherited, or familial (52). Most colorectal cancer cases are sporadic non-familial cancers and their incidence is strongly associated with age, nutrition and lifestyle (53). There are four hereditary colorectal cancer syndromes that account for less than 5% of CRC cases: familial adenomatous polyposis (FAP), hereditary nonpolyposis colorectal cancer (NHPCC), Peuts-Jeghers syndrome and juvenile polyposis (52).

Most colorectal cancers arise from adenomatous polyps. There are several types of colorectal polyps, including non-neoplastic hamartomas (juvenile polyps), hyperplastic polyps, and adenomatous polyps (54). The prevalence of adenomatous polyps is approximately 30% in the middle-aged, and around 50% in elderly persons; however, less than 1% of all adenomas develop into cancer (54). The likelihood of an adenomatous polyp transforming into a cancer depends on several characteristics, such as size, histologic features, and appearance of the lesion (54). Adenomas can be pedunculated/stalked, or sessile/flat-based (54). Histological subtypes of adenomas include tubular, villous, or tubulovillous polyps. Cancer develops more frequently from villous adenomas, most of which are sessile. It is estimated that approximately 5% of small adenomas, and 50% of large villous adenomas transform into cancer (55-57). It was also shown that 70 to 90% of all colorectal cancers develop from adenomas (54). Some data indicate that hyperplastic polyps can give rise to serrated polyps and subsequently to cancer (46, 58).

Nowadays, colorectal adenomatous polyps are well-established precursors to most colorectal cancers. As adenomas can be detected years before cancer develops, they are now the only biomarkers of risk for colorectal cancer and surrogate markers for CRC screening (59, 60). The most reliable method for diagnosing colorectal adenomas is the colonoscopy, which is labor intensive, expensive procedure poorly tolerated by patients. Following the detection of an adenoma, colonoscopy is repeated periodically (every 3-5 years) as such patients have a 30–50% probability of developing a new, recurrent adenoma and are at a higher-than-average risk for developing a colorectal cancer. Adenomatous polyps are thought to require at least 5 years of growth before becoming cancerous (54).

Currently, there are no generally accepted pre-neoplastic biomarkers of risk for colorectal cancer. Several procedures, including fecal occult blood testing and colonoscopy, are used to screen for colorectal neoplasms. Despite advances in screening and treatment, mortality due to colorectal cancer has declined only modestly over the past

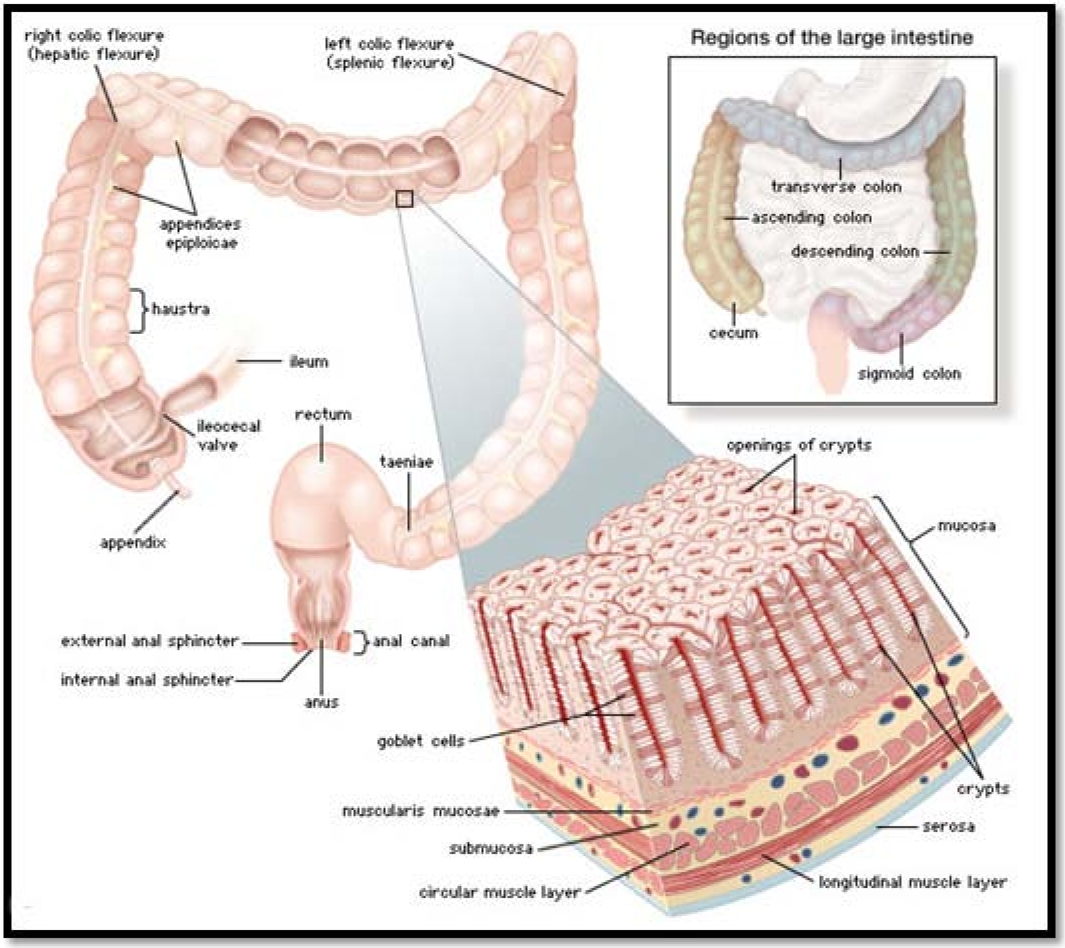
50 years, the decline probably a result of screening and polypectomy (1, 4). Development of pre-neoplastic biomarkers of risk will greatly enhance the value of screening procedures and will allow assessing individual risk for CRC like we do now for ischemic heart disease (61). The development of biomarkers in colorectal tissue and bodily fluids will also allow monitoring the response to preventive treatments (such as calcium and vitamin D), and scientific research. Using biological measurements of risk, as they have for ischemic heart disease, should likewise result in a decline in colorectal cancer incidence and mortality.

Morphology of the Colon

The colon (large intestine) serves primarily to solidify and store waste and indigestible materials prior to their elimination by defecation (62), and consists of several regions: the ascending, transverse, descending, and sigmoid colon (Figure 1.1). Furthermore, the colon contains an ecosystem consisting primarily of anaerobic symbiotic bacteria that are important contributors to whole body nutritional status (62).

The colonic mucosa is a highly organized self-renewing structure with rapid cell turnover (4-8 days), that consists of U-shaped tube-like deep invaginations into the wall of the colon called crypts (63, 64) (Figure 1.1). At the base of the crypt, stem cells divide producing new cells that migrate to the luminal surface of the crypt and differentiate into the functional cells. At the top of the crypt, differentiated cells slough off by an apoptotic mechanism (63, 65), and are replaced by the upward migrating new cells. Some cells within the crypts, such as damaged cells or stem cells at the bottom of the crypt, also undergo apoptosis (63). Epithelial cells, or colonocytes, interact with each other and the environment, and with aging, appropriate genetic background and detrimental environmental exposures, may undergo carcinogenic transformations.

Figure 1.1. Regions of the large intestine with morphology of normal colon tissue (from Encyclopaedia Britannica, Inc. 2003 (66)).



Colon Carcinogenesis

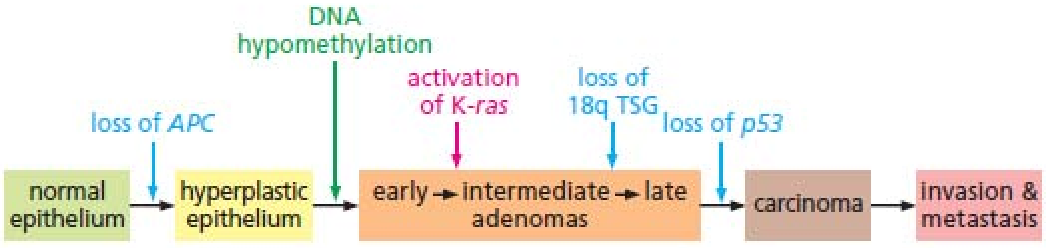
Colorectal carcinogenesis is one of the most studied carcinogenic processes and one of the classical examples of a multistage carcinogenesis (52, 67-70).

To become a malignant cell, a normal colonocyte must acquire six phenotypes: immortalization, independence from mitogenic stimulation, resistance to growth inhibition, the ability to acquire its own blood supply, ability to metastasize, and ability to suppress apoptosis (71). These phenotypes develop through mutation or silencing of key genes that regulate these functions (64). In order to accumulate genetic errors, a normal cell's genome has to become unstable, resulting in a fast accumulation of genetic errors (64). In colorectal cancer, genomic instability develops through microsatellite instability (MSI), chromosomal instability (CIN), and the CpG island methylator phenotype (CIMP) (72). Approximately 12-15% of all CRCs have MSI caused by inactivation of the DNA mismatch repair system (72). Most colorectal cancers acquire genomic instability by CIN or CIMP (72).

In 1978, Hill et al. (73) developed the adenoma-carcinoma sequence hypothesis based on a number of epidemiological and histopathological observations; a modified version is now widely accepted. The modified version of the adenoma-carcinoma hypotheses proposes that adenomatous polyps develop from abnormal highly proliferative colonocytes that result in minute lesions (resembling ACF) and microadenomas (46, 58) (Figure 1.2). And as adenomas grow in size, they undergo neoplastic transformations (46, 58). However, recent evidence indicated that sporadic MSI-High (MSI-H) colorectal cancers do not develop through the adenoma-carcinoma sequence, but rather within proximal, atypical hyperplastic polyps or sessile serrated adenomas (58). All these suggest that there are multiple pathways for developing colorectal cancer.

Several molecular events are seen during the development and growth of adenomas (Figure 1.2 (74)), and are thought to reflect the multi-step process in the transition of normal colorectal epithelium to invasive carcinoma (75). These molecular events include deletion of the *APC* tumor suppressor gene; hypomethylation of DNA, leading to gene activation; point mutations in the *K-ras* protooncogene; allelic loss of a tumor-suppressor gene on chromosome 18q; and mutations in the *p53* tumor-suppressor gene (54). These events are not entirely sequential; however, in most cases loss of *APC* function appears to be the earliest event as it results in blocking of colonocyte emigration from the crypts and the accumulation of *APC*-negative cell populations (74). These *APC*-negative colonocytes are not cleared from the crypt through emigration and apoptosis, and may acquire additional mutations leading to adenoma and carcinoma development (74). The "APC–Pathway" is one of the major pathways driving the colorectal carcinogenesis.

Figure 1.2. The adenoma-carcinoma sequence (from Weinberg, R.A. (74)).



There appears to be at least two major largely non-overlapping pathways driving colorectal tumorigenesis: the "APC– β-catenin– Tcf" and the "Mismatch Repair (MMR)" pathways.

The "APC– β-catenin– Tcf Pathway" includes a cascade of signaling molecules, silencing or activation of which leads to colorectal cancer development. This pathway includes a tumor suppressor gene, *APC* (adenomatous polyposis coli), a member of the *wnt* signaling pathway. This gene was found to be mutated in aberrant crypt foci, the earliest histologically identifiable lesions of the adenoma-carcinoma sequence(76). It regulates cellular proliferation and differentiation through the β-catenin/TCF-4 complex (77). APC functions to inhibit β-catenin, a pro-proliferative protein, and regulates E-cadherin, a cell adhesion molecule. When β-catenin is not destroyed by APC, both downstream c-myc and cyclin D_1 are up-regulated, promoting cell proliferation and decreased apoptosis and differentiation. APC may also regulate caspase activity and other apoptotic proteins by controlling their expression levels in the cell (78). The "APC– β-catenin– Tcf Pathway" accounts for FAP and approximately 80% of sporadic cancers.

The second pathway that drives colon carcinogenesis is the "Mismatch Repair (MMR) Pathway", which includes products of mismatch repair genes (*e.g.*, *MSH2* and *MLH1)* that serve to repair mismatches in paired DNA strands after replication (67, 79, 80). An inactivating mutation in one of these genes results in accumulation of mismatches in DNA and subsequent alterations in the functioning of the affected genes. The MMR pathway accounts for HNPCC and approximately 15% of sporadic cancers.

Another pathways, the serrated adenoma pathway, includes *BRAF* or *K-ras* mutations combined with extensive DNA methylation during early stages of cancer development, and yields sporadic CRCs with MSI (81).

Inflammation was relatively recently shown to play a major role in colorectal cancer development (72). Chronic inflammation in the colon leads to excessive production of reactive oxygen species (ROS) and nitric oxide (NO), which can not only cause damage to DNA and other cell molecules, but also inactivate some of the intracellular protective mechanisms that result in colorectal cancer development (72). The COX-2 enzyme promotes inflammation and cell proliferation, inhibits apoptosis, and interacts with the “APC pathway” (72, 82, 83). Moreover, in several clinical trials NSAIDs (COX-2 inhibitors) reduced recurrence of sporadic adenomas (84) and inhibited the growth of adenomatous polyps in patients with the FAP (85-90).

To understand better the causation of CRC and improve prevention and treatment, a new approach to the molecular classification of CRC was proposed (91). Based on the type of genetic instability (MSI-high (H), -low (L), or -stable (S)) and the presence of DNA methylation (CIMP-low (L), -high (H), or –negative (Neg.)), the following five major molecular subtypes of CRC, which may overlap, were suggested (Figure 1.3) (91):

Type 1: CIMP-H and MSI-H; methylation of MLH1, BRAF mutation, chromosomally stable, origin in serrated polyps, known generally as sporadic MSI-H (~12% of all CRC cases).

Type 2: CIMP-H and MSS or MSI-L; partial methylation of MLH1, BRAF mutation, chromosomally stable, origin in serrated polyps (~8%).

Type 3: CIMP-L and MSS or MSI-L; K-ras mutation, MGMT methylation, chromosomal instability, origin in adenomas or serrated polyps (~20%).

Type 4: CIMP-Neg and mainly MSS; chromosomal instability, origin in adenomas, may be sporadic, FAP-associated or MUTYH polyposis associated (~57%).

Type 5: CIMP-Neg and MSI-H, Lynch syndrome (or familial MSI-H CRC), BRAF mutation negative, chromosomally stable, , origin in adenomas (~3%).

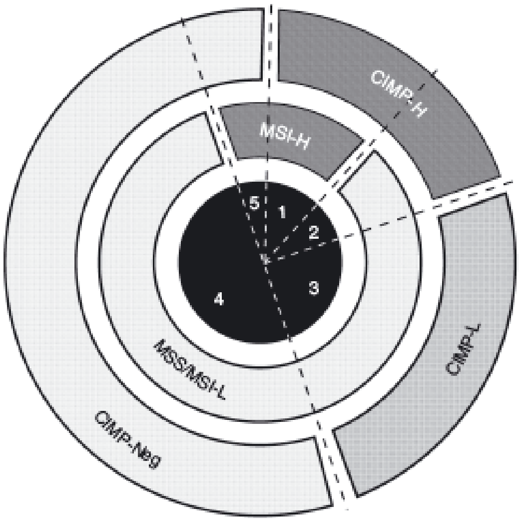


Figure 1.3. Derivation of molecular colorectal cancer groups 1–5 based on CpG island methylator phenotype (CIMP) status (H, high; L, low; Neg, negative) and DNA microsatellite instability (MSI) status (H, high; L, low; S, stable) (from Jass, J.R. (91)).

These molecular subtypes of colorectal cancer differ by various molecular, clinical, and morphological features as summarized in Table 1.1 (91).

Table 1.1. Molecular, clinical and morphological features of colorectal cancer groups 1–5 (from Jass, J.R. (91)).

Feature	Group 1	Group 2	Group 3	Group 4	Group 5
MSI status	H	S/L	S/L	S	H
Methylation	+++	+++	++	+/-	+/-
Ploidy	Dip > An	Dip > An	An >Dip	An > Dip	Dip > An
APC	+/-	+/-	+	+++	++
K-ras	-	+	+++	++	++
BRAF	+++	++	-	-	-
TP53	-	+	++	+++	+
Location	R > L	R > L	L > R	L > R	R > L
Sex	F > M	F > M	M > F	M > F	M > F
Precursor	SP	SP	SP/AD	AD	AD
Serration	+++	+++	+	+/-	+/-
Mucinous	+++	+++	+	+	++
↓differentiation	+++	+++	+	+	++

MSI, microsatellite instability; H, high; S, stable; L, low; Dip, diploid; An, aneuploid; Serration, serrated morphology; SP, serrated polyp; AD, adenoma.

From the epidemiological perspective, it is important to recognize colorectal cancer as a multipathway disease consisting of distinct subgroups with particular molecular and pathological features (91). Understanding the various pathways involved in colorectal cancer development may facilitate the identification of risk factors for colorectal neoplasms (genetic or lifestyle), and the development of pathway-oriented chemoprevention strategies.

Risk Factors for Colorectal Cancer

Risk factors associated with colorectal cancer were reviewed in detail elsewhere (4, 92, 93) and include the following categories (adopted from Weitz *et al*. (92)):

Sporadic colorectal cancer (accounts for 88-94% of all CRC cases)

- **Demographic risk factors**
  - older age, male sex
- **Environmental/lifestyle, reproductive factors**
  - high red meat and fat diet; low vegetable fruit, fiber, folate, calcium diet; low physical activity, anthropometry, smoking, obesity, high alcohol intake; hormonal factors for women (nulliparity, late age at first pregnancy, early menopause); occupational exposure to asbestos and cotton dust; infection with JC virus, *Schistosoma japonicum*, *Helicobacter pylori*, *Streptococcus bovis* bacteremia
- **Personal history of sporadic tumors**
  - history of colorectal polyps, colorectal, small bowel, endometrial, breast, or ovarian cancer
- **Medications**
  - NSAIDs, postmenopausal hormone use (HRT)
- **Family history**
  - first or second degree relatives with colorectal cancer
- **Medical (non-inflammatory) conditions**
  - cholecystectomy, previous irradiation, diabetes mellitus

Hereditary colorectal cancer (accounts for 5-10% of all CRC cases)

- **Polyposis syndromes**
  - Familial adenomatous polyposis (FAP), Gardner's syndrome, Turcot's syndrome, attenuated adenomatous polyposis coli, flat adenoma syndrome
- **Hereditary non-polyposis colorectal cancer (HNPCC)**
- **Hamartomatous polyposis syndromes**

Inflammatory conditions (accounts for 1 -2% of all CRC cases)

- **Ulcerative colitis, Crohn's colitis**

Demographic Risk Factors

Colorectal cancer incidence rates increase sharply with age (46). Approximately 90% of colorectal cancer cases occur in persons of age 50 or older (4, 54, 94, 95). Colon cancer affects men and women essentially equally (4, 46, 96); however, rectal cancer incidence in men is about twice that in women (67). Furthermore, incidence of colorectal cancer varies by race and ethnicity (97). In the United States, black men and women have the highest colorectal cancer incidence and mortality rates. White men and women have slightly lower colorectal cancer incidence rates (by 11 and 15%, respectively), but substantially lower colorectal cancer mortality rates (by 30 and 31%, respectively) than black men and women (97). American Indians and Alaska Natives have the lowest colorectal cancer incidence rates (97). During 2000–2004, colorectal cancer incidence rates decreased slightly for White, non-Hispanic, Asian/Pacific Islander men and women, and remained stable for Black, Hispanic, American Indian and Alaska native (97).

Hereditary Colorectal Cancer

The strongest known risk factors for colorectal cancers include several rare, highly penetrant hereditary conditions, such as familial adenomatous polyposis (FAP), Gardner's syndrome, Turcot's syndrome, attenuated adenomatous polyposis coli, and flat adenoma syndrome (67). However, these conditions together account for approximately 5–10% of all colorectal cancer cases (92).

Family History

Family history of colorectal cancer is positively associated with sporadic colorectal cancer risk. About 30% of sporadic colorectal cancer cases have a history of the disease in a first degree relative (67), which is associated with a 2- to 3-fold increased risk of colorectal cancer (98, 99) . Moreover, having a history of CRC in a first degree relative younger than 40 years of age is associated with a 5-fold increase in risk of the disease (99). Furthermore, having two relatives of any age with CRC is associated with a 6-fold increase in risk (98, 99).

Infection

Recent findings indicated that a virus (JCV) carried by most healthy persons and found in colon cancer tissue (100, 101) may be a possible cause of chromosomal instability (CIN). This type of chromosomal instability plays a major role in the development and progression of colorectal carcinogenesis (72). It has been hypothesized that JC virus infection is involved in the initiation of colorectal neoplasia, but further investigation is required. Persons who develop endocarditis or septicemia from *Streptococcus bovis* bacteremia have a higher incidence of colorectal tumors, but the reasons for this possible association are unknown and require investigation (54). Also, another bacteria, *Helicobacter pylori*, was proposed as a risk factor for colorectal neoplasms (102, 103); however, a recent meta-analysis of the 11 human studies suggested that there may be only a small increase in colorectal cancer risk (odds ratio (OR) = 1.4, 95% confidence interval (CI): 1.1–1.8) associated with *H. pylori* infection (104). Finally, infection with *Schistosoma japonicum* has also been associated with increased risk for rectal cancer (105).

Inflammation and NSAIDs

In response to a range of toxic and pathogenic challenges, lymphocytes infiltrating into colorectal epithelium can release proinflammatory cytokines. These may lead to increased generation of reactive oxygen species (ROS) and other genotoxic compounds in the colorectal epithelium. Continual release of proinflammatory cytokines can cause chronic inflammation, which has been reported to play a major role in colorectal tumorigenesis. Multiple observational studies and randomized clinical trials found that regular use of anti-inflammatory drugs (NSAIDs), such as aspirin and other NSAIDs, reduces the risk of colorectal neoplasms (84, 106-110). The anti-carcinogenic effects of NSAIDs are through to be largely through COX-2 inhibition; however, they can also block COX-1, which can cause gastrointestinal bleeding and renal failure. Since September 2004 several studies raised concern about potential cardiovascular toxicity associated with the use of coxibs, selective COX-2 inhibitors (111); therefore, despite irrefutable effectiveness of aspirin and NSAIDs as colorectal neoplasm chemopreventive agents, they are no longer used in colorectal cancer prevention.

Inflammatory Bowel Disease (IBD)

Persons with inflammatory bowel disease (ulcerative colitis and Crohn's disease) are at increased risk of developing colorectal cancer (112-114). In younger patients under the age of 50, approximately 5% of all colorectal cancers develop in individuals with these inflammatory conditions (46). CRC risk increases with younger age of IBD onset, longer duration of symptoms, and greater extent of disease (115). Free radicals and other processes associated with inflammation are believed to be involved in carcinogenesis in IBD patients (116-119). An increased use of anti-inflammatory drugs to control the disease has been associated with a recent decline in CRC risk among IBD patients (120).

Diabetes Mellitus

Diabetes mellitus is associated with a moderate increased risk for colorectal cancer (121-124). One epidemiologic study also found high fasting insulin and blood glucose levels to be directly associated with colorectal cancer risk among non-diabetic patients (124). A meta-analysis (125) of 15 epidemiologic studies reported that diabetes was statistically significantly associated with moderately increased risk for colorectal cancer in both men and women (summary risk ratio (RR) = 1.30, 95% CI: 1.20–1.40), and with moderately increased colorectal cancer mortality (summary RR = 1.26, 95% CI: 1.05–1.50) (125).

Occupation

Several epidemiologic studies reported an increased risk for colorectal cancer among persons exposed to asbestos (126, 127) or dyes and metals (128). Also, colon cancer is elevated in white-collar occupations, likely due to lower physical activity (129). Another report found that exposure to cotton and cotton dust is associated with a reduced risk for colorectal cancer (128).

Reproductive Factors and Postmenopausal Hormone Use in Women

More than 20 observational epidemiologic studies reported on the association of colorectal cancer risk with reproductive history in women (46) , but the results of these studies were mostly inconsistent (130). Overall, age at first birth, age at menarche, and parity were not associated with risk for colorectal cancer in women (46). A meta-analysis of 12 epidemiologic studies reported decreased colorectal cancer risk (OR = 0.82, 95%CI: 0.74–0.92) in women who were taking combination oral contraceptives

(OC) (131), but the studies reviewed were mostly in older women aged between 55 and 60 years and no information about OC type was available. Data on postmenopausal hormone use and colorectal cancer in women are not entirely consistent: nine studies reported decreased risk with HRT use (132-140), seven of which were statistically significant (132-138), two studies were null (141, 142), and one found an increased risk (143). Longer use of HRT is probably associated with lower risk, but more studies are needed to confirm this (46). As for CRC, an inverse association of HRT use with colorectal adenomas was also found (144, 145).

In a Women's Health Initiative Estrogen plus Progestin randomized clinical trial, treatment with estrogen and progestin considerably reduced invasive colorectal cancer risk (hazard ratio (HR = 0.56; 95% CI: 0.38–0.51) (146). In addition, a recent case-control study found that conjugated estrogen with progestin is more strongly associated than estrogen alone with risk for MSI-low and MSI-stable, but not MSI-high colorectal tumors (147).

Physical Activity and Anthropometry

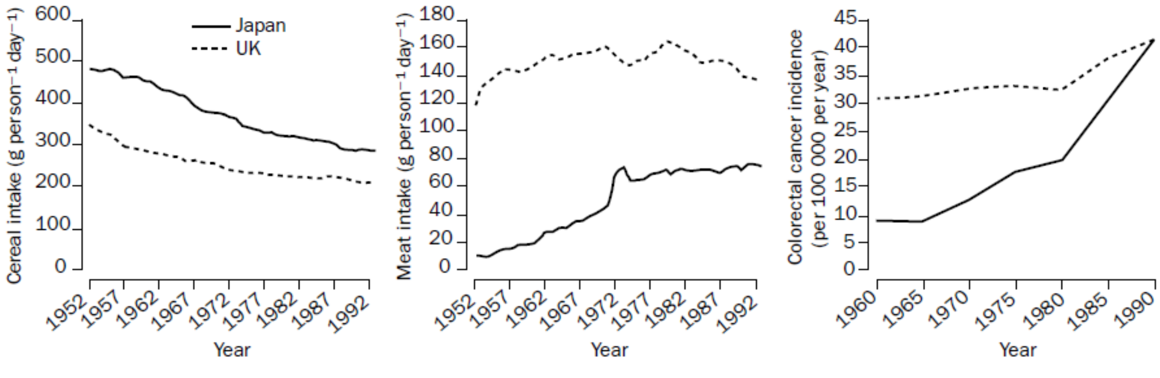
There is abundant epidemiological evidence showing a strong and consistent association of reduced risk of colon neoplasms with higher overall levels of physical activity, as well as with greater intensity and frequency (148) . Several large cohort studies and meta-analyses found a 20–29% reduction of colon cancer risk in individuals with high levels of physical activity compare to sedentary individuals (148-150). However, no association was observed for rectal cancer. There are several, likely complimentary, mechanisms by which physical activity may protect against colorectal carcinogenesis: 1) stimulation of colon peristalsis resulting in reduced gut transit time (and thus less carcinogen contact time with the colon epithelium); 2) reduction in insulin resistance; 3) favorable effects on the immune system; 4) effects on endogenous steroid hormone metabolism; and 5) reduction in body fatness (46, 148).

Obesity is strongly and consistently associated with an increased risk of colorectal cancer. A recent meta-analysis of 28 cohort studies found a statistically significant 3% increase in CRC risk per 1 kg/m^2 increase in BMI (148). This would produce a 15% increased CRC risk for each 5 kg/m^2 increase in BMI, assuming a linear relationship (148). As with physical activity, a more consistent association and a larger increase in risk was found for colon cancer than for rectal cancer, or for colorectal cancer as a whole (148). BMI may not be an ideal measurement of the adiposity in humans for CRC risk prediction for several reasons: 1) fat is not distributed equally around the body; 2) there are two patterns of fat stores in the human body (‘peripheral’ or ‘abdominal’) that are largely determined by genetic factors; and 3) the size of intra-abdominal fat stores influences several hormone systems and predicts the risk of chronic diseases better than overall indicators of body fatness, such as BMI or subcutaneous fat measures (148). A meta-analysis of four cohort studies found a statistically significant 5% increase in CRC risk per 2.5 cm (1 inch) increase in waist circumference (148). Similar results were found for a different measure of abdominal fatness, the waist-to-hip ratio (RR = 1.30, 95% CI: 1.17–1.44 per ratio increment of 0.1) (148). The main mechanisms through which body fatness and abdominal fatness may promote colon carcinogenesis include obesity-induced insulin resistance, increased circulating estrogens, and low-grade chronic inflammation (148, 151, 152).

Diet

International ecologic studies of correlations of the consumption of specific nutrients and foods with colorectal cancer incidence suggested that the diets of different populations may in part explain their rates of cancer. This hypothesis was supported by the fact that immigrants from low- to high-risk countries acquired the cancer rates of their adopted country within one generation (50, 51). In 1981 Doll and Peto (153) estimated that up to 90% of colon cancers may have a primarily dietary contribution. Colorectal cancer rates are high in almost all economically developed countries and are thought to be associated with ‘‘Westernization’’ of the society (50). The extreme example of the “westernization” – colorectal cancer association is Japan. Between the 1950s and the 1990s, consumption of meat increased ten-fold and intake of cereals decreased in Japan with a concomitant five-fold rise in colorectal cancer incidence among men (Figure 1.4) (51).

Figure 1.4. Indicators of dietary change and trends in CRC (men) in the UK and Japan (from Key *et al.* (51)).



This and other ecologic studies resulted in numerous hypotheses relating some components of a western diet, such as a higher intake of meat (red and processed) or fat, and lower intake of fruits, vegetables, and fiber with high risk for colorectal neoplasms.

Despite evidence provided by ecologic and immigrant studies on potential dietary risk factors, analytic observational epidemiologic studies have been inconsistent in many respects. Moreover, there have been very few clinical trials that tested effects of dietary components and nutrients on risk of colorectal neoplasms.

The recent report of World Cancer Research Fund and American Institute for Cancer Research (148) reviewed data on dietary or diet-related factors that modify risk for colorectal cancer and concluded that the strongest evidence exists for protective effects of physical activity and detrimental effects of high intakes of red and processed meats, alcoholic drinks (in men), high body and abdominal fatness, and greater adult attained height (Table 1.2) (148). Some of these factors are reviewed below.

Table 1.2. Nutritional risk factors, physical activity and cancers of the colon and the rectum (from WCRF/AICR (148)).

Evidence	**↓ Risk**	**↑ Risk**
Convincing	Physical activity	Red meat (↑15% per 50g/d) Processed meat (↑21% per 50g/d) Alcoholic drinks (men) Body & abdominal fatness Adult attained height
Probable	Dietary fiber Garlic Milk, calcium	Alcoholic drinks (women)
Limited-suggestive	Non-starchy vegetables Fruits, selenium Foods containing folate Foods containing selenium Fish Foods containing vitamin D	Foods containing iron Cheese Foods containing animal fats Foods containing sugars

Red and Processed Meat

A high intake of red and processed meats is a strong risk factor for colorectal cancer as concluded by the WCRF/AICR panel (148). This association may be due to several factors such as high content of fat and iron and/or meat preparation methods (154). In the human body, nitrites from processed and red meats can react with the degradation products of amino acids to form N-nitroso compounds (NOCs), which are known human carcinogens (148, 154). NOCs may also form in meat during the curing processes. Furthermore, meats cooked at high temperatures contain other potential carcinogens such as heterocyclic amines (HCAs) and polycyclic aromatic hydrocarbons (PAHs) (154, 155). Despite strong biologic plausibility, the exact association between meat consumption (or, specifically, the role of meat type, cooking methods, doneness levels) and colorectal cancer is not clear, mostly due to potential confounding by other dietary factors. Fat and meat consumption are highly correlated with total energy intake. Moreover, persons who consume high red and processed meat diets are more likely to eat less poultry, fish, and vegetables. Therefore, it is difficult to isolate the effects of meat or its compounds on colorectal cancer risk.

Meta-analyses of cohort studies found a 43% increase in CRC per each additional serving/week of red meat (RR = 1.43, 95% CI: 1.05–1.94), a 23% increase in CRC risk per 100 g/day of red meat (RR = 1.29, 95% CI: 1.04–1.60), and a 21% increase in CRC risk (RR = 1.21, 95% CI: 1.04–1.42) per 50 g/day of processed meat, with an apparent dose-response association for both types of meats (148). Another meta-analysis of 15 prospective studies reported a RR of 1.28 (95% CI: 1.18–1.39) for each 120 g/day increase of red meat, and a RR of 1.09 (95% CI: 1.05–1.13) for each 30 g/day increase of processed meat (156). However, a reduction of meat consumption in a randomized trial did not change the recurrence of colorectal adenomas (157).

Total Dietary Fat, Saturated/Animal Fat, Fatty Acids

In contrast to the international ecologic studies, epidemiologic observational studies have failed to find a consistent association between total dietary fat and colorectal cancer risk (46, 148). A meta-analysis of 7 cohort studies found no association between colorectal cancer risk and total fat intake (RR= 1.0, 95% CI: 1.00–1.00) (148). Another meta-analysis of 13 case-control studies also found no association between dietary fat intake and risk of colorectal cancer after adjustment for total energy intake (158). These analyses suggest that there is no-energy independent association between dietary fat intake and colorectal cancer. In addition, it is difficult to disentangle the contribution of specific nutrients in the diet. Finally, dietary fat is the largest source of energy. It contributes to a high energy intake and obesity. Therefore, dietary fat may appear to be associated with colorectal cancer, when in may be that increased energy intake, regardless of its source, and obesity are related to colorectal cancer risk (159).

The association of animal/saturated fats with colorectal cancer risk is very similar to that of total fat, possibly because they also contribute to obesity and high energy consumption (159). One of the proposed hypotheses is that high animal fat intake leads to an increased proportion of anaerobes in the gut microflora, resulting in higher rates of conversion of bile acids into carcinogens (54). Also, diets rich in animal (but not vegetable) fats are associated with high serum cholesterol, which is also associated with increased risk for colorectal neoplasms (54). A meta-analysis of three cohort studies found a statistically non-significant RR of 1.13 (95% CI: 0.92–1.38) per 20 g/day of animal fat (148). Another meta-analysis of 13 case-control studies also found no statistically significant association between colorectal cancer risk and intake of saturated fat (158).

It was also hypothesized that n-3 polyunsaturated fatty acids (PUFA) in fish protect against colorectal cancer. Plausible protective effects of fish oils are thought to be related to direct inhibition of COX-2 activity and reduced n-6 PUFA-derived eicosanoid biosynthesis resulting in decreased inflammation (148). Alternative effects include relatively the high selenium and vitamin D content of fish (148). Despite the biologic plausibility, epidemiologic studies investigating associations of fish/seafood or n-3 PUFA with colorectal neoplasms produced mixed results. A meta-analysis of seven cohort studies found a small, marginally statistically significant decrease in colorectal cancer risk (RR = 0.96, 95% CI: 0.92–1.00 per serving of fish per week) (148). A systematic review of cohort studies reported no significant association between omega-3 fatty acids and colorectal cancer risk and no evidence to support any benefit from high fish intake (160). However, the large EPIC (European Prospective Investigation into Cancer and Nutrition) study found a statistically significant 31% decrease in colorectal cancer risk in individuals who consume 80 g/day of fish versus 10 g/day (HR = 0.69, 95% CI: 0.54–0.88) (161). Furthermore, several observational studies that examined the association between biomarkers of PUFA intake in body fat or blood (as opposed to the FFQ-based measured) and colorectal neoplasms found more consistent evidence of inverse association (162-166).

Vegetables, Fruits and Fiber

The substantial epidemiologic evidence on the inverse association between consumption of fruit and vegetables and colorectal cancer risk was not consistent despite strong biologic plausibility. Vegetables and fruits contain numerous substances with potential antitumorigenic effects, including carotenoids, flavonoids, folate, vitamins C and E, selenium, isothiocyanates, indoles, coumarins and other bioactive compounds (3, 148, 167). Potential mechanisms for cancer prevention include binding carcinogens, inhibiting nitrosamine formation, modulating hormone levels, inducting phase II metabolizing enzymes, protecting against oxidation damage, suppressing DNA adduct formation, and effects on DNA methylation (3, 148). Results from several large meta-analyses of cohort studies provided limited evidence suggesting that non-starchy vegetables and fruits protect against colorectal cancer (148). In addition, an intervention of low-fat, high-fiber, increased fruit and vegetables diet found no effect on recurrence of colorectal adenomas over an average of four years (168). These results suggest that the inverse association of colorectal cancer with vegetable and fruit intake may be limited to specific food components, may be non-linear, or a part of a more complex dietary pattern (46).

Association of dietary fiber with risk of colorectal neoplasms is also inconsistent despite the biologic plausibility and a substantial body of epidemiologic literature. The proposed mechanisms of anticarcinogenic action in the gastrointestinal tract are dilution of fecal content, decrease in transit time, and increase of stool weight (148). Moreover, a wide range of dietary carbohydrates and mucins are metabolized by the gut flora into various fermentation products, including butyrate (148). Butyrate and other short-chain fatty acids were shown to suppress cell proliferation, increase differentiation, stimulate apoptosis, and influence the secretion of chemokines in animal and *in vitro* studies (148, 169-172). A large meta-analysis of 20 cohort studies found a 10% decrease in risk per 10 g of dietary fiber per day (RR = 0.90, 95% CI: 0.84–0.97) with an apparent dose-response association (148). Two meta-analyses of case-control studies also found a statistically significant 21–47% reduction in colorectal cancer risk with increasing dietary fiber intake (173, 174). However, a pooled analysis of 13 prospective cohort studies (8,081 colorectal cancer cases and 730,000 participants) found a statistically non-significant 6% decreased risk for those with the highest intake of dietary fiber after adjusting for other risk factors (RR = 0.94, 95% CI: 0.86–1.03) (175). The results of epidemiologic studies of a dietary fiber – CRC association are inconsistent, probably because of the heterogeneous nature of fiber itself, issues with measurement of fiber intake (46), and the presence of other compounds in fiber rich foods. Intervention studies are much less consistent with the hypothesis that dietary fiber reduces colorectal cancer risk. Three randomized clinical trials that tested the effect of high-fiber diets did not show the reduction in colorectal adenoma recurrence (168, 176, 177). However, the results of the randomized trials should be interpreted with caution, as the intervention was relatively short term (3–5 years) and was done in the patients who already had neoplastic changes such as adenoma in their colons.

Folate

Folate has been proposed to explain the possible inverse association of colorectal cancer risk with vegetables intake (167, 178). Folate is a water-soluble B vitamin that is obtainable only from diet and is essential for normal DNA repair, synthesis, and methylation. Abnormal DNA methylation leads to aberrant gene expression and is associated with cancers at several sites (148). Animal studies have provided considerable support for a causal relationship between folate depletion and colorectal carcinogenesis, for a dose-dependent protective effect, and for the importance of dose and timing of folate supplementation (179). Epidemiologic studies have also linked lower folate intake to higher risk of colorectal cancer. A meta-analysis of four cohort studies reported a 16% reduction in colorectal cancer risk (RR = 0.84, 95% CI: 0.76–0.93) per 100 µg of dietary folate per day (148). Another meta-analysis of 7 cohort studies found a statistically significant 25% reduction in colorectal cancer risk in those with the highest dietary (but not total) folate intake compared to those with the lowest intake (RR = 0.75, 95% CI: 0.64–0.89) (180). Two studies investigated serum folate levels in relation to colorectal cancer risk. Both studies of serum folate found a decreased risk for those with the highest folate level compared to those with the lowest (181, 182), but only in one study was this decrease statistically significant (OR = 0.52, 95% CI: 0.27–0.97) (181). Recently, a randomized clinical trial of folic acid supplementation found no reduction in colorectal adenoma recurrence, but statistically significant increases in the occurrence of multiple adenomas (RR = 2.32, 95% CI: 1.23–4.35) and large adenomas (RR = 1.67, 95% CI: 1.00–2.80) (183). Similar findings were seen in animal studies, where exceptionally high folic acid doses as well as a folate intervention after the establishment of microscopic neoplastic foci in the colon promoted, rather than suppressed, colon carcinogenesis (179). Recently, it was proposed that folate may play a dual role in carcinogenesis: it may act as a preventive agent during the early stages of carcinogenesis in individuals with a low folate status, and it may promote carcinogenesis during the later stages of tumorigenesis, especially if administered at very high doses (184, 185). Also, the form of folate (natural folate in food vs. synthetic folic acid in supplements) may play an important role in cancer prevention.

Tobacco and Alcohol

Cigarette and pipe smoking, especially long-term and with early onset, is linked to the development of colorectal neoplasms (186-190) . Some data indicate that tobacco smoking is more strongly associated with microsatellite unstable colorectal tumors and that approximately 21% of MSI may be attributable to cigarette smoking (191). Also, in one case-only analysis, long-term smoking was positively statistically significantly associated with lack of MLH1 expressions in colorectal tumors (192). These studies indicated that smoking may substantially contribute to MSI in colorectal tumors. There are several proposed mechanisms for an association between tobacco smoking and colorectal neoplasms. Smoking may affect methylation of the MLH1 promoter region resulting in decreased or absent MLH1 expression and deficient DNA repair (193). Moreover, tobacco smoke contains many carcinogens, including polycyclic hydrocarbons, nitrosamines, heterocyclic amines, and other blood-borne carcinogens (46), that may cause DNA mutations (e.g., in the *APC* gene (194)). When DNA repair mechanisms are altered, colonocytes may become more susceptible to mutations that may lead to neoplastic changes (192).

The epidemiologic evidence on association of alcohol consumption with colorectal cancer was not consistent. Although the majority of the observational epidemiologic studies demonstrated positive association between alcohol consumption and colorectal neoplasms, most of them yielded statistically nonsignificant results (148). Meta-analysis of six cohort studies yielded a summary effect estimate of 1.01 (95% CI: 0.95–1.08) per drink per day (148). Another meta-analysis of nine cohort studies showed a statistically significant 9% (RR = 1.09, 95% CI: 1.03–1.14) increase in CRC risk per 10 gram of alcohol (as ethanol) per day (148). Moreover, in a stratified meta-analysis, there were no statistically significant difference with cancer site; however, there was indication of a larger effect in men (RR = 1.09, 95% CI: 1.02–1.14 per 10 g/day) than in women (RR = 1.00, 95% CI: 0.89–1.40 per 10 g/day) (148). In a large pooled analysis of 8 cohort studies with more than 4,600 CRC cases and 475,000 participants, followed up for 6 to 16 years, the group that had the highest alcohol consumption (≥45 g/day) showed a 41% increased CRC risk (RR = 1.41, 95% CI: 1.16–1.72) (195). Interestingly, in this pooled study (195) no significant heterogeneity by sex was found. In human body, alcohol (ethanol) is metabolized into acetaldehyde that can be carcinogenic as it forms DNA adducts (148). Alcohol may also inhibit DNA repair and function as a solvent to other carcinogenic molecules enhancing their penetrations into the colonocytes (46, 148). Additionally, high consumption of alcohol may be associated with consumptions of diets low in essential nutrients, particularly folate (46). Lastly, alcohol consumption may interact with tobacco smoking (196-198).

Calcium and Colorectal Adenomas

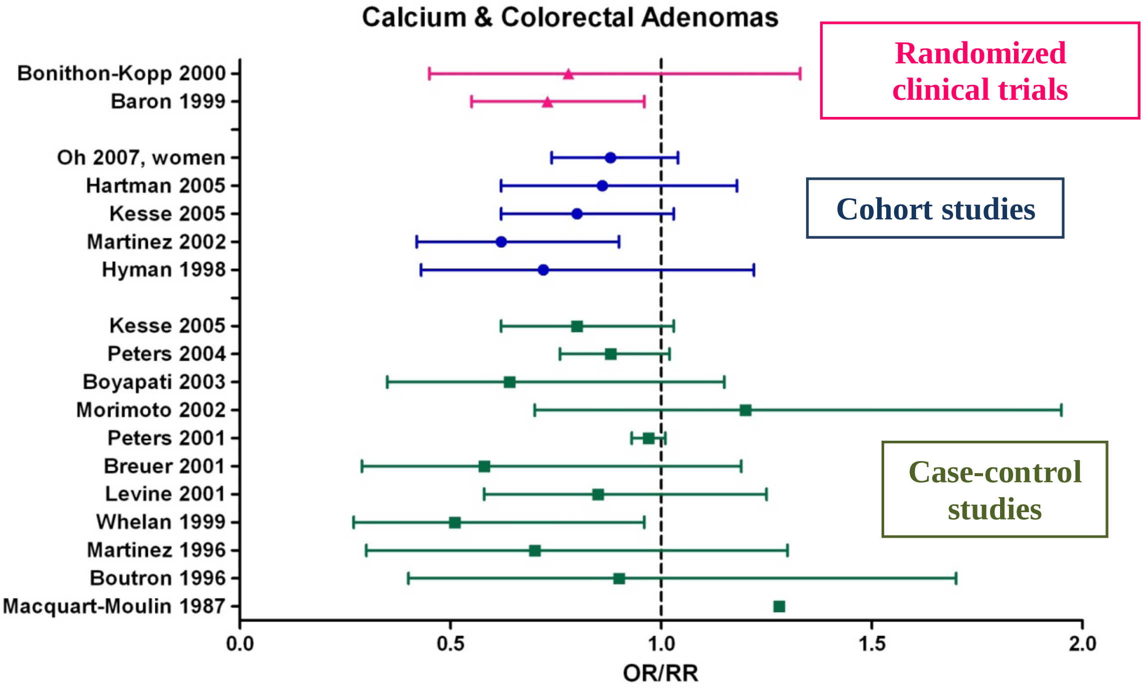
Calcium is an element that is essential for living organisms. It has a variety of functions in the body, including its “classical” functions in intracellular signaling as a second messenger (*e.g.*, muscle contraction, vesicle secretion, fertilization, signal transduction), and bone structure; and its “non-classical” functions in modulating cell proliferation and differentiation. Blood calcium levels are tightly regulated within a narrow physiological range. Most of the calcium (over 99% of the 1–2 kg) is deposited normally in bones, which act as a reservoir of calcium for use throughout the body. Calcium homeostasis is controlled by three hormones: vitamin D, parathyroid hormone

(PTH), and calcitonin (199). All calcium in the human body, whether in blood, in interstitial spaces or within the cell cytosol or organelles, originates from the diet and enters the body only through the intestines (7, 199). On average, only about 30% of ingested calcium get absorbed in the intestine through active (transcellular) and passive (paracellular) mechanisms (199, 200). Non-absorbed calcium binds with free bile and fatty acids or as free calcium and is expelled in the feces. Dietary calcium intake in the United States varies widely, ranging from 400–1500 mg/d (199). The estimated average intake of calcium in Western diets is, on average, 500–700 mg/day, which is quite low by evolutionary standards (200). The optimal intake (calculated based on the calcium intake of all mammalian species other than modern man) that corresponds to the estimated intake of Paleolithic man is 1,500–2,000 mg/day (200).

There is a large amount of evidence supporting protective effects of calcium against colorectal neoplasms; however, its exact anticarcinogenic effects are not clear. Proposed mechanisms of calcium against colorectal cancer include protection of colonocytes against bile acids and fatty acids (5, 6), direct effects on cell cycle regulation (7), promotion of colonocyte differentiation (201, 202), and modulation of E-cadherin and β-catenin expression via the calcium-sensing receptor (CaSR) (7-9). Further, there is some evidence that extracellular calcium activates protein kinase C, which is associated with the differential induction of p21 in the intestinal epithelium (7), and that an intracellular calcium gradient along the colon crypt that coincides with the differentiation compartment may also modulate differentiation of the colonocytes (203). Calcium may also act as an oxidative stress and DNA damage reducing agent in the colon. In the colon lumen, bile acids damage cell membranes, at least in part through an oxidative mechanism (204, 205), provoking an inflammatory response and causing DNA damage (206), and calcium can bind the free bile acids rendering them inert (6). Further investigations are needed to understand the role of calcium in colon carcinogenesis.

The epidemiologic evidence supports the hypothesis that higher intakes of calcium reduce risk for colorectal adenomas. Of 15 observational epidemiologic studies of calcium and colorectal adenoma (two cohort studies (24, 207), seven case-control studies (22, 40, 208-212), four case-control/cohort studies nested in randomized clinical trials (23, 38, 39, 213), two cross-sectional studies (198, 214)), 13 (93%) reported inverse associations of which only three were statistically significant (39, 40, 198), and two (211, 212) found direct associations which were not statistically significant (Figure 1.5).

Figure 1.5. Epidemiologic studies of calcium and colorectal adenoma.



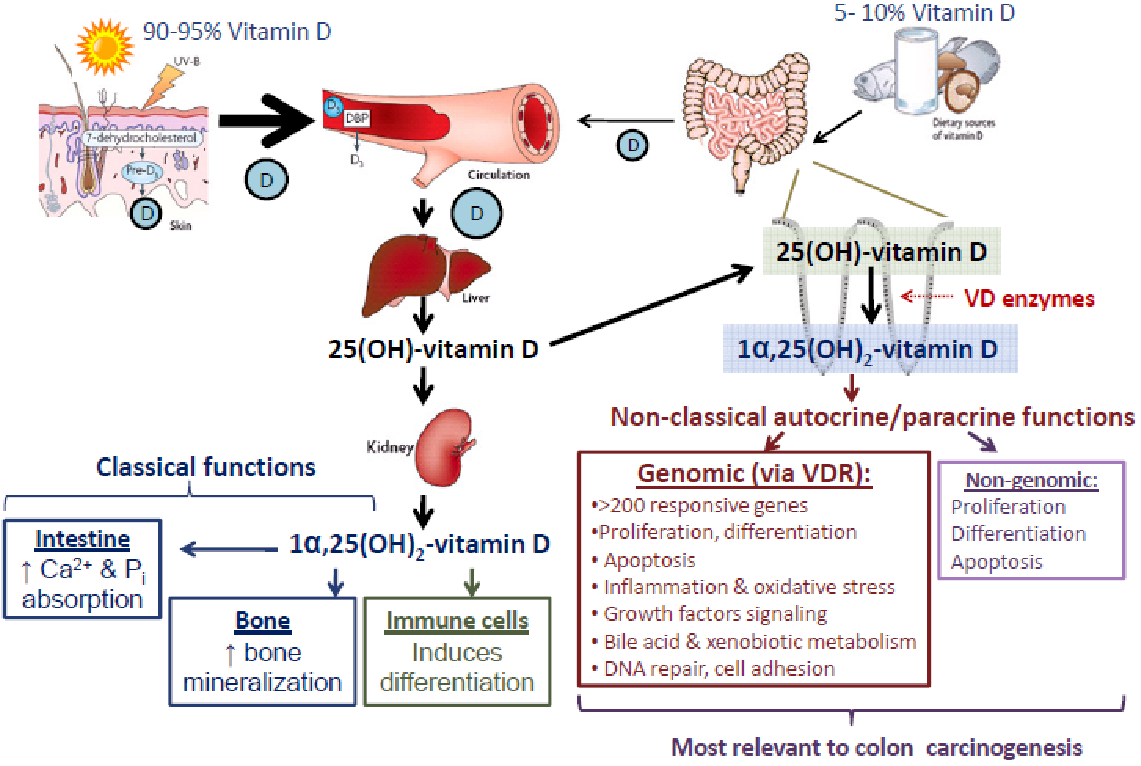
There were two randomized controlled trials (215, 216) of the effects of dietary calcium on the development of adenomas, and one trial (217) of dietary calcium and antioxidants. A systematic review of the first two randomized clinical trials (41) found a statistically significant 26% reduction in colorectal adenoma recurrence with calcium supplementation (RR = 0.74, 95% CI: 0.58–0.95). A similar reduction in colorectal adenoma recurrence was observed when all three studies were combined (RR = 0.80, 95% CI: 0.68–0.93) (218).

In summary, inverse associations have been consistently observed in calcium and colorectal adenoma observational studies, and several clinical trials found reduced colorectal adenoma recurrence with calcium supplementation. The calcium-adenoma association appears to be nearing causal status, but requires some additional large clinical trials and mechanistic confirmation.

Vitamin D and Colorectal Adenomas

Vitamin D is a group of fat-soluble pro-hormones that includes two major forms: vitamin D_2 and vitamin D_3. The active form of vitamin D, 1α,25-$(OH)_2$-vitamin D is formed during a multistep process (Figure 1.6). Persons acquire vitamin D from two sources – cutaneous synthesis after exposures to UVB and diet (219). It is estimated that the general Western European population gets most of its vitamin D (80-90%) from cutaneous production in response to sunlight exposure, and only 5-10% from intestinal absorption from dietary sources. After synthesis in the skin from 7-dehydrocholesterol or from intestinal absorption, vitamin D is transported through the circulation to the liver, where it undergoes hydroxylation to 25-(OH)-vitamin D at the 25 position by 25-hydroxylases. Production of 25-(OH)-vitamin D in the liver is not regulated and is limited only by vitamin D substrate availability. Most 25-hydroxylase activity in the liver was detected in the microsomal fraction; however, the enzyme itself was not identified. It was hypothesized that mitochondrial CYP27A1 can participate in 25-hydroxylation of vitamin D, however, its major activity is cholesterol-27-hydroxylation in the bile-acid pathway and contribution to the circulating 25-vitamin D pool is minor (220). Other enzymes with 25-hydroxylase activity include CYP2D6, CYP2R1, CYP2C11, CYP3A4, CYP2D25, and CYP2J3, and other unknown microsomal enzymes (221). 25-hydroxylase activity was also detected in other tissues, such as skin, kidney, and intestine (220). Factors influencing activity of 25-hydroxylase are unknown.

Figure 1.6. Metabolism and functions of vitamin D (adopted from Lamrecht *et al*. and Deeb *et al*. (7, 222)).



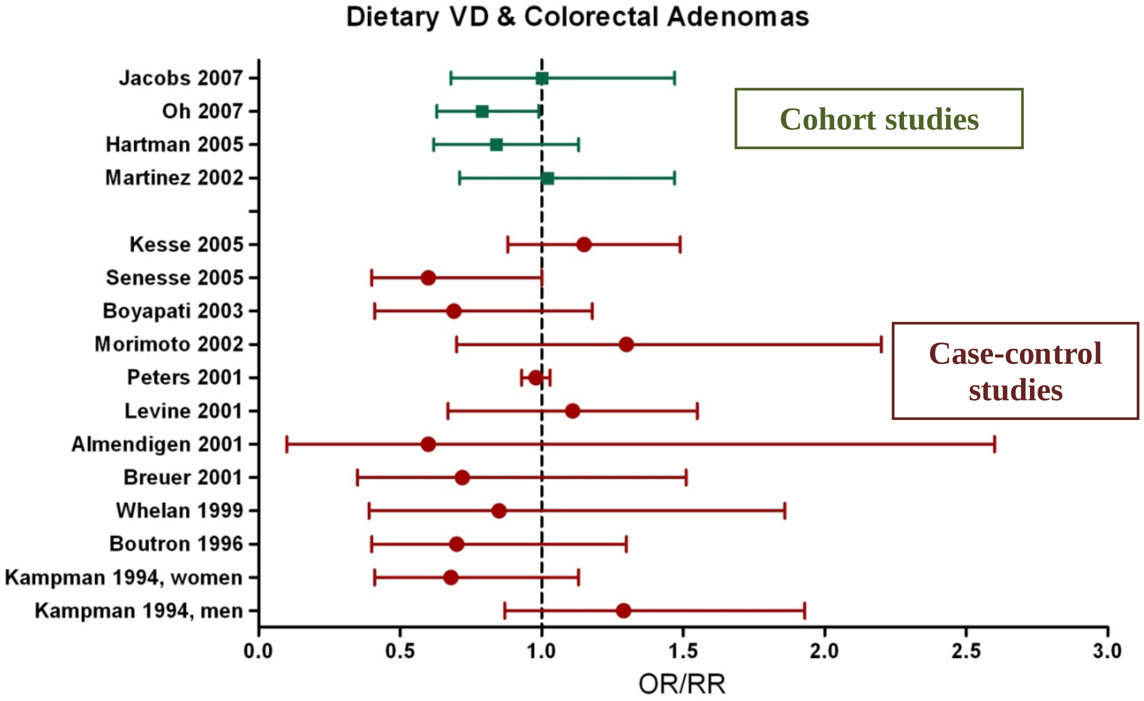
The resultant 25-(OH)-vitamin D is then absorbed into the blood, where most of it circulates bound to DBP (vitamin D binding protein). The liver synthesizes DBP, which circulates at a much higher concentration in the blood than vitamin D metabolites (219). Because of 25-(OH)-vitamin D's relatively long half-life of about 20 days, it serves as a reservoir of vitamin D for further hydroxylation in various target tissues including classically the kidney, and since the synthesis of 25-(OH)-vitamin D is not under tight homeostatic regulation, its concentration is useful as a biomarker of vitamin D exposure that reflects vitamin D from combined dietary and sunlight sources over a relatively long period of time (219). Circulating 25-(OH)-vitamin D concentrations are dependent on several factors, including: 1) the amount of vitamin D synthesized in the skin or absorbed from the intestine; 2) DBP concentration and the activity of 25-hydroxylases in the liver; 3) the volume of the extracellular compartment; 4) the amount of body fat and muscle; and 5) the efficiency of cellular uptake of 25-(OH)-vitamin D and its rate of conversion (219). Therefore, the analysis of circulating 25-(OH)-vitamin D levels should take into account several factors, such as body adiposity (BMI or WHR), season of blood draw, age, disease, increased physiologic need, *etc*. In various tissues (skin, kidney, intestine, bone, and others) 25-(OH)-vitamin D undergoes a second hydroxylation catalyzed by enzyme CYP27B1 (1-hydroxylase, P450C1) yielding an active secosteroid, 1α,25-$(OH)_2$-vitamin D. Expression of the kidney CYP27B1 enzyme is tightly regulated (220), and the half-life of 1α,25-$(OH)_2$-vitamin D in the circulation is very short, approximately 4 to 6 hours (219). Synthesized in kidneys and released into the circulation, 1α,25-$(OH)_2$-vitamin D participates in calcium and bone homeostasis (its classical endocrine functions). The synthesis of 1α,25-$(OH)_2$-vitamin D in the kidneys is stimulated by parathyroid hormone (PTH) and suppressed by Ca^{2+}, P_i and 1α,25-$(OH)_2$-vitamin D itself. Also, fibroblast growth factor 23 (FGF23) downregulates the synthesis of renal CYP27B1 resulting in lower production of 1α,25-$(OH)_2$-vitamin D in the kidneys (223). Due to its short half-life and tight regulation, level of 1α,25-$(OH)_2$-vitamin D in blood is not a good biomarker of vitamin D status. Produced in other tissues, such as colon crypts (the basic structures of the colon), 1α,25-$(OH)_2$-vitamin D exerts its other non-classical autocrine/paracrine functions locally (including modulation of cell proliferation, differentiation, and apoptosis) and generally does not reach the circulation (219). The degradation of 25-(OH)-vitamin D and 1α,25-$(OH)_2$-vitamin D to 24,25-(OH)-vitamin D and 1α,24,25-$(OH)_2$-vitamin D, respectively, occurs through 24-hydroxylation by the CYP24A1 enzyme (24-OHase, 24-hydroxylase, P450C24). CYP24A1 is expressed in various tissues, including the intestine (220). The major inducer of CYP24A1 expression is 1α,25-$(OH)_2$-vitamin D itself (220), and low calcium can also decrease the *CYP24* gene transcription in the colon (224).

The hypothesis that vitamin D plays a role in preventing cancer was first initiated by the observation in the 1930s of an inverse correlation between cancer risk and sunlight exposure. Ten years later, an association between cancer mortality and latitude was demonstrated for the first time. However, it was not until 1980 that Garland proposed the hypothesis that vitamin D status accounted for an inverse association between solar ultraviolet-B exposure and risk of colon cancer (225). At that time, the antineoplastic effects of vitamin D were unknown, and for another nearly 30 years a considerable but evolving literature on the biologic basis of this hypothesis and on a vitamin D – colorectal neoplasms association was published. This literature includes mechanistic, experimental (animal and *in vitro*) and epidemiologic studies of associations of vitamin D intake (dietary, supplemental, and total) or circulating vitamin D (plasma or serum 25-(OH)-vitamin D_2 and D_3) with risk for colorectal neoplasms.

Beyond calcium homeostasis, for its non-classical functions, vitamin D regulates proliferation, differentiation, and apoptosis; promotes bile acid degradation and xenobiotic metabolism; and influences growth factor signaling, cell adhesion, DNA repair, angiogenesis, inflammation, and immune function (reviewed in (10-13)). Moreover, vitamin D modulates more than 200 responsive genes (reviewed in (7, 14, 15, 226)). Furthermore, vitamin D may act as an oxidative stress and DNA damage reducing agent in the colon. Vitamin D activation of the ubiquitous vitamin D receptor (VDR) in the colon up-regulates CYP3A4, which in turn catabolizes the secondary bile acid, lithocholic acid (11, 227). In colonocytes, vitamin D increases expression of enzymes involved in antioxidant responses (228-230), thereby decreasing oxidative stress in the colorectal epithelium.

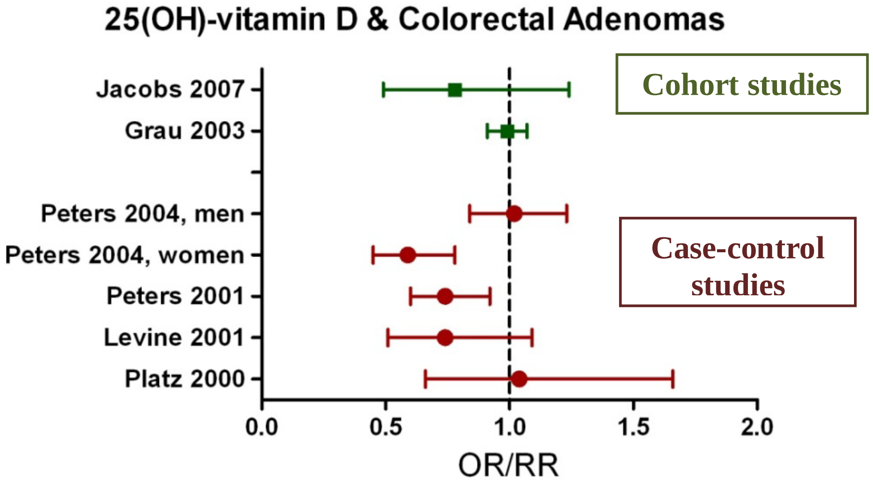
Twenty one case-control, cohort, and cross-sectional studies examined the association between vitamin D intake or circulating vitamin D and colorectal adenoma. Of at least 15 epidemiologic studies (Figure 1.7) (22-24, 39, 40, 207-211, 231-235) that investigated the possible association of vitamin D intake and colorectal adenoma, 12 suggested an inverse association either overall or in a subgroup of participants (22-24, 39, 40, 208-210, 231-234), one reported a null association (235), and two reported a positive statistically non-significant association (207, 211). In studies that considered a range of adenoma outcomes, vitamin D intake was also inversely associated with small adenomas (232), large adenomas (24), recurrent adenomas (23, 39, 40, 235), and advanced adenomas (24, 40, 198, 207).

Figure 1.7. Epidemiologic studies of vitamin D intake and colorectal adenomas.



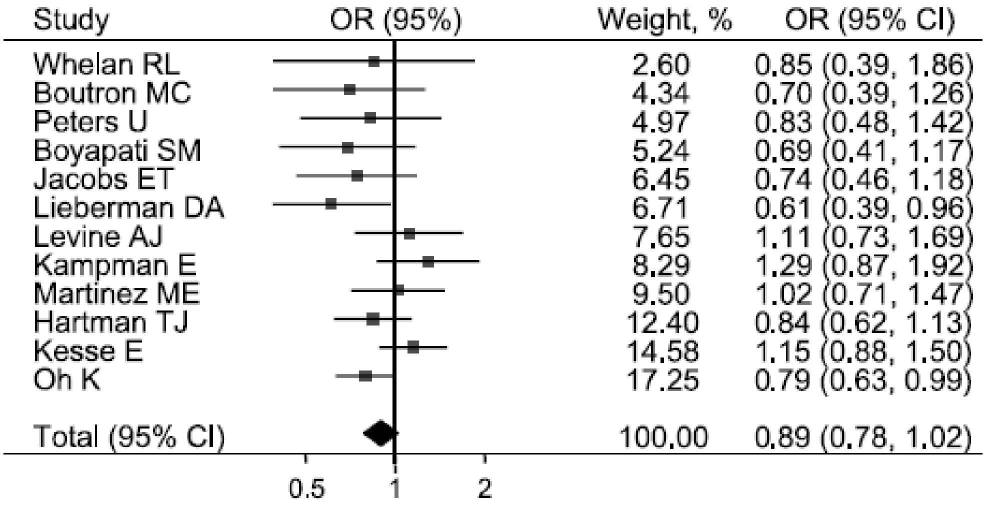
Seven studies (Figure 1.8) that examined the association between circulating 25-(OH)-vitamin D and colorectal adenomas included three case-control studies (21, 22, 210), one nested case-control study (236), two cohort studies based on the data from randomized clinical trials (235, 237), and one cross-sectional study (214). Six studies included in their analysis the season of blood draw, either by matching by date of blood draw (236) or by including month of blood draw in the model (21, 22, 214, 235, 237). All studies, except one (236), found inverse associations of 25-(OH)-vitamin D with colorectal adenomas. Four studies (21, 22, 214, 237) reported statistically significant results overall (22, 214), in women (21), or in patients randomized to receive calcium supplementation (237). Furthermore, one study reported a statistically significant 42% reduction in advanced adenomas among individuals with high 25-(OH)-vitamin D levels and randomized to receive calcium supplementation (237).

Figure 1.8. Epidemiologic studies of circulating 25-(OH)-vitamin D and colorectal adenomas.



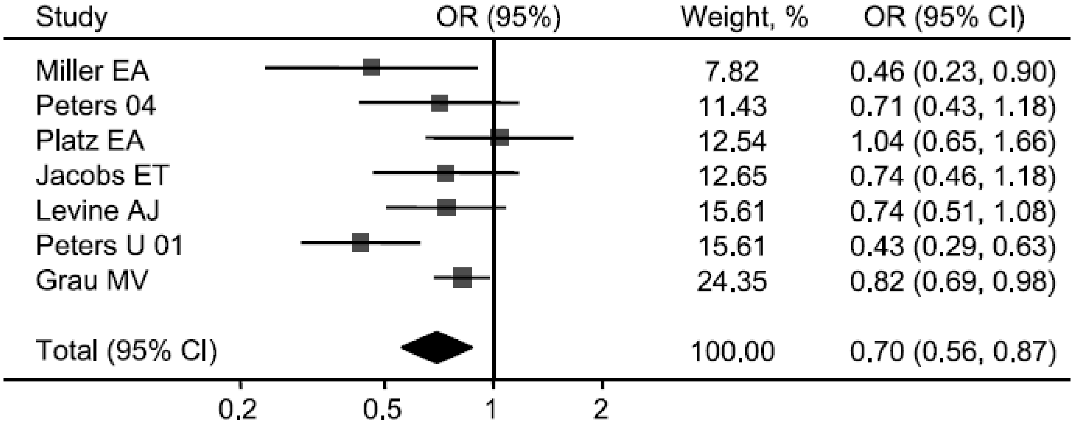
A recent meta-analysis of the overall relationship between circulating 25-(OH)-vitamin D or vitamin D intake and colorectal adenoma found that both blood 25-(OH)-vitamin D and vitamin D intake were inversely associated with colorectal adenoma incidence and recurrent adenomas (Figures 1.9 and 1.10 from ref. (238)). However, high compared to low vitamin D intake was associated with an 11% statistically non-significant decreased risk of colorectal adenomas; whereas, high versus low circulating 25-(OH)-vitamin D was associated with a statistically significant 30% decreased risk (Figures 1.10 and 1.11 from ref. (238)). Furthermore, individuals with high versus low vitamin D intake were less likely to develop a recurrent adenoma (OR = 0.88, 95% CI: 0.72–1.07) (238). In this meta-analysis, the inverse association of vitamin D intake with recurrent adenomas was not statistically significant (OR = 0.88, 95% CI: 0.72–1.07), whereas for advanced adenomas it was (OR = 0.77, 95% CI: 0.63–0.95). Consistent with the data for vitamin D intake, high 25-(OH)-vitamin D was associated with a 36% decreased risk of advanced adenomas (OR = 0.64, 95% CI: 0.45–0.90) (238). Finally, a subgroup analysis by sex suggested that there was a more consistent inverse association among women (238).

Figure 1.9. Vitamin D intake (dietary, supplemental, or total) and risk of colorectal adenoma for the highest compared to the lowest quintile of vitamin D intake (from Wei *et al*. (238)).



Study	Weight, %	OR (95% CI)
Whelan RL	2.60	0.85 (0.39, 1.86)
Boutron MC	4.34	0.70 (0.39, 1.26)
Peters U	4.97	0.83 (0.48, 1.42)
Boyapati SM	5.24	0.69 (0.41, 1.17)
Jacobs ET	6.45	0.74 (0.46, 1.18)
Lieberman DA	6.71	0.61 (0.39, 0.96)
Levine AJ	7.65	1.11 (0.73, 1.69)
Kampman E	8.29	1.29 (0.87, 1.92)
Martinez ME	9.50	1.02 (0.71, 1.47)
Hartman TJ	12.40	0.84 (0.62, 1.13)
Kesse E	14.58	1.15 (0.88, 1.50)
Oh K	17.25	0.79 (0.63, 0.99)
Total (95% CI)	100.00	0.89 (0.78, 1.02)

Figure 1.10. Circulating 25-(OH)-vitamin D and risk of colorectal adenoma for the highest compared to the lowest quintile of 25-(OH)-vitamin D (from Wei *et al*. (238)).



Study	Weight, %	OR (95% CI)
Miller EA	7.82	0.46 (0.23, 0.90)
Peters 04	11.43	0.71 (0.43, 1.18)
Platz EA	12.54	1.04 (0.65, 1.66)
Jacobs ET	12.65	0.74 (0.46, 1.18)
Levine AJ	15.61	0.74 (0.51, 1.08)
Peters U 01	15.61	0.43 (0.29, 0.63)
Grau MV	24.35	0.82 (0.69, 0.98)
Total (95% CI)	100.00	0.70 (0.56, 0.87)

Inadequate blood vitamin D levels are common in the U.S. and in many populations worldwide (239). From the evolutionary perspective, Paleolithic humans were primarily outdoor hunter-gatherers exposed to a lot of sunlight (200). Studies of lifeguards and dark skinned persons from sub-Saharan regions who spent most of their times outdoors showed that 25-(OH)-vitamin D blood levels in these groups are greater than 60 ng/mL (240). Based on several lines of evidence, vitamin D insufficiency was defined as having blood vitamin D levels less than 33 ng/ml, and deficiency as < 20 ng/mL (Table 1.3). There is no clear definition of "normal" or adequate or sufficient levels with respect to blood 25-(OH)-vitamin D (240) ; and the optimal vitamin D dose or blood levels for anti-neoplastic effects in humans is not known. However, one pooled study suggested that a serum 25-(OH)-vitamin D level of 33 ng/mL or higher relative to <12 ng/mL is associated with a 50% decrease in colorectal cancer incidence (241).

Table 1.3. Health implications of various levels of blood vitamin D, and their approximate, average associated vitamin D exposures (from Grant *et al.* (242)).

	25-OH-D blood levels		**Vitamin D exposures**
	ng/ml	nmol/L	IU/day
Suggested guidelines			
Deficiency	< 20	< 50	< 600
Insufficiency	20 - 32	50 - 79	600 - 999
Sufficiency	33 - 100	80 - 250	1,000 - 4,000
Suprafficiency	101 - 150	251 - 375	4,000 - 10,000
Toxicity	> 150	> 375	> 10,000
Perspectives			
Normal in sunny countries	54 - 90	135 - 225	2,000 - 4,000
Estimated to ↓ CRC risk by 50%	33	82	1,000 - 2,000

In summary, based on biological and epidemiologic evidence, vitamin D is a promising dietary chemopreventive agent that was found in some epidemiologic studies to be associated with lower risk for colorectal cancer (16-20) and adenoma (21-24). In studies that investigated dietary vitamin D intake without considering exposure to UVB light, the association between vitamin D intake and colorectal adenoma/cancer was not consistent. This inconsistency between these studies can be explained by misclassification of actual vitamin D exposure, which leads to an underestimation of the association. In those few studies that assessed the main form of circulating vitamin D, 25-(OH)-vitamin D (collective term for 25-(OH)-vitamin D_2 and D_3), an inverse association was observed between 25-(OH)-vitamin D levels and colorectal cancer (25-27) or adenomas (21, 22). The results of these studies suggest that circulating vitamin D level is a better marker of vitamin D exposure than indirect estimates of vitamin D exposure based solely on a diet due to its long half-life in the circulation and lack of tight homeostatic regulation of its concentration. 25-(OH)-vitamin D reflects vitamin D supply and usage over a period of time (reviewed in ref. (219)). However, the use of circulating 25-(OH)-vitamin D levels as vitamin D exposure has its own complications due to seasonal variations in vitamin D levels and assay sensitivity/variability that should be kept in mind during data analysis.

Calcium, Vitamin D and Colorectal Adenomas

Evidence exists that calcium and vitamin D are closely inter-related beyond their ‘classical’ (*e.g.*, calcium homeostasis) functions: in animal models the expression of the VDR is dose-dependent on the calcium concentration; extracellular calcium modulates renal CYP27B1 expression through a PTH-independent mechanism, likely through the CaSR (220); low dietary calcium inhibits colonic CYP24 mRNA expression and does not affect the expression of colonic CYP27B1 or VDR mRNA (224); and colon tissue expresses VDR and CaSR, as well as CYP27B1 and CYP24, *etc*. Furthermore, most animal studies that investigated the combination of calcium and vitamin D reported that supplemental vitamin D has stronger anti-neoplastic effects in animals given relatively high-calcium diets (243-245). However, at least one 2x2 factorial experiment of calcium and vitamin D in rodents found substantial treatment effects on proliferation markers for the individual agents but not for the combination (246). Overall, these findings indicate potential synergistic anti-neoplastic effects of calcium and vitamin D that are worth further examination in animal and human studies.

Although there have been numerous epidemiologic studies of calcium and vitamin D and risk for colorectal adenomas, very few addressed the combined effects of these agents. The modifying effect of calcium on the vitamin D–colorectal adenoma association was reported on in only six studies that either examined calcium supplementation in a randomized clinical trial (237) or stratified by calcium status in case-control or cohort studies (22, 24, 208, 210, 214). In the study by Grau *et al*. (237), an inverse association between 25-(OH)-vitamin D and recurrent adenoma (RR = 0.88, 95% CI: 0.77–0.99 per 12 ng/mL increase of 25-(OH)-vitamin D) was observed only among patients receiving calcium supplementation, but not in the placebo group ($P_{\text{interaction}}$ =0.006). In the four observational studies (22, 210, 214), participants were stratified by calcium intake (total, dietary, or supplemental). In the Miller *et al.* study, high 25-(OH)-vitamin D among subjects with high dietary calcium intake was associated with a 68% lower prevalence of adenomas, whereas there was no association for high 25-(OH)-vitamin D levels among those with low calcium intakes ($P_{\text{interaction}}$ = 0.30) (214). Peters *et al*. also found a greater decreased risk of adenomas for each 10 ng/mL increase in 25-(OH)-vitamin D among subjects with high calcium intakes (OR = 0.56, 95% CI: 0.37–0.85, $P_{\text{interaction}}$ = 0.13) (22). Furthermore, in the Oh *et al.* study, participants who had both the highest total calcium and vitamin D intakes had the lowest adenoma risk when compared with the opposite extreme (RR = 0.75, 95% CI: 0.62–0.92, $P_{\text{interaction}}$ = 0.27) (24). Contrary to these results, Levine *et al.* found that subjects with high 25-(OH)-

vitamin D but low calcium intake had a greater decreased risk of colorectal adenomas (OR = 0.40, 95% CI: 0.22–0.71) compared to those with high calcium intake (OR = 1.17, 95% CI: 0. 69–1.99) (210). Furthermore, one case-control study by Boyapati *et al*. found no evidence that the association between calcium intake and colorectal adenoma differed according to levels of total vitamin D intake (208). In a meta-analysis (238), compiling four 25-(OH)-vitamin D studies (22, 210, 214, 237) and stratifying by calcium intake yielded an inverse vitamin D – adenoma association for both high (OR = 0.67, 95% CI: 0.46–0.97) and low calcium intakes (OR = 0.78, 95% CI: 0.54–1.12), with a stronger association among those with high calcium intakes (238).

In summary, a large number of studies investigated associations or treatment effects of vitamin D and calcium separately from each other, and the few studies that addressed the interaction of these two agents found inconclusive results. Moreover, despite a vast number of publications, the biological mechanisms through which calcium and vitamin D together exert their anti-neoplastic effects on the normal colon mucosa are not clear. One of the goals of this dissertation is to begin to understand the combined effects of calcium and vitamin D.

Modifiers of Calcium and Vitamin D Effects

It is biologically plausible that there are multiple agents or conditions that can modify the association of vitamin D and calcium with colorectal adenoma risk. Furthermore, these potential effect modifiers have not been fully considered in the previous epidemiologic studies. One potential effect modifier is retinol, which may antagonize the actions of vitamin D by competing for the same substrate, retinoid X receptor protein (247-249); therefore, a high dietary intake of retinol may diminish protective effects of vitamin D. At least one observational study(24) found that the risk of colorectal adenoma was lowest in persons with high vitamin D/low retinol intake compared with those with low vitamin D/high retinol intake (RR = 0.55, 95% CI: 0.28–1.10, $P_{\text{interaction}} = 0.02$). Also, high levels of the pro-inflammatory marker TNFα may inhibit vitamin D actions through an NFκB - mediated mechanism, resulting in a decrease in the transcription efficiency of vitamin D-stimulated genes (250). In animal studies estrogen increased VDR expression in the colon mucosa (53); and one human study found that an estrogen intervention activated the VDR pathway, and downregulated inflammatory and immune signaling pathways in the rectal mucosa of postmenopausal women (251). Furthermore, in animal studies soy products (containing phytoestrogens) were found to upregulate colonic CYP27B1 and downregulate colonic CYP24; and folate may regulate the expression of VDR, CYP27B1, and CYP24 (reviewed in ref. (53)). Therefore, one of the goals of this dissertation project is to investigate hypotheses that increased systemic inflammation, HRT use in women, obesity, high dietary retinol and soy product intakes, and low folate intakes modify the vitamin D/calcium and colorectal adenoma association.

Effects of Calcium and/or Vitamin D on Cell Proliferation

Cell proliferation can be defined as a process involving a sequential pattern of repeating changes in gene expression leading to the physical division of the cells (252). Hyperproliferation in the colorectal mucosa is thought to be a phenotypic biomarker of risk for colorectal neoplasms, and may be modulated by multiple interacting genetic, epigenetic, and environmental factors. Increased proliferation may increase the rate of DNA damage and decrease the rate of repair, thus facilitating colon carcinogenesis.

Numerous experimental and animal studies demonstrated anti-proliferative effects of calcium and/or vitamin D (227, 244, 253-273). However, fewer studies investigated modulation of proliferation markers by calcium supplementation in humans, and almost none by vitamin D.

The most commonly used laboratory methods of measuring colorectal epithelial cell proliferation have been the [^{3}H]dThd (tritiated thymidine), BrdUrd (bromodeoxyuridine), PCNA (proliferating cell nuclear antigen), and more recently, MIB-1 immunohistochemical methods of labeling cells in and around the S-phase of the cell cycle. Monoclonal antibody to detect MIB-1 is raised against the recombinant parts of the proliferation-associated KI-67 antigen, which is expressed in all cells not in G_0 phase of the cell cycle (274). Another potential biomarker of cell proliferation is telomerase, which functions to regenerate telomeres on the end of chromosomes. Normally, in most cells, with repeated replication, telomeres “wear down” resulting in a senescent cell that undergoes apoptosis. Telomerase expression in colon crypt cells may be more reflective of average, long term proliferative activity than are “snapshot” proliferative indicators, such as the S-phase markers PCNA and MIB-1, which demonstrate rapid, large responses to short term physiologic stimuli.

Traditionally, in human studies two basic measurements of colorectal epithelial cell proliferation kinetics, one to indicate the rate of proliferation of colon crypt epithelial cells and the second to indicate the distribution of proliferating cells within the colon crypts have been used (275). Hyperproliferation of the colorectal mucosa with a shift of the proliferative zone to the upper portion of the crypt is thought to be an early step of a complex transition from normal mucosa to adenoma to carcinoma (276, 277).

There have been two large clinical trials of calcium and colorectal epithelial cell proliferation (278, 279) as well as several smaller trials (reviewed in ref. (275), also (43, 44, 280-282)). One large full-scale clinical trial found no evidence for a reduction in the overall PCNA labeling index, but a marked statistically significant shift of the colon crypt proliferative zone downwards (278). However, the second large study (279) found no effect on proliferation (as measured by the PCNA or BrdUrd methods), but, in contrast to the first, this study was multi-centered and had no standardized procedures for bowel preparation, biopsy collection or handling procedures, as well as poor biopsy reader-reliability (reviewed in ref. (275)). Of the seventeen small controlled studies, twelve [six statistically significant] suggested decreases (1–42%) in the overall labeling index (LI), and five suggested either no change or statistically non-significant increases (6–36%). Of the five that measured it (42, 43, 45, 283, 284), all except one (42) suggested decreases in the proportion of labeled cells in the crypt that are in the upper 40% of the crypt (φ_h). Also, five out of eight small studies that reported labeling indices for the upper two quintile compartments demonstrated a decrease in proliferation at the top of the crypt relative to the entire crypt (reviewed in ref. (275), also (281, 282)), two studies reported an increase in the LI of the upper crypt compartments, and one study reported no change. All of these studies indicated that calcium supplementation can substantially lower proliferation rates in the upper parts of the colorectal crypts where adenomas are thought to form.

There have been no published human studies that tested the effect of vitamin D alone or combined with calcium on the hTERT marker of long-term proliferation despite evidence from experimental studies that 1,25-$(OH)_2$-vitamin D downregulates expression of hTERT *in vitro* (285-287). One small study (n = 21) explored potential effects of a 6-month chemopreventive regimen of calcium and vitamin D on biomarkers of risk in normal rectal mucosa and polyps that had been left *in situ* (45). MIB-1 immunostaining in this study decreased significantly in flat mucosa and resected polypoid tissue after supplementation with calcium and vitamin D (45).

In summary, the exact synergistic and separate anti-neoplastic effects of vitamin D and calcium on cell proliferation markers are not fully elucidated. However, experimental and human evidence points to the use calcium and vitamin D as promising anti-proliferative agents against colorectal tumorigenesis. Thus, one of the goals of this dissertation project is to clarify the possible effects of calcium and vitamin D on tissue markers of cell proliferation in the normal human colon.

Effects of Calcium and/or Vitamin D on Cell Apoptosis

Programmed cell death, apoptosis, is a genetically regulated process of cell suicide that plays a central role in the development, homeostasis and integrity of multicellular organisms (288). Apoptosis is characterized by stereotypical morphological features including cell shrinkage, plasma membrane blebbing, mitochondrial outer membrane permeabilization, nuclear chromatin condensation and fragmentation, genomic DNA fragmentation, cytoskeletal modifications, and segmentation of the cell into apoptotic bodies (288, 289). The mechanism of apoptosis is highly conserved. There are two pathways of apoptosis – intrinsic (activated by various cellular stresses) and extrinsic (activated by the binding of ligands to “death receptors”). The key regulators of apoptosis are Bcl-2 family proteins (*e.g.*, the pro-apoptotic proteins Bak and Bax, and the anti-apoptotic protein Bcl-2). This family of proteins controls a crucial step in the intrinsic pathway – the release of cytochrome *c* (290, 291).

Apoptosis is difficult to detect in tissues because the morphological changes occur rapidly (over 2-4 hours) and the apoptotic bodies are rapidly phagocytosed and removed from the tissue (289). There are several methods to detect apoptotic activity in normal human colon tissue including morphology by light microscopy, TUNEL (terminal deoxynucleotidyl transferase-mediated nick end labeling), ISEL (in situ end labeling), and immunohistochemistry using antibodies against proteins involved in apoptotic events (CK18, Bax, Bcl-2, *etc*.). All of these methods have their advantages and disadvantages and are discussed elsewhere (292).

Failure of cells with accumulated genetic and epigenetic changes to slough into the lumen as a result of impaired apoptosis is an important step in colon carcinogenesis that may lead to adenoma development, and thus colorectal cancer (63, 65). Abnormal rates of apoptosis can be a consequence of multiple genetic, epigenetic, and environmental disturbances. Pro- or anti-apoptotic tendencies in the normal colon mucosa are reflected by the expression of Bax and Bcl-2 proteins, respectively. Thus, measuring both of these proteins in colon crypts provides a good measure of propensity to apoptosis.

Evidence from *in vitro* (254, 261, 265, 266) and animal studies (227, 273) shows that calcium and vitamin D enhance apoptosis in colonocytes. There are several hypotheses as to how calcium and vitamin D might promote apoptosis in colonocytes and, thus, decrease risk for colorectal cancer: 1) direct effects on apoptotic proteins, mediated in part by the VDR and the CaSR (11, 293); 2) induction of differentiation with subsequent promotion of apoptosis (254); 3) indirect effects on apoptosis as a result of decreased inflammation (64); and others.

All previous studies of calcium and vitamin D and markers of apoptosis were small, and most were uncontrolled (44, 45, 294). Apoptosis in these studies was measured by the TUNEL method and/or as expression of Bak or Bcl-2. None of these studies measured Bax expression. Only one of these studies found an effect of calcium and/or vitamin D on apoptosis (45).

In summary, experimental and human evidence supports calcium and vitamin D as promising pro-apoptotic agents against colorectal tumorigenesis; but there have been very few human studies that investigated the local effects of these two agents on markers of apoptosis. Therefore, one of the goals of this dissertation project is to clarify the potential pro-apoptotic effects of calcium and vitamin D in the normal human colorectal mucosa.

Effects of Calcium and/or Vitamin D on Cell Differentiation

Differentiation can be defined as a qualitative change in the cellular phenotype that is the consequence of the onset of synthesis of new gene products that lead to functional ability (252). One of these new gene products in differentiated colonocytes is a $p21^{waf1/cip1}$ protein (p21).

$p21^{waf1/cip1}$, a cyclin-dependent kinase (CDK) inhibitor (295, 296), plays an important role in cell cycle control, apoptosis modulation, and cell differentiation (297, 298). It inhibits the activity of cyclin/cdk2 complexes thereby blocking cell cycle progression; blocks DNA replication through binding to PCNA (proliferating cell nuclear antigen); and as an adaptor protein, promotes the association of cdk4 with the D-type cyclins (reviewed in ref. (299)). Also, p21 expression is directly induced by p53 (295). In addition to multiple cell cycle roles in the cell, p21 interacts with transcription regulatory proteins and several other regulatory proteins, among which is calmodulin (300); and it participates in the control of DNA methylation (301). Furthermore, p21 is a potent inducer of differentiation in intestinal colonocytes, and its expression is known to be downregulated during the early stages of colon tumorigenesis (302, 303). In rodent studies, *p21* dose-dependently suppressed tumor formation initiated by *APC,* and the loss of a single or both *p21* alleles potentiated the tumor-promoting effects of a Western-style diet characterized by low calcium and vitamin D (304).

In colon cancer cells *in vitro* (201, 202, 261, 305, 306), vitamin D and calcium increased p21 expression. Moreover, vitamin D and its analogues increased p21 expression in other cancerous tissues, such as prostate cancer cells (307-310), a xenograft model of human retinoblastoma (311), breast cancer cells (312-315), head and neck squamous carcinoma cell lines (316), human osteosarcoma (317), human epidermal keratinocytes (247), leukemia cells (318-320), and osteoblast cells (321), but not in ovarian cancer cells (322). The plausibility of this observation is supported by the fact that the p21 gene is a primary 1,25-$(OH)_2$-vitamin D_3-responsive gene with at least three vitamin D response element (VDRE)-containing regions within its promoter (323, 324); and that calcium, through the calcium-sensing receptor (CaSR), promotes differentiation in colorectal epithelial cells (201, 202). However, there is little literature regarding direct regulation of p21 by calcium, but there is some evidence that extracellular calcium activates protein kinase C, which is associated with the differential induction of p21 in the intestinal epithelium (7). Also, an intracellular calcium gradient along the colon crypt that coincides with the differentiation compartment may modulate differentiation of the colonocytes, thus, regulating p21 expression (203).

There have been no previous human studies that tested the effects of calcium and/or vitamin D supplementation on p21 expression in the normal colorectal mucosa, but three small studies (43-45) investigated the effects of these agents or low fat dairy foods on other markers of differentiation (acidic mucins and/or cytokeratin AE1) in the normal colorectal mucosa with inconsistent results. Two small studies found no changes in the normal rectal crypt differentiation markers after supplementation with calcium and vitamin D_3 (45), or with calcium or low fat dairy foods (44); but a third, larger (n = 70), randomized, placebo-controlled trial reported significant changes in differentiation markers after supplementation with low fat dairy foods, which are rich in calcium and vitamin D, but contain other components that may also exert pro-differentiative effects (43).

Taken together, calcium and vitamin D are promising pro-differentiative chemopreventive agents against colorectal neoplasms that require further investigation. One of this dissertation’s projects aims is to clarify the effect of these agents on the marker of differentiation, p21, in the normal colorectal epithelium.

Calcium, Vitamin D, and Oxidative Stress

Oxidative stress, a condition characterized by an imbalance of pro-oxidants to antioxidants which results in macromolecular damage and disruption of redox signaling and control (325), may play a role in colon carcinogenesis, inducing protein and DNA damage and lipid peroxidation, and impairing intracellular signaling. Under normal conditions, reactive oxygen species (ROS) have an important role as intracellular signaling molecules that regulate many genes (118). However, under inflammatory conditions, increased generation of ROS products leads to cell molecule damage such as oxidation of DNA (118). The most abundant product of oxidative DNA modifications by ROS is 8-hydroxy-2'-deoxyguanosine (8-OH-dG) (326). This oxidized base is a useful biomarker of oxidative stress that can be measured in urine, blood, and tissues (327, 328). Several studies demonstrated increased levels of oxidatively modified DNA in colorectal adenocarcinomas when compared to adenomas and adjacent normal epithelium (329, 330). This suggests that inhibition of oxidative stress in the normal colorectal epithelium may slow down or even prevent carcinogenesis, and prompts the development of chemopreventive agents, such as calcium and vitamin D, that target oxidative stress in the colon.

There are several lines of evidence to support our hypotheses that calcium and vitamin D may act as antioxidants and DNA damage reducing agents in the colon. Bile acids damage cell membranes, at least in part through an oxidative mechanism (204, 205), provoking an inflammatory response and causing DNA damage (206), and both calcium and vitamin D can reduce the free bile acid load in the colon lumen. Calcium directly binds bile acids, rendering them inert (6). Vitamin D activation of the ubiquitous vitamin D receptor (VDR) in the colon up-regulates CYP3A4, which in turn catabolizes the secondary bile acid, lithocholic acid (11, 227). Furthermore, high blood 25-(OH)-vitamin D levels provide a pool of vitamin D that is available for various tissues, such as the colorectal epithelium. In colonocytes, vitamin D increases expression of enzymes involved in antioxidant response, inhibits iron-dependent lipid peroxidation in liposomes, lowers glutathione reductase levels, induces glutathione peroxidase and manganese dependent superoxide dismutase activity, and elevates glutathione levels ((228, 229), also reviewed in ref. (230)), thereby decreasing oxidative stress in the colorectal epithelium. Furthermore, the complete loss of VDR significantly increased 8-OHdG labeling in the mouse colon (331, 332) . All these data indicate important roles of vitamin D and calcium in modulating oxidative stress; however, no reported human studies explored this novel hypothesis of oxidative stress reduction by higher calcium and vitamin D intakes in reducing risk for colorectal neoplasms. This dissertation project contributes to the understanding of anti-oxidative properties of calcium and vitamin in the normal-appearing colorectal mucosa.

Hypotheses

1. I hypothesize that subjects with low levels of 25-(OH)-vitamin D are at high risk of developing colorectal adenoma and that this association is modified by inflammation status, obesity, HRT use in women, and dietary intakes of retinol, soy products, and folate. Also, I hypothesize that I will find a synergistic effect of high vitamin D exposure and high calcium intake such that the subjects who have both the highest intakes of calcium and the highest serum/plasma levels of 25-(OH)-vitamin D are at the lowest risk of developing colorectal adenomas.

2. I hypothesize that calcium and vitamin D_3, alone and in combination, can modulate the expression of apoptosis markers in the normal colon mucosa of patients with at least one pathology-confirmed adenomatous colorectal polyp.

3. I hypothesize that calcium and vitamin D_3, alone and in combination, can modulate the expression of cell proliferation and differentiation markers in the normal colon mucosa of patients with at least one pathology-confirmed adenomatous colorectal polyp.

4. I hypothesize that vitamin D_3 and/or calcium supplementation decreases oxidative stress in colorectal crypts (as indicated by reduced 8-OH-dG content in the normal colorectal crypts).

Objectives

My primary objective is to investigate associations of circulating 25-(OH)-vitamin D levels with risk of incident, sporadic colorectal adenomas. Moreover, based on basic science observations, I will investigate if these associations are modified by obesity, HRT use in women, dietary retinol, folate, soy products, and systemic inflammation status. Further, I will test hypotheses that vitamin D_3 and calcium supplementation, alone or in combination, in normal colorectal tissue, increases markers of apoptosis and differentiation, and decreases markers of proliferation and oxidative DNA damage. The objectives of this study will be addressed through the following specific aims.

Specific Aims

Aim #1: Investigate whether high 25-(OH)-vitamin D levels alone or in combination with high calcium intake reduce risk for colorectal adenomas in a pooled analysis of three case-control studies (combined N=1,901) of incident, sporadic colorectal adenomas.

a) Measure serum/plasma 25-(OH)-vitamin D levels in all controls (n=1,074) and cases with colorectal adenomas (n=827);

b) Estimate the association of serum/plasma 25-(OH)-vitamin D with risk of colorectal adenomas;

c) Estimate the combined association of 25-(OH)-vitamin D levels and intake of calcium with risk of colorectal adenomas;

d) Investigate the association of calcium and vitamin D with colorectal adenomas by inflammation status, HRT use in women, BMI, and high/low dietary intakes of retinol, folate, and soy products;

e) Investigate the association of calcium/vitamin D with colorectal adenomas stratified by systemic inflammation status. Systemic inflammation status will be defined based on C-reactive protein (CRP) levels, which were shown to correlate well with TNFα levels (333).

Aim #2: Using data from a randomized, double-blind, placebo-controlled 2x2 factorial clinical trial (N=92) of calcium and/or vitamin D_3 in incident sporadic adenoma patients, determine whether supplementation with calcium and vitamin D_3, alone or in combination, alters expression of biomarkers of apoptosis in normal rectal mucosa.

a) Estimate the effect of calcium and vitamin D_3 supplementation on expression of biomarkers of apoptosis (Bax, Bcl-2) in normal-appearing colon crypts in patients with previously removed sporadic adenoma.

Aim #3: Using data from a randomized, double-blind, placebo-controlled 2x2 factorial clinical trial (N=92) of calcium and/or vitamin D_3 in incident sporadic adenoma patients, determine whether supplementation with calcium and vitamin D_3, alone or in combination, alters expression of biomarkers of cell proliferation and differentiation in normal rectal mucosa.

a) Estimate the effect of calcium and vitamin D_3 supplementation on expression of biomarkers of proliferation (hTERT, MIB-1/Ki-67) in normal-appearing colon crypts in patients with previously removed sporadic adenoma;

b) Estimate the effect of calcium and vitamin D_3 supplementation on expression of biomarker of differentiation (p21$^{waf1/cip1}$) in normal-appearing colon crypts in patients with previously removed sporadic adenoma.

Aim #4: Using data from a randomized, double-blind, placebo-controlled 2x2 factorial clinical trial (N=92) of calcium and/or vitamin D_3 in sporadic adenoma patients, determine whether supplementation with calcium and vitamin D_3, alone or in combination, reduces oxidative DNA damage in the normal colorectal mucosa:

a) Estimate the effect of calcium and vitamin D_3 supplementation on levels of a DNA damage marker (8-OH-dG) in normal-appearing colon crypts in patients with previously removed sporadic adenoma.

Methods

Pooled Case-Control Study Protocol

To address the first question (Aim #1), I used the data from a large pooled case-control study of incident, sporadic colorectal adenomatous polyps. This pooled study combined three methodologically very similar colonoscopy-based case-control studies of incident, sporadic colorectal adenomas conducted by the same PI in three different states. The first study, conducted in Minnesota, included 574 cases and 707 controls (CPRU study). The second study, conducted in North Carolina, included 204 cases and 213 controls (MAPI study). The third study, done in South Carolina, enrolled 49 cases and 154 controls (MAPII study). All three studies used the same questionnaires, and had nearly identical data collection protocols and recruitment procedures. The total number of cases and controls in the pooled study was 827 and 1,074, respectively. The following data were collected for this pooled case-control study of colorectal adenomas: dietary variables from Willett food frequency questionnaires (FFQ) (334), demographic characteristics, medical history, medications and nutritional supplements, polyp pathology (*e.g.*, size and histology), lifestyle/behavior variables, family history of cancer, and reason for colonoscopy. Blood levels of 25-(OH)-vitamin D_3 and D_2 were measured by liquid chromatography/tandem mass spectrometry (LC/MS/MS). The detailed case-control study protocol is described below in the Methods section of Chapter 2.

Clinical Trial Protocol

To address the last three questions (Aims #2, #3 and #4), I used the data from a pilot, randomized, double-blind, placebo-controlled, 2 x 2 factorial chemoprevention trial (n =

92) of calcium 2.0 g/day and vitamin D_3 800 IU/day, alone and in combination, vs. placebo over 6 months in patients with recently removed sporadic colorectal adenomatous polyps. Participants in this study were recruited from the patient population attending the Digestive Diseases Clinic of the Emory Clinic, of Emory University. Of patients who passed initial chart eligibility, 42% were contacted and 20% were eligible and consented to participate. Participants (n = 92) were randomly assigned to the following four treatment groups: a placebo control group (n = 23), a 2.0 g elemental calcium (as calcium carbonate in equal doses twice daily) supplementation group (n = 23), an 800 IU vitamin D_3 supplement group (400 IU twice daily) (n = 23), and a calcium plus vitamin D supplement group taking 2.0 g elemental calcium plus 800 IU of vitamin D_3 daily (n = 23). Seven people (8%) were lost to follow-up due to perceived drug intolerance (n = 2), unwillingness to continue participation (n = 3), physician's advice (n = 1), and cardiovascular death (n = 1). Dropouts included one person from the vitamin D supplementation group, and two persons from each of other three groups. The following data were collected at baseline and 6-months follow-up for each participant in this chemoprevention trial: dietary variables from a Willett FFQ, medical history, medications and nutritional supplements, lifestyle/behavior variables, plasma 25-(OH)- and 1,25-$(OH)_2$- vitamin D levels, immunohistochemically detected biomarkers in "non-prep" biopsies of normal-appearing rectal mucosa (see Appendix for detailed description of laboratory procedures). Demographic characteristics, polyp pathology (*e.g.*, size and histology), family history of cancer, and *VDR BsmI* genotyping data were also available for each participant. The detailed clinical trial protocol is described below in the Methods section of Chapter 3.

Data Analysis Plan

The proposed data analysis plan for the *pooled case-control study* is as follows: 1) the case and control groups will be evaluated for comparability with respect to important covariates, including demographics, lifestyle, and other risk factors, using chi square or Fisher's exact tests and analysis of variance and covariance methods; 2) linear regression and/or mixed linear model methods and correlation analyses will used to evaluate associations among continuous variables; and 3) multiple logistic regression methods will be used to calculate and assess strengths of association (odds ratio; with 95% confidence intervals and tests for trend) for main effects and interactions adjusted for confounding variables. Finally, sensitivity analyses will be performed.

The proposed data analysis plan for the *chemoprevention trial* is as follows: 1) treatment groups will be assessed for comparability of characteristics at baseline and at final follow-up by the Fisher's exact test for categorical variables and analysis of variance (ANOVA) for continuous variables; 2) for each biomarker we will have the raw measurements of the total expression of each biomarker along the colon hemicrypt as a continuous variable; the normality of these variables will be assessed and appropriate transformations (e.g., natural log) will be applied to normalize the data; 3) if required, each biomarker measurement will be standardized or adjusted for batch to account for potential batch effects; 4) variables to summarize the quantity of expression and the distribution of the expression in the colon crypts will be created; 5) crypt distributions of biomarker expression will be examined using graphical methods (LOESS procedure), and then variables to describe differences seen graphically will be created; 6) treatment effects on the tissue and blood biomarkers across the four treatment groups, the placebo and the three supplementation groups, will be compared by a general mixed linear models procedure for repeated measures data as implemented in SAS Institute's Mixed Procedure. In addition, the sensitivity of the study results to potential biases and modeling assumptions and approaches will be examined.

Statistical Power Considerations

Pooled case-control study: The merged data set from the three case-control studies contains data on 785 cases of incident sporadic adenomas, 535 healthy community and 966 clinic controls, and 291 controls with incident hyperplastic polyps. Power calculations were performed using SAS PROC POWER (SAS Institute, version 9.3.1), with a significance level of 0.05. The prevalence of vitamin D insufficiency (<33 ng/ml) in the general population was estimated to vary from 11% to 42% at the end of the summer and winter seasons (335-340), respectively. Approximately 785 cases and 785 controls will be suitable for laboratory analyses. Power to identify the association between low vitamin D and colorectal adenomas with an odds ratio of 1.4 varies from 57-91%, depending on prevalence assumptions (Table 1.4).

Table 1.4. Statistical power for pooled case-control study (785 cases and 785 controls, and α=0.05, 2-sided).

Odds Ratio	**Prevalence of vitamin D insufficiency in controls**	**Power**
1.2	0.1	0.20
	0.2	0.32
	0.3	0.39
	0.4	0.43
1.4	0.1	0.57
	0.2	0.80
	0.3	0.88
	0.4	0.91

Chemoprevention controlled trial: This study was a pilot study designed primarily to provide preliminary estimates of treatment effect size and variability in order to calculate the needed sample size for a larger trial. It includes 92 patients, 23 patients in each treatment group (calcium, vitamin D, calcium & vitamin D, and placebo). Power calculations were conducted using PASS 2008 statistical software (NCSS, Kaysville, Utah). The results of the power calculations are presented in Table 1.5. Estimates of the means and standard deviations were obtained from our previously conducted case-control study (Markers of Adenomatous Polyps II, MAPII) (341, 342) and calcium and colorectal epithelial cell proliferation trial (278). As can be seen from Table 1.5, there will be enough power to detect a major change in the biomarkers' optical density means or moderate change with relatively small between-subject variability.

Table 1.5. Statistical power, calculated to detect a given range of changes in biomarker expression means, in a chemoprevention controlled trial with 4 randomization groups, 23 participants in each group.

SD	**Change in means**	**Power**
0.87	0.80	0.89
0.87	0.40	0.32
0.50	0.80	0.99
0.50	0.40	0.79

Two-sided type I error $\alpha = 0.05$

Student Contribution to Data Collection

My role in the proposed research includes: 1) developing an immunohistochemistry (IHC) staining protocol for a marker of oxidative DNA damage in colon tissues (8-Hydroxy-2'-deoxyguanosine, 8-OH-dG; Appendix A); 2) assisting in IHC staining procedures; 3) quantifying the staining density of immunohistochemically detected biomarkers (Bax, Bcl-2, 8-OH-dG) in normal colon crypts (“scoring”); 4) scanning all immunohistochemically stained for 8-OH-dG slides (184 sets of slides x 5 slides in each set = 920 slides) using the new ScanScope slide scanner (Aperio Technologies, Inc., CA); 5) participation in the development and testing of a new software designed to analyze scanned images and quantify staining optical density of immunohistochemically detected biomarkers in normal colon crypts (DivEyes Software, DivEyes LLC, GA); 6) developing a protocol for quantifying the staining optical density of immunohistochemically detected biomarkers in normal colon crypts (“scoring” procedures) using the newly developed software and updating older versions of ‘scoring’ protocols; 7) coordinating blood shipping, and assisting with blood and tissue sample processing; 8) extensive cleaning, error-checking, and verification of raw data obtained from questionnaires and laboratory data; 9) combining data from three case-control studies; and 10) merging all data into one analytical database and conducting data analyses.

Study Strengths

The first study is the largest pooled case-control study of incident colorectal adenomas. In this study, the adenoma-free status of gastrointestinal (GI) clinic controls was verified by colonoscopy. Also, the extensive questionnaires provided data on demographic characteristics, lifestyle and behavior, medical history, medications, family history of cancer, diet, and polyp pathology, which were combined with genotyping and blood assay data. Moreover, in this study we will use plasma or serum 25-(OH)-vitamin D_3 levels as the main vitamin D exposure instead of poorly measured dietary vitamin D intake. Few studies explored the synergistic effect of calcium and vitamin D on colorectal adenoma risk. Finally, this study provides an opportunity to explore the impact of potential effect modifiers of the colorectal adenoma – vitamin D association.

The second study is the largest pilot chemoprevention controlled trial to test the effect of supplemental calcium and vitamin D_3, alone and in combination vs. placebo on tissue/blood biomarkers of colorectal adenoma risk. Few human studies investigated the effect of calcium and vitamin D supplementation on the expression of apoptotic, differentiation and proliferative markers in normal colon mucosa. Moreover, there have been no randomized clinical trials to assess the efficacy of combined supplementation with calcium and vitamin D on markers of apoptosis in the colon. None of the previously published human studies determined whether supplementation with calcium and vitamin D, alone or in combination, reduces oxidative DNA damage in the normal colorectal mucosa. In addition, this study had high adherence to visit attendance (92%) and only seven (8%) people were lost to follow-up. Also, on average, at least 80% of pills were taken by 93% of participants at the first follow-up visit and 84% at the final follow-up visit. Furthermore, all tissue biomarkers were stained using automated immunohistochemistry methods that guaranteed the high reproducibility and reliability of the assay, and semi-automated methods were used for quantifying the staining densities of the immunohistochemically detected biomarkers (‘scoring’). Three biomarkers (Bax, Bcl-2, and 8-OH-dG) were scored by one slide reader, and the other biomarkers (p21, hTERT and MIB-1) were scored by another slide reader. Intra-class correlation coefficients for biopsy “scoring” reliability were 0.93–0.98, and the average intra-assay coefficient of variation for plasma 25-(OH)-vitamin D was 2.3%, and for 1,25-$(OH)_2$-vitamin D, 6.2%. Demographic, lifestyle, diet, physical activity, medical history, medication, polyp pathology, plasma vitamin D, and *VDR BsmI* genotyping data are available for each study participant.

CHAPTER 2. BLOOD 25-HYDROXYVITAMIN D_3 CONCENTRATIONS AND INCIDENT, SPORADIC COLORECTAL ADENOMA RISK: A POOLED CASE-CONTROL STUDY

Veronika Fedirko[1,2], Roberd M. Bostick[1,2], Michael Goodman[1,2], W. Dana Flanders[1,3],
Myron D. Gross[4]

[1]Department of Epidemiology, Rollins School of Public Health, Emory University,
Atlanta, GA 30322
[2]Winship Cancer Institute, Emory University, Atlanta, GA 30322
[3]Department of Biostatistics and Bioinformatics, Rollins School of Public Health, Emory
University, Atlanta, GA 30322
[4]Department of Laboratory Medicine and Pathology, University of Minnesota Medical
School, Minneapolis, MN 55455

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Abstract

Low vitamin D exposure has been implicated in colorectal carcinogenesis. However, the observational evidence for a protective effect of vitamin D against colorectal neoplasms is not very strong, probably because most of the epidemiologic studies of vitamin D and colorectal neoplasms investigated only poorly measured vitamin D exposure from diet.

The purpose of this analysis was to examine the association between circulating 25-hydroxyvitamin D_3, the best indicator of total vitamin D exposure, and colorectal adenoma risk. We pooled the primary data from three colonoscopy based case-control studies of incident, sporadic colorectal adenomas conducted in Minnesota, North and South Carolinas between 1991 and 2002. The pooled study included 1,386 White individuals, among whom 616 were colorectal adenoma cases and 770 were polyp-free controls. Circulating 25-(OH)-vitamin D_3 concentrations were measured by a liquid chromatography/tandem mass spectrometry method. Multivariable logistic regression was used to estimate the association between circulating 25-hydroxyvitamin D_3 and colorectal adenoma risk. Stratified analyses and the likelihood ratio test were used to examine effect modification by various lifestyle, demographic, and dietary factors.

In the pooled analysis, higher circulating 25-(OH)-vitamin D_3 concentrations were statistically significantly associated with decreased colorectal adenoma risk (highest *versus* lowest quartile: odds ratio (OR) = 0.59, 95% confidence interval (CI): 0.41–0.84). The observed inverse association was stronger among participants who took aspirin or other non-steroidal anti-inflammatory drugs regularly (highest versus lowest quartile, OR = 0.33, 95% CI: 0.19–0.56). Inverse associations between 25-(OH)-vitamin D_3 and

colorectal adenoma did not differ substantially by adenoma characteristics, and by other risk factors.

These findings support the hypothesis that greater vitamin D exposure reduces risk for colorectal adenoma, and suggest that this protective effect of vitamin D may be more pronounced in combination with anti-inflammatory agents.

Introduction

In 1980, based on ecologic observations of a correlation between sun exposure and reduced colorectal cancer incidence, Garland and Garland proposed the hypothesis that vitamin D (collective term for vitamin D_2 and D_3) status accounted for this inverse association (225). At that time, the antineoplastic effects of vitamin D were unknown, and for another several decades, literature on the biologic basis of this hypothesis and on a vitamin D – colorectal neoplasms association evolved considerably.

Recent studies found that colon epithelium expresses vitamin D receptor (VDR) and several vitamin D metabolizing enzymes that are responsible for the autocrine/paracrine synthesis and degradation of the active metabolite of vitamin D, 1,25-$(OH)_2$-vitamin D (262, 343, 344). In the colon, 1,25-$(OH)_2$-vitamin D exerts its anti-neoplastic effects by both genomic (mediated by the VDR) and non-genomic (mediated by a membrane receptor) mechanisms (345). These include regulation of more than 200 vitamin D-responsive genes, and rapid activation of intracellular signaling pathways resulting in induction or inhibition of synthesis of new proteins involved in the control of cell cycle events, degradation of bile acids, and modulation of immune response and growth factor signaling (7, 226).

The majority of observational studies investigated the association between dietary vitamin D intake and risk for colorectal neoplasms, whereas fewer studies assessed the association with the main form of circulating vitamin D, 25-(OH)-vitamin D (221, 238, 346). The proportion of vitamin D obtained from the diet is very small compared with that synthesized in the skin during exposure to sunlight. Therefore, dietary vitamin D intake, unlike circulating 25-(OH)-vitamin D, may not reflect the actual vitamin D exposure. Of the seven epidemiologic studies (21, 22, 210, 214, 235-237), all, except one (236), found inverse associations of 25-(OH)-vitamin D with colorectal adenomas; however, of all studies only four (21, 22, 214, 237) reported statistically significant results overall (22, 214), or in women (21), or in patients randomized to receive calcium supplementation (237). We also hypothesized that several *a-priori* selected risk factors may modify the association between 25-(OH)-vitamin D and colorectal neoplasms. These factors include inflammation-related conditions and agents, exposure to estrogen, and dietary intakes of calcium, retinol, and folate. High levels of the pro-inflammatory cytokine TNFα may inhibit vitamin D genomic actions, resulting in decreased transcriptional efficiency of vitamin D-responsive genes (250). In mice, phytoestrogens, as well as folic acid, may up-regulate colonic enzyme involved in conversion of 25-(OH)- to 1,25-$(OH)_2$-vitamin D (CYP27B1), and down-regulate colonic enzyme responsible for deactivating vitamin D (CYP24A1) (53). Also, calcium and vitamin D, highly physiologically inter-related agents, may synergistically protect against colorectal carcinogenesis by influencing bile-acid metabolism and modulating genes or proteins in colon carcinogenic pathways (221). Finally, retinol may antagonize the actions of vitamin D by competing for the same substrate, retinoid X receptor (247-249). However, for many of these potential interactions, few human observational data are available (24, 210, 237).

To investigate the association between circulating 25-(OH)-vitamin D_3 and risk for colorectal neoplasms, we conducted a pooled colonoscopy based case-control study of incident, sporadic colorectal adenomas. We also examined hypotheses that the vitamin D – colorectal adenoma association differs by age, sex, regular use of non-steroidal anti-inflammatory drugs (NSAIDs) or aspirin, obesity, hormone replacement therapy (HRT) use in women, and by levels of physical activity, systemic inflammation marker (C-reactive protein, CRP), and dietary intakes of calcium, retinol, soy, and folate.

Materials and Methods

Case-Control Studies

We pooled data from three methodologically very similar colonoscopy-based case-control studies of incident, sporadic colorectal adenomas conducted by the same principal investigator in three different states.

The first case-control study (**the Cancer Prevention Research Unit study**, **CPRU**) was conducted between 1991 and 1994 as a part of the Minnesota Cancer Prevention Research Unit, an NCI-funded program project that combined several units within the University of Minnesota and Digestive Healthcare, PA (DH), a large multi-clinic private gastroenterology practice. The detailed study protocol was described elsewhere (347). Briefly, participants for this case-control study were recruited from patients with no prior history of colorectal neoplasms who were scheduled to undergo outpatient, elective colonoscopy in one of 10 hospitals in the Minneapolis metropolitan area. Of the 3,126 colonoscopy patients identified, 2,771 (89%) were eligible on initial screening, and of these, 1,890 (68%) agreed to participate and signed consent. Of the 1,886 (99%) participants who met final eligibility criteria, 574 (30%) had a colorectal adenoma, 219 (12%) had a hyperplastic polyp but no adenoma, and 707 (37%) were free of any polyps of any type.

The second case-control study (**the Markers of Adenomatous Polyps study, MAPI**) was conducted from 1994 to 1997 to assess the validity of colonic epithelial cell proliferation as a biomarker of risk for sporadic colorectal adenomas. The study protocol was described in detail elsewhere (208, 348). Briefly, participants were recruited from patients with no prior history of colorectal neoplasms who were scheduled for outpatient, elective colonoscopy visit by community gastroenterology practices in Winston-Salem and Charlotte, North Carolina. Of the 2,246 colonoscopy patients identified among three clinical sites, 669 (30%) were eligible on initial screening, and of these, 617 (92%) were contacted. Of the 417 (68%) patients who signed consent and had study colonoscopies, nine were subsequently determined ineligible for the study, and an additional eight patients had incident colon cancer and were not eligible for the primary case-control analyses. Of the remaining 400 participants, 174 (44%) had a colorectal adenoma, 49 (12%) had a hyperplastic polyp but no adenoma, and 177 (44%) were free of any polyps of any type.

The third case-control study (**the Markers of Adenomatous Polyps II study, MAPII**) was identical in design to the MAPI study and was conducted in 2002 to investigate whether the expression patterns of various genes and cell cycle markers in the normal-appearing colorectal mucosa are associated with incident, sporadic adenomas. The study protocol was described in detail elsewhere (349, 350). Briefly, participants with no prior history of colorectal neoplasms were recruited upon referral for routine outpatient, elective colonoscopy at Consultants in Gastroenterology, PA, a large private practice gastroenterology group in Columbia, SC. Of the 351 patients identified over a 5-month period, 305 (87%) were eligible to participate upon initial recruitment screening. Of these, 232 (76%) were successfully contacted and provided informed consent before colonoscopy. Of the 203 (88%) who met final eligibility criteria, 49 (24%) were colorectal adenoma cases, 38 (19%) were controls with at least one hyperplastic polyp, and 116 (57%) were controls free of polyps of any type.

The participants' initial eligibility assessment was identical in the three case-control studies and evaluated whether patients were aged 30–74 years, English speaking, willing to participate and able to understand informed consent, had no contraindications to endoscopy, free of known genetic syndromes associated with predisposition to colonic neoplasia (*e.g.*, familial polyposis coli or Gardner's syndrome) and an individual history of ulcerative colitis, Crohn's disease, colorectal adenomas, and cancer (except non-melanoma skin cancer). Because 96% of all study participants were White, we excluded all participants of other races/ethnicities from the pooled analysis.

Data Collection

Before undergoing colonoscopy, all patients completed mailed questionnaires regarding demographic characteristics, personal medical history, reproductive history (women only), family history of polyps or colon cancer, anthropometrics, diet (via a semi-quantitative Willett 153-item Food Frequency Questionnaire (334)), lifestyle, alcohol and tobacco use, usual physical activity, and reasons for colonoscopy. Because participant selection, study questionnaires, and protocols were identical for the MAPI and

MAPII studies, we combined data from these studies and hereafter refer to them as the **MAP** (Markers of Adenomatous Polyps) study.

For all studies, preparation for colonoscopy included a 12-h fast and bowel cleansing with polyethylene glycol. At the clinic visit, the signed consent form and completed questionnaires were collected, and venous blood was drawn from each participant and stored at -70°C until further analyses. Plasma and serums were separated according to a standardized protocol. The colonoscopy findings were recorded on standardized forms to record colon site and *in vivo* size and shape of any polyps. Upon removal, polyps were examined histologically by an index study pathologist using diagnostic criteria established for the National Polyp Study (351). Only participants with a complete colonoscopy reaching the cecum were eligible. The presence or absence of pathology was determined, and based on colonoscopy and pathology findings, participants were assigned to one of the following three groups: (*a*) an adenomatous polyp group (defined as either adenomatous or mixed pathology); (*b*) a hyperplastic polyp-only group; and (*c*) a colonoscopy-negative control group. Participants with polyps with invasive carcinoma were excluded. The hyperplastic polyp group was treated as a separate group and was excluded from this pooled analysis. A total of 630 cases and 787 controls had plasma (CPRU) or serum (MAP) samples available for 25-(OH)-vitamin D assays.

The final sample size for this pooled case-controls study included 1,386 White participants with measured circulating 25-(OH)-vitamin D, among whom 616 were incident, sporadic colorectal adenoma cases, and 770 were endoscopy controls without hyperplastic polyps.

The protocols of each study were approved by the Institutional Review Boards of the corresponding institutions, the University of Minnesota and each DH colonoscopy site for the CPRU study, Wake Forest University School of Medicine for the MAPI study, and the University of South Carolina for the MAPII study. Informed consent was obtained from each participant.

Laboratory Methods

Serum samples were not available for the CPRU study, and plasma samples were not available for some participants of the MAP study. Therefore, the 25-(OH)-vitamin D assays were conducted in serum samples for the MAP study, and in plasma samples for the CPRU study. To check the comparability of serum and plasma concentrations of 25-(OH)-vitamin D, we analyzed serum and plasma samples from 20 participants. The means (standard deviations) for 25-(OH)-vitamin D_2 were 11.1 (6.4) in plasma, and 9.6 (5.6) in serum (Spearman's rank correlation coefficient (ρ) = 0.9, $P < 0.001$). For 25-(OH)-vitamin D_3, the means (standard deviations) were 21.3 (6.7) in plasma, and 23.9 (8.8) in serum ($\rho \geq 0.8$, $P < 0.001$); and for total 25-(OH)-vitamin D ($D_2 + D_3$), 32.4 (9.2) in plasma, and 33.5 (10.2) in serum ($\rho \geq 0.8$, $P < 0.001$). All laboratory assays for blood 25-OH-vitamin D_2 and D_3 were performed at the University of Minnesota Medical Center, Fairview using a liquid chromatography/tandem mass spectrometry (LC/MS/MS) method as previously described (352). Serum/plasma samples for all subjects were assayed together, ordered randomly, and labeled to mask case-control status, and quality control replicates. The average intra-assay coefficient of variation for serum/plasma 25-(OH)-vitamin D_2 was 80%, and for 25-(OH)-vitamin D_3, 3%.

Laboratory assays for serum CRP (C-reactive protein) were also performed at the University of Minnesota Medical Center, Fairview using a Tina-quant® C-reactive protein (latex) high sensitive assay (Roche, product # 1972855). The CRP assay was performed only for participants in the MAP study.

Statistical Analysis

Standard techniques for case-control analyses were used. The case and control groups were evaluated for comparability with respect to important covariates, including demographics, lifestyle, and other risk factors, using the chi square test for categorical variables and the *t*-test for continuous variables.

Only the analyses for serum/plasma 25-(OH)-vitamin D_3, the primary exposure variable of interest, are presented for the following reasons: 1) the poor performance of the vitamin D assay in detecting 25-(OH)-vitamin D_2 (intra-assay coefficient of variation was 80%); 2) 64% of participants had 25-(OH)-vitamin D_2 measurements below the limit of assay sensitivity of 5 ng/mL (and 87% had a level of <10 ng/mL); 3) vitamin D_2 may be less effective than vitamin D_3 in raising 25-(OH)-vitamin D levels (353, 354); 4) the vitamin D 25-hydroxylase, CYP27A1 enzyme, may selectively 25-hydroxylate vitamin D_3, but not vitamin D_2 (355); and 5) vitamin D_2 may have less bioefficacy than vitamin D_3, which may be due to lower binding affinity of vitamin D_2 metabolites to the vitamin D receptor (VDR), and of vitamin D_2 to vitamin D binding protein (DBP) (reviewed in ref. (356)). Accordingly, we included 25-(OH)-vitamin D_2 measurements only in sensitivity analyses in which we combined 25-(OH)-vitamin D_2 in different weights with 25-(OH)-vitamin D_3. For these additional analyses, we calculated total circulating 25-(OH)-vitamin D as the sum of 25-(OH)-vitamin D_2 and D_3, and weighted total circulating

25-(OH)-vitamin D as [k*25-(OH)-vitamin D_2 + 25-(OH)-vitamin D_3], where $k = 0.25$ based on our assumptions for bioefficacy of the two vitamin D forms (356).

Unless indicated otherwise, study-specific quartiles of circulating 25-(OH)-vitamin D_3 concentrations were calculated based on the distribution in control subjects by month of blood draw as described in reference (357). Unconditional logistic regression models were used to assess the association between quartiles of blood vitamin D concentrations and risk of colorectal adenoma, with appropriate control for confounding. In addition, we investigated the association between 25-(OH)-vitamin D quartiles and adenoma characteristics by classifying adenoma cases into subgroups based on multiplicity, size, location, and pathological subtype. We also examined associations stratified by age, sex, family history of colorectal cancer (CRC) in a first degree relative, regular use (≥ once a week) of aspirin, regular use (≥ once a week) of other nonsteroidal anti-inflammatory drugs (NSAIDs), physical activity, body mass index (BMI), calcium, retinol, folate, and soy intake. Cut points for continuous variable effect modifiers were calculated based on the study-specific median distributions in the control subjects. In addition to comparing stratum-specific ORs, we included the interaction terms in the model, and tested the significance of the estimates with the log-likelihood ratio test.

Study site, age, sex, education, regular use of NSAIDs and/or aspirin, family history of CRC in a first degree relative, physical activity, smoking status (current, ever, or never), BMI, total energy intake, total (dietary and supplemental) intakes of calcium, folate, soy, fiber, alcohol, red and processed meats, and, among women, hormone replacement therapy and menopausal status were considered as established and suspected confounding variables. Several techniques were used to assess confounding factors: 1) biological plausibility; 2) whether the variable of interest was associated with the outcome and exposure; and 3) whether the logistic regression coefficient of the exposure variable substantially changed (by >10%) after adding the potential confounding variable in the model. We built the most parsimonious model with adequate control for confounding using the following steps: 1) we ranked all potential confounding variables based on published literature on their hypothesized relative contributions, and strengths of their associations with colorectal adenoma risk; 2) a summary rank was calculated and potential confounders were added to the study-, age-, and sex-adjusted model one at a time according to their rank; and 3) the model that had the smallest number of parameters and with adequate control for confounding was selected as the final multivariable adjusted model. Final covariates included in multivariate-adjusted models were age, sex, study site (CPRU or MAP), BMI, physical activity, smoking, regular aspirin or NSAID use, family history of CRC in a first degree relative, and dietary intakes of alcohol, calcium, retinol, folate, and red and processed meats.

The odds ratio (OR) was the measure of association. For each OR, a 95% confidence interval (95%CI) was calculated. A test for trend was calculated based on the median of each quartile of blood 25-(OH)-vitamin D concentration included in the model as a continuous variable. All statistical tests were two-sided, and *P*-values < 0.05 were considered to be statistically significant. All statistical analyses were conducted using SAS version 9.2 software (SAS Institute, Inc., Cary, NC).

In additional analyses, we included all participants of any race and ethnicity and controls with and without hyperplastic polyps. Also, we used total 25-(OH)-vitamin D and weighted total 25-(OH)-vitamin D as the main vitamin D exposure variable.

Furthermore, we calculated 25-(OH)-vitamin D_3 season- and study-specific quintiles, and used suggested reference values for defining vitamin D deficiency and sufficiency (< 20 and ≥ 32 ng/mL, respectively) (241, 358). The season categories were defined as winter: December, January, February; spring: March, April, May; summer: June, July, August; fall: September, October, November (357). Some study participants had missing dietary data (2%), or missing data on NSAID use (1%), aspirin use (1%), family history of CRC in a first degree relative (1%), BMI (2%), or smoking (2%). To assess the effect of missing data on main estimates, we used multiple imputation techniques as implemented in SAS procedures PROC MI and PROC MIANALYZE. The results from these additional analyses did not differ materially from those reported.

We did a probabilistic sensitivity analysis to assess the potential effects of non-differential and differential misclassifications of an exposure (limited to highest and lowest 25-(OH)-vitamin D_3 quartiles) on the odds ratio for colorectal adenoma by varying the sensitivity and specificity drawn from trapezoidal distributions in 10,000 simulations (359). To evaluate the effect of selection bias, we conducted analyses stratified by reason for colonoscopy (*e.g.*, routine screening, family history of CRC in a first degree relative). A sensitivity analysis for unmeasured confounding was adopted from ref.(360), and was conducted by varying the prevalence of the unmeasured binary confounder in the exposure and reference group, and the strength of the association between the unmeasured confounder and outcome.

Results

Selected characteristics of cases and controls by study are shown in **Table 2.1**. Cases and controls did not differ considerably with regard to most risk factors; however, there were more males in the case group than in the control group, and controls were more likely to be younger, to regularly take an NSAID or aspirin, to have positive family history of colorectal cancer, to take multivitamins, less likely to be a current smoker, and tended to have lower intakes of red and processed meats and alcohol. Among women, cases were more likely to be postmenopausal, and among postmenopausal women, controls were more likely to use hormone replacement therapy. In the CPRU and MAP studies, mean plasma 25-(OH)-vitamin D_3 concentrations were slightly statistically non-significantly higher in controls than in adenoma cases (**Table 2.1**).

Among all cases, 32% had at least one adenoma located in the right colon, 32% had multiple adenomas, and 32% had an adenoma that was ≥ 1 cm in diameter. In 24% of cases the largest or most advanced adenoma was located in the right colon. The largest or most advanced adenoma had a pedunculated shape in 28% of cases, and villous or tubulovillous histology in 30% of all cases (data now shown).

Vitamin D deficiency, defined as 25-(OH)-vitamin D_3 of < 20 ng/mL, was relatively common in both studies. In the CPRU study, 33.3% of participants were vitamin D deficient, and only 23.0% had sufficient levels of 25-(OH)-vitamin D_3 above 32 ng/mL. In the MAP study, 29.8% of participants were vitamin D deficient, and 31.8% were vitamin D sufficient. When we pooled the studies, 32.4% and 25.2% of participants were vitamin D deficient and sufficient, respectively.

The multivariable-adjusted study-specific and pooled ORs for the association of blood 25-(OH)-vitamin D_3 with incident, sporadic, colorectal adenoma are shown in **Table 2.2**. The median with the lower 25th and upper 75th percentiles of study- and month-specific 25-(OH)-vitamin D_3 values among controls (**Figure 2.1**) were used to define 25-(OH)-vitamin D_3 quartiles. In the pooled analysis, higher levels of 25-(OH)-vitamin D_3 were associated with a statistically significant 41% reduction in colorectal adenoma risk after multivariable adjustment (highest *versus* lowest month- and study-specific quartile, odds ratio [OR] = 0.59, 95% confidence interval [CI]: 0.41–0.84; P_{trend} = 0.01). Similar inverse associations were observed in the separate analyses of the CPRU and the MAP studies. However, the association was the strongest in the MAP study (OR = 0.35, 95% CI: 0.17–0.70; P_{trend} = 0.003). Additional analyses using study- and season-specific quintiles, or the categories defined based on suggested guidelines (< 20 ng/mL for deficiency and≥ 33 ng/mL for sufficiency) did not differ substantially from those reported (data not shown). We also analyzed circulating 25-(OH)-vitamin D_3 as a continuous variable in the multivariable model (adjusted additionally for season of blood draw) and found a marginally statistically significant 11% decrease in colorectal adenoma risk per 10 ng/mL increment in 25-(OH)-vitamin D_3 in the pooled study (OR = 0.89, 95% CI: 0.79–1.00, P = 0.06). The inverse association of circulating 25-(OH)-vitamin D_3 with adenoma did not differ substantially according to adenoma characteristics (**Table 2.2**); however, the sample size was relatively small for these analyses.

We also examined whether the 25-(OH)-vitamin D_3–adenoma association was modified by various demographic/lifestyle (**Table 2.3**), and dietary risk factors (**Table**

2.4) for colorectal neoplasms. The inverse association of circulating 25-(OH)-vitamin D_3 with colorectal adenomas was stronger among those who took regularly aspirin or other non-steroidal anti-inflammatory drugs (NSAIDs); for those in the upper quartile of circulating 25-(OH)-vitamin D_3, there was a statistically significant, approximately 67% lower risk for colorectal adenoma (OR = 0.33, 95% CI: 0.19–0.56; $P_{\text{interaction}} = 0.04$; **Table 2.3**). Further, higher levels of circulating 25-(OH)-vitamin D_3 were associated with statistically significant 53% and 48% reductions in adenoma risk among older and more physically active participants, respectively (**Table 2.3**). The associations of higher 25-(OH)-vitamin D_3 with adenoma did not differ substantially by sex and obesity, and among women, by menopausal status and hormone replacement therapy (HRT), and in the MAP study, by levels of C-reactive protein (data not shown). There were no substantial differences in the 25-(OH)-vitamin D_3 –adenoma association according to strata (< or ≥median) of dietary intakes of calcium, retinol, folate, and soy (**Table 2.4**). The investigation of the joint and combined effects of 25-(OH)-vitamin D_3 with dietary intakes on adenoma risk yielded similar results (data not shown).

Further, we examined whether the association between the highest versus lowest quartiles of 25-(OH)-vitamin D_3 and colorectal adenoma is sensitive to differential and/or non-differential misclassification of exposure (**Table 2.5**). Assuming non-differential misclassification of 25-(OH)-vitamin D_3 status (with minimum 75%, modes of 85 and 95%, and a maximum of 100% for specificity and sensitivity), the median corrected odds ratio comparing the highest *versus* lowest quartile of 25-(OH)-vitamin D_3 was 0.52 with the 95% simulation limits 0.40 to 0.67. When we included random error, the 95% simulation limits were 0.33 and 0.82. Under differential misclassification (sensitivity and specificity among cases: min = 75%, mode = 85 to 95%, max = 100%; sensitivity and specificity among controls: min = 70%, mode = 80 to 90%, max = 95%), the median corrected odds ratio comparing the highest *versus* lowest quartile of 25-(OH)-vitamin D_3 was 0.51 with the 95% simulation limits 0.31 to 0.79. When we included random error, the 95% simulation limits were 0.28 and 0.89. The results of the selection bias analysis suggested that the 25-(OH)-vitamin D_3–colorectal adenoma association was stronger among participants who underwent routine screening colonoscopy in the MAP study compared to those who underwent non-routine screening colonoscopy (**Table 2.6**); however, the sample size for this analysis was very small, and data on routine screening colonoscopy were not available in the CPRU study. Unmeasured confounder could account for the observed 25-(OH)-vitamin D_3 – colorectal adenoma association only if it is strongly associated with the colorectal adenoma risk, and if its distribution is highly unbalanced among participants with the highest and the lowest levels of 25-(OH)-vitamin D_3 (**Table 2.7**). For example, if there was a strong association between unmeasured binary confounder and colorectal adenoma risk (OR = 0.5), and the prevalence of the unmeasured confounder variable was 90% among those with the highest levels of 25-(OH)-vitamin D_3 (quartile 5), and 10% among those with the lowest levels of 25-(OH)-vitamin D_3 (quartile 1), only then the corrected odds ratio for colorectal adenoma risk for the high 25-(OH)-vitamin D_3 group would become greater than 1 and insignificant.

Discussion

In this pooled study, the largest colorectal adenoma case-control study to date, we observed a substantial statistically significant inverse association between circulating 25-(OH)-vitamin D_3 concentrations and risk of incident, sporadic colorectal adenomas. Our results also suggested that this association may be stronger in participants who regularly take NSAID or aspirin, but that the association did not substantially differ by other demographic, lifestyle, or dietary risk factors, or according to adenoma characteristics.

There is strong biologic plausibility and animal experimental and human evidence for protection against colorectal neoplasms by vitamin D. Proposed mechanisms for vitamin D involve bile acid catabolism, direct effects on the cell cycle, growth factor signaling, and immunomodulation (7, 221). Anti-neoplastic effects of vitamin D on colon tissue are also supported by recent findings that normal colorectal epithelium expresses the vitamin D receptor (VDR) and vitamin D metabolizing enzymes (CYP27B1 and CYP24A1) and therefore can locally produce and degrade the active form of vitamin D, 1,25-(OH)-vitamin D, from 25-(OH)-vitamin D (262, 343, 344).

In epidemiologic studies that investigated dietary vitamin D intake without considering exposure to UVB light, the association between vitamin D intake and colorectal neoplasms was not consistent (221). This inconsistency between these studies may be explained by an underestimation of the main effect as a result of misclassification of actual vitamin D exposure. All epidemiologic studies that examined the circulating vitamin D levels (21, 22, 210, 214, 235-237), except one (236), found inverse associations of 25-(OH)-vitamin D with colorectal adenomas. Four studies (21, 22, 214, 237) reported statistically significant results overall (22, 214), or in women (21), or in patients randomized to receive calcium supplementation (237). A recent meta-analysis of the overall associations of circulating 25-(OH)-vitamin D and vitamin D intake with colorectal adenoma found that both 25-(OH)-vitamin D and vitamin D intake were inversely associated with incident and recurrent colorectal adenomas (238), but only the finding for 25-(OH)-vitamin D was strong and statistically significant. High versus low circulating 25-(OH)-vitamin D was associated with a statistically significant 30% decreased risk of colorectal adenomas (OR = 0.70, 95% CI: 0.56–0.87); whereas high compared to low vitamin D intake (*i.e.*, from diet and supplements) was associated with an 11% statistically non-significant decreased risk (OR = 0.89, 95% CI: 0.78–1.02) (238). Consistent with these data, we found that high *versus* low 25-(OH)-vitamin D_3 concentration was statistically significantly associated with a 41% decreased risk of incident, sporadic colorectal adenoma.

Circulating vitamin D level is a better marker of vitamin D exposure than indirect estimates of vitamin D exposure based solely on a diet due to its long half-life in the circulation and lack of tight homeostatic regulation of its concentration (219). 25-(OH)-vitamin D reflects vitamin D supply and usage over a period of time (219). However, the use of circulating 25-(OH)-vitamin D levels as vitamin D exposure must take into account seasonal variations in vitamin D levels. Previously discussed epidemiologic studies of 25-(OH)-vitamin D and colorectal adenoma tried to avoid potential bias from such seasonal variation by including in their analysis the season of blood draw either by matching on the date of blood draw (236), or including the month of the blood draw in the model (21, 22, 214, 235, 237). In our analyses we used study- and month-specific 25-(OH)-vitamin D_3 cut-points, which were found in simulation studies to be a preferred method for accounting for seasonal variability in 25-(OH)-vitamin D levels (357).

Inadequate blood vitamin D levels are common in the U.S. and in many populations worldwide (239, 361). From the evolutionary perspective, Paleolithic humans were primarily outdoor hunter-gatherers exposed to a lot of sunlight (200). Lifeguards and dark skinned persons from sub-Saharan regions who spend most of their times outdoors were found to have 25-(OH)-vitamin D blood levels greater than 60 ng/mL (240). Based on several lines of evidence, vitamin D insufficiency was defined as having 25-(OH)-vitamin D blood levels less than 33 ng/ml, and deficiency as < 20 ng/mL (358). By these definitions, in our study, 32% of participants were vitamin D deficient (< 20 ng/mL), and only 25% were vitamin D sufficient (≥ 33 ng/mL). There is no clear definition of "normal" or adequate or sufficient levels with respect to blood 25-(OH)-vitamin D (240); and the optimal vitamin D dose or blood level for anti-neoplastic effects in humans is unknown. However, consistent with our results, one pooled study suggested that a serum 25-(OH)-vitamin D level of 33 ng/mL or higher is associated with a 50% decrease in colorectal cancer incidence when compared to serum 25-(OH)-vitamin D < 12 ng/mL (241).

Regular aspirin or NSAID use reduces colorectal neoplasms risk (107, 362, 363). The major mechanism of their anti-neoplastic action is inhibition of the pro-inflammatory COX-2 pathway. Pro-inflammatory markers such as TNFα may interfere with vitamin D signaling through the NFκB pathway by decreasing the transcription efficiency of vitamin D-responsive genes (250) and down-regulating the human 1α-hydroxylase (CYP27B1) promoter (364). In turn, the active form of vitamin D, 1,25-$(OH)_2$-vitamin D, and/or its analogs may inhibit the activity of COX-2 (365, 366), modulate arachidonic acid release, decrease PGE_1 and E_2 levels (367), and induce expression of 15-prostaglandin dehydrogenase (366). There were no human clinical trials testing the combined effect of vitamin D and NSAIDs on colorectal neoplasms incidence or recurrence. In the previously published results from the MAPI case-control study, a statistically non-significant 16% reduction in colorectal adenoma risk was found among NSAID users with the highest total vitamin D intake; whereas among NSAID non-users with the highest total vitamin D intake a statistically non-significant 26% increase in colorectal adenoma risk was found (208). Consistent with the synergistic effects of higher 25-(OH)-vitamin D_3 and NSAIDs use on colorectal adenoma risk, in our pooled study we found that the inverse association between circulating 25-(OH)-vitamin D_3 and colorectal adenoma was stronger among persons who regularly take aspirin or other NSAIDs ($P_{\text{interaction}} = 0.04$); however, this interaction was marginally significant and requires further research.

Obese individuals have a chronic low-grade inflammation, which is characterized by production of pro-inflammatory cytokines (*e.g.*, TNFα and IL-6) by adipose tissue (368). Furthermore, adipose tissue stores fat-soluble vitamin D_3 resulting in lower circulating 25-(OH)-vitamin D_3 levels. In addition, more frequent physical activity may be associated with decreased inflammation, as indicated by decreased C-reactive protein (CRP) levels, among U.S. adults (369, 370). Consistent with our results for NSAIDs, we also found that the 25-(OH)-vitamin D_3–colorectal adenoma association was suggestively stronger in non-obese ($P_{\text{interaction}} = 0.14$), and more physically active participants ($P_{\text{interaction}} = 0.06$). However, in the MAP study, we did not observe a stronger inverse association between 25-(OH)-vitamin D_3 and colorectal adenoma among persons with low *versus* high CRP levels; but the sample size for this analysis was relatively small.

Vitamin D and calcium are highly physiologically inter-related, and both agents are thought to be important in colorectal carcinogenesis. They both influence bile-acid metabolism, and modulate multiple proteins and genes involved in colorectal carcinogenesis (221). Despite the biologic plausibility, few observational epidemiologic studies investigated whether vitamin D and calcium synergistically modify risk for colorectal adenoma, and among them very few presented complete data for assessing a potential interaction (24, 210, 237). In a secondary analysis of a calcium and adenoma recurrence trial (n = 803), a statistically significant 12% reduction in adenoma recurrence per 12 ng/mL increase of 25-(OH)-vitamin D was found among patients who received calcium supplements, but not among those who received the placebo ($P_{\text{interaction}} = 0.006$) (237). In the Nurses' Health Study (n = 2,747), women who had both the highest total calcium and vitamin D intakes had the lowest adenoma risk when compared with the opposite extreme (24). However, in one large sigmoidoscopy-based case-control study (n = 980), participants with high circulating 25-(OH)-vitamin D and low calcium intake had a statistically significant 60% decrease in colorectal adenoma risk, whereas those with high circulating 25-(OH)-vitamin D and high calcium intake a statistically non-significant 17% increase in colorectal adenoma risk (210). Furthermore, in a meta-analysis of four 25-(OH)-vitamin D epidemiologic studies (22, 210, 214, 237) that stratified by calcium intake, an inverse 25-(OH)-vitamin D–adenoma association was found for both, high (OR = 0.67, 95% CI: 0.46–0.97) and low calcium intakes (OR = 0.78, 95% CI: 0.54–1.12), with a stronger association among those with high calcium intake (238). Our findings did not support the hypothesis that the 25-(OH)-vitamin D_3–adenoma association differs by calcium intake.

There are other agents or conditions for which there are biologically plausible reasons to suggest that they may modify the association of vitamin D with colorectal neoplasms. Retinol may antagonize the actions of vitamin D by competing for the same substrate, the retinoid X receptor (247-249), and thus, a high dietary intake of retinol may diminish protective effects of vitamin D. At least one observational study, the Nurses' Health Study (*n* = 48,115) (24), found that risk of colorectal adenoma was lowest in persons with high vitamin D/low retinol intake compared with those with low vitamin D/high retinol intake (RR = 0.55, 95% CI: 0.28–1.10, $P_{\text{interaction}} = 0.02$). However, consistent with another report (371), we also found no evidence for a vitamin D–retinol interaction. In mice studies, soy products, which contain phytoestrogens, were found to up-regulate CYP27B1 and down-regulate CYP24 in colon (53); and in human studies, hormone replacement therapy with estrogens was found to increase vitamin D – binding protein levels (372-374). Moreover, one human study found that an estrogen intervention activated the VDR pathway in the rectal mucosa of postmenopausal women (251). However, findings from the reanalysis of the Women's Health Initiative trial data (*n* = 36,282) indicated that calcium plus vitamin D supplementation statistically non-significantly reduced colorectal cancer risk by 29% among women not taking hormone replacement therapies (HRTs), whereas among women concurrently assigned to estrogen-progestin or estrogen-only therapies, calcium and vitamin D supplementation statistically non-significantly increased colorectal cancer risk ($P_{\text{interaction}} = 0.04$) (375). In a nested case-control study (*n* = 791) within the Prostate, Lung, Colorectal and Ovarian Cancer Screening (PLCO) trial, serum 25-(OH)-vitamin D levels were significantly higher in current users of HRT than in former or never HRT users, however the inverse association between 25-(OH)-vitamin D and colorectal adenoma risk was similar in both groups ($P_{\text{interaction}} = 0.43$) (21). In our study the inverse association between 25-(OH)-vitamin D_3 and colorectal adenoma was also consistent across strata of soy intake, and by menopausal and HRT status in women. Though mice studies found that folic acid supplementation may epigenetically regulate the colonic expression of the VDR and vitamin D-metabolizing enzymes (53), and, therefore, influence vitamin D metabolism and function, we found no evidence that the 25-(OH)-vitamin D_3–adenoma association varied by various levels of folate intake.

Out of two 25-(OH)-vitamin D forms (D_2 and D_3) measured in blood, we used 25-(OH)-vitamin D_3 as a primary measure of vitamin D status in our analysis. The primary reason for this was the poor intra-assay reproducibility for 25-(OH)-vitamin D_2. Although 25-(OH)-vitamin D_2 contributes to total circulating 25-(OH)-vitamin D, we expect this to be minimal. In our data, the vast majority of participants had undetectable or very low levels of 25-(OH)-vitamin D_2. Unlike vitamin D_3, vitamin D_2 cannot be synthesized by humans and is present mostly in fungus/yeast-derived products. In addition, vitamin D_2 may have lower bioefficacy compared to vitamin D_3, which may be due to lower binding affinities of vitamin D_2 and its metabolites to the vitamin D receptor (VDR), vitamin D binding protein (DBP), and CYP27A1 enzyme (reviewed in ref. (356)); however, more research is needed to understand the biological differences between the two vitamin D forms. Our additional analyses of total 25-(OH)-vitamin D and weighted total 25-(OH)-vitamin D were consistent with those reported for 25-(OH)-vitamin D_3. Therefore, the potential misclassification of participants' vitamin D status due to excluding circulating 25-(OH)-vitamin D_2 concentrations appears negligible.

An active metabolite of vitamin D, 1,25-$(OH)_2$-vitamin D, was not measured and investigated in this study as it has a short half-life in the circulation, its production in the body is tightly regulated according to serum calcium levels, and its normal concentrations are maintained even with vitamin D deficiency. Moreover, none of the previous studies that examined it (21, 236, 237, 376) found an association between 1,25-$(OH)_2$-vitamin D and colorectal neoplasms (reviewed in ref. (221)).

Strengths of this study include: 1) verification of the adenoma- and hyperplastic polyp-free status of controls by colonoscopy, resulting in reduction of the outcome misclassification; 2) in each study, cases and controls came from the same population; 3) collection of information on potential confounders/effect modifiers before case-control status was ascertained, thereby reducing recall bias; 4) collection of detailed data on demographic characteristics, lifestyle and behavior, medical history, medications, family history of cancer, diet, and polyp pathology, thereby reducing unmeasured confounding; and 5) the use of circulating 25-(OH)-vitamin D_3 levels as the main vitamin D exposure instead of poorly measured dietary vitamin D intake, thereby reducing misclassification of the true vitamin D exposure. Finally, this study is the largest pooled case-control study of incident colorectal adenomas reported to date.

Because the study population included only older White individuals who underwent colonoscopy, results from this analysis may not be representative of the general population. Colonoscopy controls in the CPRU study (which was conducted between 1991 and 1994, before the use of colonoscopies for routine screening purposes) represent a highly selected group of participants, 44% of whom had gastrointestinal (GI) bleeding, 26% had family history of colorectal cancer in a first degree relative, and 30% had gastrointestinal symptoms (*e.g.*, abdominal pain, constipation, diarrhea). Data on routine screening as indication for colonoscopy were not collected in the CPRU study. In the MAP study, 25% of controls had colonoscopy for routine screening purposes, and 37%, 21%, and 17% of controls had colonoscopy due to GI bleeding, family history of CRC, or GI symptoms, respectively. Because majority of the participants in these studies had an indication for undergoing a colonoscopy, vitamin D status and exposure to some lifestyle and dietary risk factors may have been similar between cases and controls, resulting in attenuation of the results toward the null. Therefore, in asymptomatic individuals undergoing colonoscopy for routine purposes, the association between circulating vitamin D and colorectal neoplasms risk may be stronger than the association found in this study. The latter was consistent with our analyses stratified by reason for colonoscopy, in which the 25-(OH)-vitamin D_3–colorectal adenoma association was stronger among participants who were asymptomatic or underwent routine screening colonoscopy in the MAP study. Circulating 25-(OH)-vitamin D_3 concentration may fluctuate because of seasonal variation in sun exposure; therefore measuring 25-(OH)-vitamin D concentration from a single sample as in the current study may yield some exposure misclassification. To minimize this, in our analyses we used study- and month-specific 25-(OH)-vitamin D_3 cut-points as described in ref. (357). Although, this pooled study had 616 colorectal adenoma cases and 770 controls, the sample size for some subgroup analyses was still small.

The results of sensitivity analyses suggested that the association between high circulating 25-(OH)-vitamin D_3 levels and colorectal adenoma was robust to differential or non-differential misclassification of exposure, and unmeasured confounding.

In conclusion, our findings strongly support the hypothesis that higher vitamin D_3 exposures may reduce risk for incident, sporadic colorectal adenoma. Our findings also suggest that vitamin D exposures may synergize with anti-inflammatory agents to more markedly reduce risk for colorectal neoplasms. This potential interaction, as well as potential interactions with genetic variants in vitamin D pathway related genes and the various other potential modifying factors investigated in this study need to be investigated in other studies and populations.

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Notes

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Tables and Figures

Figure 2.1. Circulating 25-(OH)-vitamin D_3 concentrations by month of blood draw in the CPRU and MAP studies among colonoscopy-negative controls. The solid (▬) and dashed (▪▪▪) lines connect median month-specific 25-(OH)-vitamin D_3 values for the CPRU and MAP studies, respectively. Vertical bars represent the lower 25th and upper 75th percentiles of month-specific 25-(OH)-vitamin D_3 values in the CPRU (■, square) and MAP (●, circle) studies.

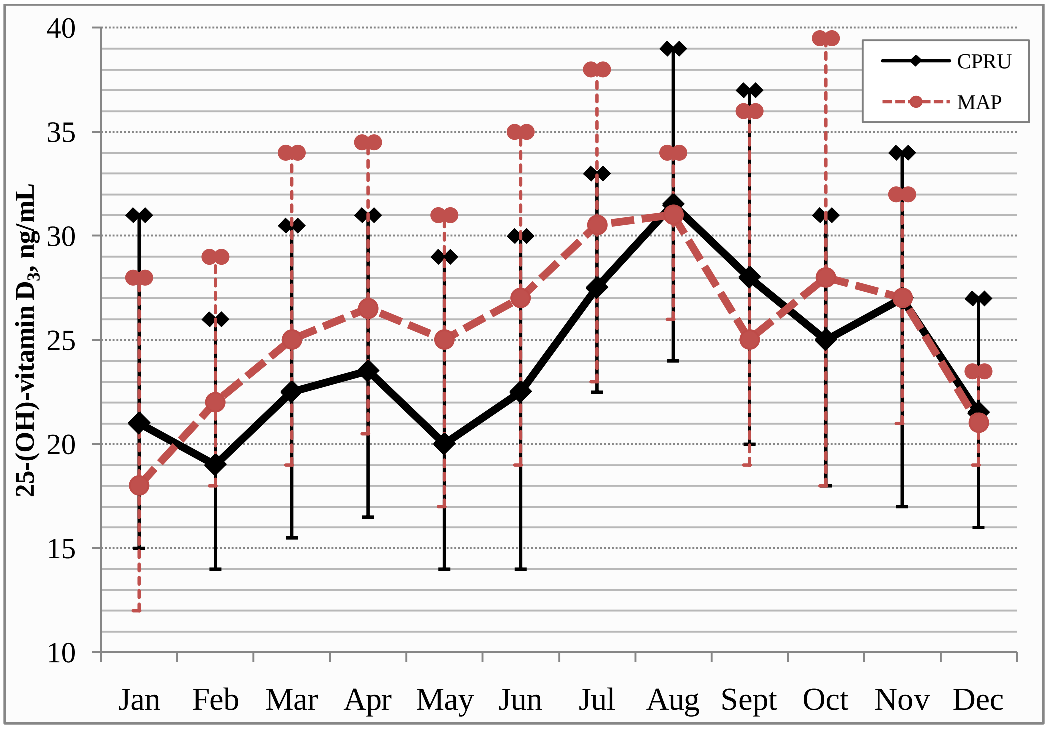

Table 2.1. Selected characteristics and mean circulating 25-(OH)-vitamin D_3 concentrations in cases and controls in three case-control studies of incident, sporadic colorectal adenomas.

Characteristic*	**CPRU study, MN**		**MAP study, NC and SC**		**Pooled**	
	Cases (*n* = 474)	Controls (*n* = 563)	Cases (*n* = 142)	Controls (*n* = 207)	Cases (*n* = 616)	Controls (*n* = 770)
Age, y	58.2 (9.7)	52.7 (11.0)°	57.5 (8.3)	55.9 (9.4)	58.0 (9.4)	53.6 (10.7)°
Male, %	62	39°	56	44°	60	39°
College graduate, %	30	28	22	31	28	29
25-(OH)-vitamin D_3, ng/mL	24.0 (9.7)	24.9 (10.5)	26.1 (11.6)	27.3 (11.4)	24.5 (10.2)	25.5 (10.8)
Season of blood donation, %**						
Winter	27	23	18	19	25	22
Spring	27	27	37	38	30	30
Summer	21	23	24	28	22	24
Fall	24	27	21	15	23	24
C-reactive protein (hs-CRP), mg/L	N/A	N/A	5.8 (6.4)	4.4 (5.5)$^{\bullet}$	N/A	N/A
Family history of CRC$^{\$}$, %	13	30°	18	30°	14	30°
Regular take NSAID$^{¥}$, %§	12	20°	23	35$^{\bullet}$	15	24°
Regular take aspirin, %§	28	31	37	38	30	33
If a woman:						
Postmenopausal, %	84	68°	83	81	83	71°
HRT user, %$^{¤}$	44	67°	71	71	51	68°
Current smoker, %	20	16°	34	14°	23	15°
Body mass index (BMI), kg/m^2	27.4 (4.8)	26.9 (5.0)	27.8 (6.2)	27.4 (6.0)	27.5 (5.1)	27.1 (5.3)
Physical activity, MET-hr/wk	270.4 (283.1)	233.4 (218.9)$^{\bullet}$	193.8 (139.4)	183.5 (128.4)	253.0 (259.5)	220.2 (200.2)$^{\bullet}$

(Table continues)

Table 2.1 (continued).

Characteristic*	CPRU study, MN		MAP study, NC and SC		Pooled	
	Cases (*n* = 474)	Controls (*n* = 563)	Cases (*n* = 142)	Controls (*n* = 207)	Cases (*n* = 616)	Controls (*n* = 770)
Multivitamin supplement use, %	23	31	33	39	26	33
Vitamin D supplement user, %	19	25•	30	36	22	28°
Dietary intakes per day:						
Total energy intake, kcal	2,115 (789)	2,007 (711)•	2,069 (862)	1,780 (923)°	2,104 (806)	1,946 (780)°
Dietary vitamin D, IU	236 (154)	226 (143)	202 (124)	164 (111)°	228 (148)	209 (138)•
Total vitamin D, IU[§§]	327 (256)	328 (243)	349 (270)	345 (300)	331 (259)	332 (259)
Calcium, mg[§§]	962 (529)	987 (523)	856 (458)	893 (492)	937 (515)	962 (516)
Retinol, IU[§§]	3,039 (2,981)	3,327 (3,772)	3,474 (3,512)	3,752 (4,913)	3,140 (3,115)	3,442 (4,112)
Folate, µg[§§]	400 (232)	408 (240)	466 (246)	471 (279)	414 (237)	425 (253)
Red and processed meats intake, servings	7.5 (6.3)	6.6 (5.1)•	8.4 (8.8)	6.9 (6.4)	7.7 (7.0)	6.6 (5.5)
Dietary fiber, gm	21.9 (9.8)	21.6 (9.6)	21.9 (9.8)	19.2 (11.1)•	21.9 (9.8)	20.9 (10.1)
Alcohol, g	10.0 (16.6)	6.5 (13.4)°	7.2 (14.6)	4.5 (9.6)•	9.4 (16.2)	6.0 (12.6)°

* Data are given as means (SD) unless otherwise specified.

** Seasons defined as winter: December, January, February; Spring: March, April, May; Summer: June, July, August; Fall: September, October, November

$ Family history of colorectal cancer in a first degree relative

¥ Nonsteroidal anti-inflammatory drug.

§ At least once a week.

§§ Diet plus supplements.

¤ Percentage of women using HRT was calculated among postmenopausal women only

• Indicates $P < 0.05$ compared with adenoma cases; by Fisher's exact test for categorical variables, and t-test for continuous variables

° Indicates $P < 0.01$ compared with adenoma cases; by Fisher's exact test for categorical variables, and t-test for continuous variables

Table 2.2. Multivariable-adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for associations of circulating 25-(OH)-vitamin D_3 concentrations with colorectal adenoma overall and by adenoma characteristics in the CPRU, MAP (combined MAPI and MAPII), and pooled studies.

Adenoma characteristic	Quartile of $25(OH)D_3$	**CPRU**		**MAPs**		**Pooled analysis**	
		n (cases/controls)	Multivariate OR (95%CI)*	*n* (cases/controls)	Multivariate OR (95%CI)*	*n* (cases/controls)	Multivariate OR (95%CI)*
All colorectal adenomas	1	110/126	1.00 (ref)	44/44	1.00 (ref)	154/170	1.00 (ref)
	2	130/150	0.85 (0.57-1.27)	29/54	0.61 (0.30-1.22)	159/204	0.77 (0.55-1.09)
	3	132/136	0.99 (0.66-1.48)	35/48	0.55 (0.27-1.10)	167/184	0.85 (0.60-1.20)
	4	102/151	0.69 (0.45-1.06)	34/61	0.35 (0.17-0.70)	136/212	0.59 (0.41-0.84)
$P_{trend}^{\perp}$			*0.16*		*0.003*		*0.01*
Location							
Right colon†	1	36/126	1.00 (ref)	19/44	1.00 (ref)	55/170	1.00 (ref)
	2	43/150	0.76 (0.42-1.38)	13/54	0.81 (0.32-2.07)	56/204	0.76 (0.46-1.24)
	3	31/136	0.68 (0.36-1.31)	10/48	0.34 (0.12-0.97)	41/184	0.58 (0.34-0.98)
	4	27/151	0.52 (0.27-1.03)	17/61	0.44 (0.18-1.09)	44/212	0.50 (0.29-0.85)
$P_{trend}^{\perp}$			*0.06*		*0.04*		*0.007*
Left colon‡	1	94/126	1.00 (ref)	36/44	1.00 (ref)	130/170	1.00 (ref)
	2	102/150	0.82 (0.54-1.26)	24/54	0.57 (0.27-1.19)	126/204	0.76 (0.53-1.08)
	3	120/136	1.06 (0.70-1.62)	30/48	0.58 (0.28-1.23)	150/184	0.92 (0.63-1.32)
	4	89/151	0.71 (0.45-1.11)	24/61	0.30 (0.14-0.65)	113/212	0.58 (0.40-0.85)
$P_{trend}^{\perp}$			*0.30*		*0.003*		*0.02*
Multiplicity							
Multiple adenomas	1	35/126	1.00 (ref)	17/44	1.00 (ref)	52/170	1.00 (ref)
	2	35/150	0.70 (0.37-1.30)	11/54	0.61 (0.23-1.67)	46/204	0.69 (0.41-1.15)
	3	41/136	1.00 (0.53-1.87)	13/48	0.53 (0.19-1.44)	54/184	0.85 (0.51-1.43)
	4	29/151	0.57 (0.29-1.13)	18/61	0.46 (0.18-1.17)	47/212	0.55 (0.32-0.93)
$P_{trend}^{\perp}$			*0.25*		*0.11*		*0.06*

(Table continues)

Table 2.2 (continued).

Adenoma characteristic	Quartile of 25(OH)D_3	**CPRU**		**MAPs**		**Pooled analysis**	
		n (cases/controls)	Multivariate OR (95%CI)*	*n* (cases/controls)	Multivariate OR (95%CI)*	*n* (cases/controls)	Multivariate OR (95%CI)*
Single adenoma	1	75/126	1.00 (ref)	29/44	1.00 (ref)	104/170	1.00 (ref)
	2	95/150	0.97 (0.63-1.49)	18/54	0.59 (0.27-1.32)	113/204	0.83 (0.57-1.21)
	3	91/136	1.04 (0.66-1.62)	22/48	0.54 (0.24-1.18)	113/184	0.87 (0.60-1.27)
	4	73/151	0.75 (0.47-1.20)	16/61	0.26 (0.11-0.60)	89/212	0.60 (0.40-0.89)
P_{trend}[⊥]			*0.29*		*0.002*		*0.02*
Size[§§]							
Large adenoma	1	33/126	1.00 (ref)	13/44	1.00 (ref)	46/170	1.00 (ref)
≥ 1 cm	2	38/150	0.87 (0.48-1.59)	9/54	0.69 (0.24-2.00)	47/204	0.82 (0.49-1.37)
	3	44/136	1.26 (0.69-2.30)	10/48	0.54 (0.18-1.62)	54/184	0.98 (0.59-1.63)
	4	39/151	0.99 (0.53-1.85)	10/61	0.34 (0.12-0.99)	49/212	0.75 (0.44-1.26)
P_{trend}[⊥]			*0.71*		*0.04*		*0.40*
Small adenoma	1	77/126	1.00 (ref)	31/44	1.00 (ref)	108/170	1.00 (ref)
< 1 cm	2	92/150	0.88 (0.56-1.36)	20/54	0.63 (0.29-1.39)	112/204	0.79 (0.54-1.15)
	3	88/136	0.92 (0.58-1.45)	25/48	0.61 (0.29-1.32)	113/184	0.83 (0.57-1.22)
	4	63/151	0.55 (0.34-0.90)	24/61	0.36 (0.17-0.78)	87/212	0.52 (0.34-0.77)
P_{trend}[⊥]			*0.03*		*0.01*		*0.003*
Shape							
Pedunculated	1	30/126	1.00 (ref)	11/44	1.00 (ref)	41/170	1.00 (ref)
	2	25/150	0.53 (0.27-1.05)	6/54	0.44 (0.12-1.64)	31/204	0.53 (0.30-0.96)
	3	33/136	1.11 (0.57-2.16)	5/48	0.38 (0.10-1.49)	38/184	0.81 (0.46-1.44)
	4	22/151	0.58 (0.28-1.20)	6/61	0.28 (0.07-1.06)	28/212	0.47 (0.25-0.86)
P_{trend}[⊥]			*0.50*		*0.65*		*0.06*
Sessile	1	51/126	1.00 (ref)	32/44	1.00 (ref)	83/170	1.00 (ref)
	2	80/150	1.21 (0.74-1.95)	22/54	0.69 (0.32-1.49)	102/204	1.01 (0.68-1.50)
	3	70/136	1.20 (0.73-1.97)	29/48	0.66 (0.31-1.40)	99/184	1.01 (0.67-1.51)
	4	52/151	0.78 (0.46-1.33)	27/61	0.38 (0.18-0.81)	79/212	0.65 (0.42-0.99)
P_{trend}[⊥]			*0.31*		*0.01*		*0.05*

(Table continues)

Table 2.2 (continued).

Adenoma characteristic	Quartile of 25(OH)D_3	CPRU		MAPs		Pooled analysis	
		n (cases/controls)	Multivariate OR (95%CI)*	*n* (cases/controls)	Multivariate OR (95%CI)*	*n* (cases/controls)	Multivariate OR (95%CI)*
Histological type							
Villous or tubulovillous	1	42/126	1.00 (ref)	6/44	1.00 (ref)	48/170	1.00 (ref)
	2	42/150	0.75 (0.43-1.33)	4/54	0.43 (0.08-2.28)	46/204	0.73 (0.43-1.24)
	3	43/136	0.95 (0.53-1.69)	3/48	0.23 (0.04-1.42)	46/184	0.84 (0.49-1.44)
	4	28/151	0.69 (0.38-1.27)	6/61	0.36 (0.08-1.63)	44/212	0.66 (0.38-1.14)
$P_{trend}^{\perp}$			*0.39*		*0.20*		*0.22*
Tubular	1	68/126	1.00 (ref)	38/44	1.00 (ref)	106/170	1.00 (ref)
	2	87/150	0.94 (0.60-1.48)	25/54	0.63 (0.31-1.30)	112/204	0.82 (0.56-1.18)
	3	89/136	1.08 (0.68-1.71)	32/48	0.62 (0.30-1.26)	121/184	0.91 (0.62-1.33)
	4	64/151	0.70 (0.43-1.13)	27/61	0.32 (0.16-0.68)	91/212	0.56 (0.38-0.84)
$P_{trend}^{\perp}$			*0.22*		*0.004*		*0.01*
Degree of atypia of the worst adenoma							
Mild	1	45/126	1.00 (ref)	15/44	1.00 (ref)	60/170	1.00 (ref)
	2	67/150	1.05 (0.64-1.74)	10/54	0.85 (0.31-2.35)	77/204	1.00 (0.64-1.54)
	3	65/136	1.19 (0.71-2.00)	16/48	1.01 (0.38-2.69)	81/184	1.17 (0.75-1.83)
	4	35/151	0.53 (0.30-0.95)	7/61	0.24 (0.07-0.77)	42/212	0.49 (0.30-0.80)
$P_{trend}^{\perp}$			*0.06*		*0.04*		*0.02*
Moderate/severe	1	65/126	1.00 (ref)	29/44	1.00 (ref)	94/170	1.00 (ref)
	2	63/150	0.73 (0.45-1.20)	19/54	0.51 (0.23-1.17)	82/204	0.66 (0.44-1.00)
	3	67/136	0.86 (0.52-1.41)	19/48	0.32 (0.14-0.75)	86/184	0.67 (0.44-1.02)
	4	67/151	0.77 (0.47-1.28)	26/61	0.34 (0.16-0.73)	93/212	0.63 (0.42-0.95)
$P_{trend}^{\perp}$			*0.50*		*0.005*		*0.05*

* OR – odds ratio with 95% confidence interval; adjusted for age (continuous), sex, family history of colorectal cancer in a first degree relative, regular use of aspirin or NSAIDs, smoking (current, ever, or never), physical activity (continuous), BMI (continuous), total red and processed meat intake (continuous), alcohol intake (continuous), calcium intake (continuous), retinol intake (continuous), and folate intake (continuous). Pooled odds ratio adjusted for study (CPRU versus MAP) in addition to other covariates.

⊥ P_{trend} values (two-sided) calculated by including the median of each quartile of blood 25-(OH)-vitamin D_3 as a continuous variable in addition to all above mentioned covariates in the multivariable models.

† At least one adenoma in right colon; right colon includes cecum, ascending colon, hepatic flexure, and transverse colon.

‡ At least one adenoma in left colon; left colon includes splenic flexure, descending colon, sigmoid colon, and rectum.

§ Adenoma size from in vivo comparison of maximum diameter to fully opened endoscope forceps.

Table 2.3. Multivariable-adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for association of study- and month-specific quartile of circulating 25-(OH)-vitamin D_3 concentrations with colorectal adenoma by demographic and lifestyle characteristics in the pooled CPRU and MAP studies.

Characteristic	Quartile of $25(OH)D_3$	*n* (cases/controls)	Multivariate OR (95%CI)*	$P_{interaction}$
Age**				
< median	1	42/69	1.00 (ref)	
	2	38/96	0.58 (0.32-1.06)	
	3	54/93	0.85 (0.48-1.53)	
	4	44/98	0.67 (0.36-1.22)	
$P_{trend}^{\perp}$			*0.44*	
≥ median	1	112/101	1.00 (ref)	
	2	121/108	0.84 (0.55-1.28)	
	3	113/91	0.79 (0.51-1.21)	
	4	91/114	**0.47 (0.30-0.73)**	
$P_{trend}^{\perp}$			*0.001*	*0.35*
Sex				
Men	1	67/39	1.00 (ref)	
	2	106/79	0.82 (0.47-1.43)	
	3	108/81	0.77 (0.45-1.33)	
	4	91/102	0.54 (0.31-0.94)	
$P_{trend}^{\perp}$			*0.02*	
Women	1	87/131	1.00 (ref)	
	2	53/125	0.65 (0.41-1.04)	
	3	59/103	0.90 (0.56-1.46)	
	4	45/110	0.60 (0.36-1.01)	
$P_{trend}^{\perp}$			*0.14*	*0.53*
Obesity[†]				
No	1	108/108	1.00 (ref)	
	2	102/151	0.60 (0.40-0.91)	
	3	121/143	0.73 (0.48-1.09)	
	4	116/177	0.55 (0.36-0.82)	
$P_{trend}^{\perp}$			*0.02*	
Yes	1	41/58	1.00 (ref)	
	2	54/49	1.16 (0.60-2.23)	
	3	44/36	1.10 (0.54-2.23)	
	4	16/30	0.46 (0.20-1.06)	
$P_{trend}^{\perp}$			*0.14*	*0.14*

(Table continues)

Table 2.3 (continued).

Characteristic	Quartile of 25(OH)D_3	*n* (cases/controls)	Multivariate OR (95%CI)*	$P_{interaction}$
Physical activity‡				
< median	1	87/110	1.00 (ref)	
	2	84/99	1.00 (0.63-1.58)	
	3	76/81	1.14 (0.71-1.84)	
	4	51/93	0.55 (0.33-0.91)	
P_{trend} $^{\perp}$			*0.06*	
≥ median	1	67/59	1.00 (ref)	
	2	73/103	0.50 (0.30-0.86)	
	3	90/102	0.58 (0.34-0.97)	
	4	84/118	**0.52 (0.30-0.88)**	
P_{trend} $^{\perp}$			*0.05*	*0.06*
NSAID$^{¥}$ **or aspirin use**				
< once/week	1	77/84	1.00 (ref)	
	2	90/111	0.86 (0.54-1.38)	
	3	109/94	1.33 (0.83-2.14)	
	4	86/105	0.92 (0.56-1.50)	
P_{trend} $^{\perp}$			*0.80*	
≥ once/week	1	77/85	1.00 (ref)	
	2	68/92	0.68 (0.41-1.13)	
	3	56/88	0.47 (0.27-0.79)	
	4	49/106	0.33 (0.19-0.56)	
P_{trend} $^{\perp}$			*0.0001*	*0.04*

[*] OR – odds ratio with 95% confidence interval; adjusted for age (continuous), sex, family history of colorectal cancer in a first degree relative, regular use of aspirin or NSAIDs, study (CPRU versus MAP), smoking (current, ever, or never), physical activity (continuous), BMI (continuous), total red and processed meat intake (continuous), alcohol intake (continuous), calcium intake (continuous), retinol intake (continuous), and folate intake (continuous). Stratification variable not included in the model.

[**] Cut-points calculated based on median distribution in control subjects and were defined as follows for age: CPRU: <52 versus ≥52 years; MAP: <55 versus ≥55 years.

[⊥] P_{trend} values (two-sided) calculated by including median of each quartile of blood 25-(OH)-vitamin D_3 as a continuous variable in addition to all above mentioned covariates in the multivariable models.

[†] Obesity defined as body mass index (BMI) ≥ 30 kg/m^2.

[‡] Cut-points calculated based on median distributions in control subjects and defined as follows for physical activity: CPRU: <166 versus ≥166 METs-hrs/wk; MAP: <165 versus ≥165 METs-hrs/wk.

[¥] Nonsteroidal anti-inflammatory drug.

Table 2.4. Multivariable-adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for association of study- and month-specific quartile of circulating 25-(OH)-vitamin D_3 concentrations with colorectal adenoma by dietary intakes in the pooled CPRU and MAP studies.

Characteristic	Quartile of 25(OH)D_3	*n* (cases/controls)	Multivariate OR (95%CI)*	$P_{\text{interaction}}$
Calcium intake[†]				
< median	1	99/99	1.00 (ref)	
	2	86/108	0.75 (0.48-1.17)	
	3	82/74	0.95 (0.59-1.54)	
	4	68/93	0.58 (0.35-0.95)	
$P_{trend}^{\perp}$			*0.08*	
≥ median	1	54/65	1.00 (ref)	
	2	71/89	0.80 (0.47-1.38)	
	3	84/106	0.84 (0.50-1.42)	
	4	66/116	0.62 (0.36-1.06)	
$P_{trend}^{\perp}$			*0.10*	*0.94*
Retinol intake[‡]				
< median	1	91/91	1.00 (ref)	
	2	91/108	0.81 (0.51-1.28)	
	3	82/82	0.97 (0.60-1.56)	
	4	62/93	0.59 (0.35-0.98)	
$P_{\text{trend}}^{\perp}$			0.10	
≥ median	1	62/73	1.00 (ref)	
	2	66/89	0.75 (0.44-1.26)	
	3	84/98	0.77 (0.46-1.30)	
	4	72/116	0.59 (0.35-1.00)	
$P_{\text{trend}}^{\perp}$			0.07	0.87
Folate intake[§]				
< median	1	81/86	1.00 (ref)	
	2	74/104	0.71 (0.44-1.15)	
	3	79/81	0.92 (0.56-1.51)	
	4	59/101	0.52 (0.31-0.87)	
$P_{\text{trend}}^{\perp}$			0.04	
≥ median	1	72/78	1.00 (ref)	
	2	83/93	0.82 (0.50-1.33)	
	3	87/99	0.78 (0.48-1.28)	
	4	75/108	0.63 (0.38-1.05)	
$P_{\text{trend}}^{\perp}$			0.08	0.64

(Table continues)

Table 2.4 (continued).

Characteristic	Quartile of $25(OH)D_3$	*n* (cases/controls)	Multivariate OR (95%CI)*	$P_{\text{interaction}}$
Soy intake$^{\$}$				
< median	1	59/61	1.00 (ref)	
	2	61/75	0.77 (0.45-1.33)	
	3	73/72	1.02 (0.59-1.76)	
	4	55/85	0.55 (0.31-0.97)	
$P_{\text{trend}}^{\perp}$			0.10	
≥ median	1	94/103	1.00 (ref)	
	2	96/122	0.75 (0.48-1.17)	
	3	93/108	0.73 (0.46-1.15)	
	4	78/124	0.61 (0.38-0.98)	
$P_{\text{trend}}^{\perp}$			0.05	0.58

[*] OR – odds ratio with 95% confidence interval; adjusted for age (continuous), sex, family history of colorectal cancer in a first degree relative, regular use of aspirin or NSAIDs, study (CPRU versus MAP), smoking (current, ever, or never), physical activity (continuous), BMI (continuous), total red and processed meat intake (continuous), alcohol intake (continuous), calcium intake (continuous), retinol intake (continuous), and folate intake (continuous). Stratification variable not included in the model.

[⊥] P_{trend} values (two-sided) calculated by including the median of each quartile of blood 25-(OH)-vitamin D_3 as a continuous variable in addition to all above mentioned covariates in the multivariable models.

[†] Cut-points calculated based on median distribution in control subjects and defined as follows for total (dietary and supplemental) calcium intake: CPRU: <917 versus ≥917 mg/day; MAP: <763 versus ≥ 763 mg/day.

[‡] Cut-points calculated based on median distribution in control subjects and defined as follows for total (dietary and supplemental) retinol intake: CPRU: <2,245 versus ≥2,245 IU/day; MAP: <2,089 versus ≥2,089 IU/day.

[§] Cut-points calculated based on median distribution in control subjects and defined as follows for total (dietary and supplemental) folate intake: CPRU: <327 versus ≥270 µg/day; MAP: <389 versus ≥389 µg/day.

[\$] Cut-points calculated based on median distribution in control subjects and defined as follows for total soy intake: CPRU: <2 versus ≥2 servings/day; MAPI: <3.5 versus ≥3.5 servings/day; MAPII: <2 versus ≥2 servings/day. Cut-points calculated for MAPI and MAPII studies separately since soy intake was assessed differently in these two studies.

Table 2.5. Results of sensitivity analyses correcting for non-differential and differential misclassification of 25-(OH)-vitamin D_3 status.

Misclassification analysis	OR* 2.5th percentile	OR* median	OR* 97.5th percentile	Proportion of simulations with OR < 1
Non-differential misclassification sensitivity analysis				
Sensitivity only	0.40	0.52	0.67	0.999
Sensitivity and random error	0.33	0.52	0.82	0.998
Differential misclassification sensitivity analysis				
Sensitivity only	0.31	0.51	0.79	0.999
Sensitivity and random error	0.28	0.51	0.89	0.990

* OR – odds ratio with 95% confidence interval; adjusted for age (continuous), sex, family history of colorectal cancer in a first degree relative, regular use of aspirin or NSAIDs, study (CPRU versus MAP), smoking (current, ever, or never), physical activity (continuous), BMI (continuous), total red and processed meat intake (continuous), alcohol intake (continuous), calcium intake (continuous), retinol intake (continuous), and folate intake (continuous).

Table 2.6. Multivariable-adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for association of study- and month-specific quartile of circulating 25-(OH)-vitamin D_3 concentrations with colorectal adenoma stratified by reason for colonoscopy in the pooled CPRU and MAP studies.

		Pooled analysis		**MAPs**	
Colonoscopy indication	Quartile of 25(OH)D_3	*n* (cases/controls)	Multivariate OR* (95% CI)	*n* (cases/controls)	Multivariate OR* (95% CI)
Routine screening**					
	1	--	--	16/10	1.00 (ref)
	2	--	--	13/14	0.40 (0.09-1.74)
	3	--	--	17/12	0.55 (0.13-2.36)
	4	--	--	12/16	0.13 (0.03-.59)
P_{trend} [⊥]					*0.015*
Non-routine screening					
	1	138/160	1.00 (ref)	28/34	1.00 (ref)
	2	146/190	0.78 (0.55-1.12)	16/40	0.54 (0.23-1.28)
	3	150/172	0.87 (0.61-1.26)	18/36	0.47 (0.20-1.12)
	4	124/196	0.63 (0.43-0.91)	22/45	0.37 (0.16-0.86)
P_{trend} [⊥]			*0.035*		*0.022*
Diagnostic/therapeutic, abnormal enema, gastrointestinal bleeding					
	1	117/126	1.00 (ref)	19/27	1.00 (ref)
	2	131/128	0.89 (0.60-1.33)	13/17	1.17 (0.40-3.43)
	3	135/120	1.06 (0.71-1.58)	14/25	0.68 (0.24-1.90)
	4	99/136	0.62 (0.41-0.95)	17/31	0.54 (0.20-1.47)
P_{trend} [⊥]			*0.071*		*0.174*
Family history in a first degree relative, routine screening (asymptomatic participants)					
	1	34/39	1.00 (ref)	23/14	1.00 (ref)
	2	26/69	0.41 (0.19-0.88)	14/33	0.18 (0.05-0.57)
	3	29/58	0.44 (0.20-0.95)	19/20	0.33 (0.10-1.06)
	4	36/70	0.49 (0.23-1.03)	16/26	0.17 (0.06-0.55)
P_{trend} [⊥]			*0.130*		*0.014*

* OR – odds ratio with 95% confidence interval; adjusted for age (continuous), sex, family history of colorectal cancer in a first degree relative, regular use of aspirin or NSAIDs, smoking (current, ever, or never), physical activity (continuous), BMI (continuous), total red and processed meat intake (continuous), alcohol intake (continuous), calcium intake (continuous), retinol intake (continuous), and folate intake (continuous). Pooled odds ratio adjusted for study (CPRU versus MAP) in addition to other covariates.

** Data available for the MAP study only.

[⊥] P_{trend} values (two-sided) calculated by including the median of each quartile of blood 25-(OH)-vitamin D_3 as a continuous variable in addition to all above mentioned covariates in the multivariable models.

Table 2.7. Sensitivity of the odds ratio between the highest *versus* lowest month- and study-specific quartile of 25-(OH)-vitamin D_3 and colorectal adenoma risk to an unmeasured binary confounder.

Prevalence of unmeasured confounder among those with the lowest 25(OH)D_3 levels (Quartile 1)	Prevalence of unmeasured confounder among those with the highest 25(OH)D_3 levels (Quartile 4)	OR between the unmeasured binary confounder and case-control status	Corrected OR between highest *vs*. lowest quartile of 25(OH)D_3 with colorectal adenoma risk*
0.1	0.9	0.5	1.36 (0.77-2.41)
0.5	0.9	0.5	0.78 (0.52-1.19)
0.7	0.9	0.5	0.65 (0.44-0.97)
0.1	0.9	0.8	0.84 (0.48-1.47)
0.5	0.9	0.8	0.61 (0.40-0.92)

* OR – odds ratio with 95% confidence interval; adjusted for age (continuous), sex, family history of colorectal cancer in a first degree relative, regular use of aspirin or NSAIDs, study (CPRU versus MAP), smoking (current, ever, or never), physical activity (continuous), BMI (continuous), total red and processed meat intake (continuous), alcohol intake (continuous), calcium intake (continuous), retinol intake (continuous), and folate intake (continuous), unmeasured binary confounder.

CHAPTER 3. EFFECTS OF VITAMIN D AND CALCIUM SUPPLEMENTATION ON MARKERS OF APOPTOSIS IN NORMAL COLON MUCOSA: A RANDOMIZED, DOUBLE-BLIND, PLACEBO-CONTROLLED CLINICAL TRIAL

Veronika Fedirko[1,2], Roberd M. Bostick[1,2], W. Dana Flanders[1,2,3], Qi Long[2,3], Aasma Shaukat[4], Robin E. Rutherford [5], Carrie R. Daniel[1,2,6], Vaunita Cohen[1,2], Chiranjeev Dash[1]

[1]Department of Epidemiology, Rollins School of Public Health, Emory University, Atlanta, GA 30322

[2]Winship Cancer Institute, Emory University, Atlanta, GA 30322

[3]Department of Biostatistics and Bioinformatics, Rollins School of Public Health, Emory University, Atlanta, GA 30322

[4]Department of Medicine, GI Division, University of Minnesota, Minneapolis, MN 55455

[5]Emory University School of Medicine, Division of Digestive Diseases, Atlanta, GA 30322

[6]Nutrition and Health Sciences Program, Graduate Division of Biological and Biomedical Sciences, Emory University, Atlanta, GA 30322

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Abstract

Background: To further clarify and/or develop calcium and vitamin D as chemopreventive agents against colorectal cancer in humans, understand the mechanisms by which these agents reduce risk for the disease, and develop ‘treatable’ biomarkers of risk for colorectal cancer, we conducted a pilot, randomized, double-blind, placebo-controlled, 2x2 factorial clinical trial to test the effects of calcium and vitamin $D_{3,}$ alone and in combination on markers of apoptosis in the normal colorectal mucosa.

Methods: Ninety-two men and women with at least one pathology-confirmed colorectal adenoma were treated with calcium 2.0 g/day or vitamin D_3 800 IU/day, alone or in combination vs. placebo over six months. Overall expression and colorectal crypt distributions of Bcl-2 (an apoptosis inhibitor) and Bax (an apoptosis promoter), in biopsies of normal-appearing rectal mucosa were detected by automated immunohistochemistry and quantified by image analysis.

Results: After six months treatment, Bax expression along the full lengths of crypts increased 56% (p=0.02) in the vitamin D group, and 33% in both the calcium (p=0.31) and calcium plus vitamin D (p=0.36) groups relative to the placebo group. The vitamin D treatment effect was more pronounced in the upper 40%, or differentiation zone, of crypts (80%; p=0.01). There were no statistically significant treatment effects on Bcl-2 expression.

Conclusions: Overall, these preliminary results suggest that calcium and vitamin D, individually or together, may enhance apoptosis in the normal human colorectal epithelium, and the strongest treatment effects may be vitamin D related and in the upper sections of the colorectal crypts.

Introduction

Despite advances in screening and treatment, mortality due to colorectal cancer, the second leading cause of cancer deaths in the US (1, 4), has declined only modestly over the past 50 years, the decline probably a result of screening and polypectomy (4). This situation recalls an analogous one with ischemic heart disease three decades ago. With the advent of biological measurements as markers of risk for the disease, including lipid profiles and blood pressures, plausible preventive interventions could be readily investigated, response to preventive treatment could be monitored, and subsequently, with individual and population control of the “biomarkers”, mortality rates from the disease began a dramatic decline which continues today (61). Using biological measurements of risk, as they have for ischemic heart disease, should likewise result in a decline in colorectal cancer incidence and mortality. Based on this vision, this study has intertwined missions of exploring the efficacy of two plausible, and evidentially well-supported dietary agents, calcium and vitamin D, on modulating plausible molecular phenotypic biomarkers of risk for colorectal neoplasia.

There is strong biologic plausibility and animal experimental and human evidence for protection against colorectal neoplasms by calcium and vitamin D. Proposed mechanisms of calcium against colorectal cancer include protection of colonocytes against bile acids and fatty acids (5, 6), direct effects on cell cycle regulation (7), and modulation of E-cadherin and β-catenin expression via the calcium-sensing receptor (CaSR) (7-9). Proposed mechanisms for vitamin D involve bile acid catabolism, direct effects on the cell cycle, growth factor signaling, and immunomodulation (5, 7, 10). Although calcium and, especially, vitamin D have pro-apoptotic effects on colonocytes *in* *vitro* and in animal models (227, 254, 261, 265, 266, 273), this has not been sufficiently confirmed in humans (44, 45, 294). Higher total calcium intakes are associated with reduced risk for colorectal adenoma (378-380), and calcium supplementation reduces adenoma recurrence (39, 41, 215). Also, higher serum 25-OH-vitamin D levels in a limited number of studies were associated with reduced risk for colorectal adenoma (21, 22). However, the independent and combined anti-neoplastic effects of calcium and vitamin D in humans are unclear, and there have been no colorectal cancer-related chemoprevention trials of vitamin D individually or jointly with calcium.

There are no generally accepted pre-neoplastic biomarkers of risk for colorectal cancer. Colorectal cancer, like ischemic heart disease (IHD), is a complex, multi-factorial disease, which, like IHD will require a multi-factorial preventive approach and a panel of biomarkers to describe phenotypes from which to categorize and quantify risk. Whereas for IHD risk markers the obvious place to look was in the vascular system, an obvious place to look for risk markers for colon cancer is in the tissue in which it forms: the colorectal epithelium. Although at first glance this would appear impractical, we have shown that the clinical procedures required are similar in ease, time, invasiveness, and discomfort as a digital rectal/prostate exam or a PAP smear (381). Although a urine or blood test would be more practical, it is most likely that such surrogate marker tests can eventually be more readily and rationally developed guided by the results from studying tissue markers. Phenotypic biomarkers are attractive biomarkers since they "summarize" the result of complex interactions among genotype, gene-gene interactions, epigenetic phenomenon, environmental exposures, and gene-environment interactions.

This is certainly true of lipid profiles (61), and should be no less so for the “molecular state”, or phenotype, of the colorectal epithelium.

To address these issues, we conducted a pilot, randomized, double-blind, placebo-controlled, 2 x 2 factorial chemoprevention clinical trial of supplemental calcium and vitamin D_3, alone and in combination vs. placebo over six months, to estimate the efficacy of these agents on modulating the expression of apoptotic biomarkers (pro-apoptotic Bax and anti-apoptotic Bcl-2) of risk in the normal colorectal mucosa.

Patients and Methods

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

Participant Population

Participants were recruited from the patient population attending the Digestive Diseases Clinic of Emory University. Eligibility included age 30 – 75 years, in general good health, capable of informed consent, and a history of at least one pathology-confirmed sporadic colon or rectal adenoma within the past 36 months. Specific exclusions were supplemental intake of calcium and/or vitamin D greater than the recommended daily allowance (RDA); supplemental daily intake of vitamin A greater than 10,000 IU/day; a major diet change within the previous six months; an inability to refrain from aspirin use for seven days; current, planned or recent participation in another clinical trial; pregnancy, trying to get pregnant, or breast-feeding; familial adenomatous polyposis; an elevated serum calcium or creatinine; supraphysiologic levels of 25-OH vitamin D at their study eligibility visit; kidney stones or sarcoidosis within the previous 20 years; a history of a bleeding disorder or current use of anticoagulant medication; use of a thiazide diuretic in an amount greater than the equivalent of 50 mg of hydrochlorothiazide daily; immunosupression; a history of osteoporosis; use of lithium, an ion exchange resin, tetracycline, or indomethacin; renal insufficiency; dementia; cardiovascular disease that moderately or severely limited activity; inflammatory bowel disease; a malignancy other than nonmelanoma skin cancer within the previous five years; hyperparathyroidism or hypoparathyroidism; uncontrolled hypothyroidism or hyperthyroidism; enema or laxative dependence; active peptic ulcer disease; gastrectomy; bowel resection; active liver or pancreatic disease; intestinal malabsorption syndromes; narcotic or alcohol dependence; on a weight loss diet; and a nondeliberate weight loss of 10% or more in previous three months.

Clinical Trial Protocol

Participant recruitment and flow is depicted in **Figure 3.1**. All age-eligible practice patients diagnosed with at least one pathology-confirmed adenomatous colonic or rectal polyp within the previous 36 months were identified as potential study participants. Medical charts were screened, and potentially eligible patients were sent an introductory letter followed by a telephone interview during which willingness to participate and further eligibility was assessed, and, if appropriate, an in-person eligibility visit scheduled. During the eligibility visit, potential participants were interviewed and signed a consent form, their medication and nutritional supplement bottles were reviewed, and they completed questionnaires (on socio-demographics, medical history, medication and nutrition supplement use, lifestyle, family history, and others) and provided a blood sample. Diet was assessed with a semi-quantitative food frequency questionnaire (382). Medical and pathology records were reviewed. Those still eligible and willing to participate then entered a 30-day placebo run-in trial. Only participants without significant perceived side effects and took at least 80% of their tablets were randomized. Adherence for the run-in trial was assessed by questionnaire, interview, and pill count. Eligible participants then underwent a baseline rectal biopsy and were randomly assigned (stratified by sex and nonsteroidal anti-inflammatory drug [NSAID] use) over nine months to treatment group. Of those who passed initial chart eligibility, 42% were contacted and 20% were eligible and consented to participate.

Participants (n=92) were randomly assigned to the following four treatments: placebo (n=23), 2.0 g elemental calcium supplementation (as calcium carbonate in equal doses twice daily) (n=23), 800 IU vitamin D_3 supplementation (400 IU twice daily) (n=23), and 2.0 g elemental calcium plus 800 IU of vitamin D_3 (n=23).

Study tablets were custom manufactured by Tishcon Corporation, NY, USA. The corresponding supplement and placebo pills were identical in size, appearance, and taste. The placebo was free of calcium, magnesium, vitamin D, and chelating agents.

Calcium carbonate was used for elemental calcium delivery in this trial since it was also successfully used in the Calcium Polyp Prevention adenoma recurrence (383) and Calcium and Colorectal Epithelial Cell Proliferation trials (384); it was used in most large studies using calcium long term for other reasons and therefore had the most established safety record; it is inexpensive; and it delivers more elemental calcium per tablet than other forms, thus fewer tablets are required, enhancing adherence.

Vitamin D_3 was chosen as our form of vitamin D for several reasons, including avoidance of the toxicity risks associated with 1,25-$(OH)_2$-vitamin D or 25-OH-vitamin D. Supplementation with vitamin D_3 (a pro-hormone), takes advantage of natural metabolism to generate the most active moiety. Supplementation with even large doses of vitamin D_3 does not increase total 1,25-$(OH)_2$-vitamin D levels in individuals who are not vitamin D deficient (385). Multivitamins and calcium/vitamin D supplements typically provide 400 IU of vitamin D_3 daily, but numerous intervention studies (reviewed in (386),(387)) show that this dose will not suppress PTH in most North American adults; however, 800 IU daily raises 25-OH-vitamin D levels toward the desired range, and leaves a substantial margin of safety, even when combined with dietary intake.

Over the six-month treatment period participants attended follow-up visits at 2 and 6 months after randomization and were contacted by telephone at monthly intervals between the second and final follow-up visits (**Figure 3.1**). At follow-up visits, pill-taking adherence was assessed by questionnaire, interview, and pill count. Adverse events were monitored by interview at each study visit and interim telephone call and two weeks after the last visit, questionnaire (included questions about hospitalizations, medical visits and diagnoses, medication changes, and symptoms) at each study visit, and by participant-initiated telephone calls, and graded according to NIH Common Toxicity Criteria and likelihood that they were study-related. Participants were instructed to remain on their usual diet and not take any nutritional supplements not in use on entry into the study. At each follow-up visit participants were interviewed and completed questionnaires. At the first and last visits all participants underwent venipuncture and a rectal biopsy procedure. Participants were asked to abstain from aspirin use for seven days prior to each biopsy visit. All visits for a given participant were scheduled at the same time of day to control for possible circadian variability in the outcome measures.

Factors hypothesized to be related to the expression of apoptosis markers in normal colon mucosa (e.g., diet, medications, etc.) were assessed at baseline, several were reassessed at the first follow-up visit, and all were reassessed at the final follow-up visit. Participants did not have to be fasting for their visits and did not take a bowel cleansing preparation or enema.

Six 1-mm thick biopsy specimens were taken from the rectal mucosa 10 cm proximal to the external anal aperture through a rigid sigmoidoscope with a jumbo cup flexible endoscopic forceps mounted on a semi-flexible rod, teased off the forceps with and onto a strip of bibulous paper, then immediately placed in phosphate buffered saline and oriented under a dissecting microscope to ensure that they were not twisted or curled on the bibulous paper, and then immediately placed in 10% normal buffered formalin.

Immunohistochemistry Protocol

The biopsies in formalin were left undisturbed for at least six hours, transferred to 70% ethanol 24 hours after being placed in formalin, embedded in paraffin blocks within two weeks of the biopsy procedure, cut and stained within another four weeks, and analyzed within another four weeks. Five slides with four section levels each taken 40 microns apart were prepared for each biomarker, yielding a total of 20 levels per biomarker. Heat-mediated antigen retrieval was accomplished by steaming the slides for 40 minutes using a Pretreatment (PT) Module (Lab Vision Corp., CA) with 100x Citrate Buffer pH 6.0 (DAKO S1699, DAKO Corp., Carpinteria, CA; further referred to as DAKO). Next, immunohistochemical (IHC) processing by a labeled streptavidin-biotin method was accomplished using a DAKO Automated Stainer (DAKO). The following reagents were used: antibody (Bcl-2 antibody, Santa Cruz Biotechnology, Inc., CA, catalog no. sc-509, dilution 1:100; or Bax antibody, DAKO, catalog no. A3533, dilution 1:200) diluted with Antibody Diluent (DAKO SS0809, DAKO), LSAB2 Detection System (DAKO K0675, DAKO), DAB (DAKO K3466, DAKO), and TBS buffer (DAKO S1968, DAKO). The slides, which were not counterstained, were coverslipped automatically with a Leica CV5000 Coverslipper (Leica Microsystems, Inc., IL) and placed in opaque slide folders. In each staining batch of biopsy slides, positive and negative control slides were included. Tonsil, used as a control tissue for both apoptosis biomarkers, was processed in the same manner as the patient's tissue except that antibody diluent was used rather than primary antibody on the negative control slide.

Protocol for Quantifying Staining Density of Immunohistochemically Detected Biomarkers in Normal Colon Crypts ("Scoring")

The method ("scoring") used to describe and quantify various characteristics of the labeled antigens in the colon crypts was a quantitative image analysis procedure for antigens that are labeled with a wide range of intensities in gradient distributions along the crypt axis—something that cannot be done manually. The unit of analysis was the "hemicrypt", defined as one-half of a crypt bisected from crypt base to colon lumen. A "scorable" hemicrypt was defined as an intact hemicrypt that extended from the muscularis mucosa to the colon lumen.

The major equipment and software for the image analysis procedures ("scoring") were: personal computer, light microscope with appropriate filters and attached digital light microscope camera, digital drawing board, ImagePro Plus image analysis software (Media Cybernetics, Inc., MD), our custom developed plug-in software for colorectal crypt analysis, and Microsoft Access (Microsoft Corporation, WA). Equipment and imaging software settings were standardized. Slides were oriented in a standard manner and the section levels on the slides viewed in sequence using light microscopy at 200x magnification. The reader created a slide background correction image for the slide to be analyzed, and, focusing on the first hemicrypt, captured and transferred the image as a 16-bit per pixel grayscale image from the camera to the image analysis program. Next, the hemicrypt was analyzed by precisely tracing the borders of the hemicrypt using a digital drawing board. The program then created a crypt length line midway along the hemicrypt axis, and then drew equally spaced perpendicular lines to the crypt length line at intervals to yield segments with the average widths of normal colonocytes. Finally, the program adjusted for any background levels on the slide, measured the optical density of the labeling across the entire hemicrypt as well as within each segment, and entered the resulting data into the database automatically. Then, the reader moved to the next hemicrypt on the same or next image, section level, biopsy, and/or slide and repeated all the previously described analysis steps. The goal was to analyze a minimum of 16 hemicrypts on each of two biopsies, for a total of 32 hemicrypts.

One slide reader analyzed all of the Bax and Bcl-2 stained slides throughout the study. Blinded subsets of previously analyzed slides were resubmitted to the reader during the study to assess intra-reader reliability, which was found to range from 0.95 – 0.98 throughout.

Protocol for Measuring Plasma 25-OH- and 1,25-$(OH)_2$-Vitamin D Levels

Laboratory assays for plasma 25-OH-vitamin D and 1,25-$(OH)_2$-vitamin D were performed by Dr. Bruce Hollis at the Medical University of South Carolina using a radioimmunoassay method as previously described (388, 389). Plasma samples for baseline and follow-up visits for all subjects were assayed together, ordered randomly, and labeled to mask treatment group, follow-up visit, and quality control replicates. The average intra-assay coefficient of variation for plasma 25-OH-vitamin D was 2.3 %, and for 1,25-$(OH)_2$-vitamin D, 6.2 %.

Statistical Analysis

Treatment groups were assessed for comparability of characteristics at baseline and final follow-up by the Fisher's exact test for categorical variables and analysis of variance (ANOVA) for continuous variables. Slide "scoring" reliability was analyzed using intra-class correlation coefficients.

The mean density of staining for Bax, Bcl-2, and the Bax/Bcl-2 ratio in normal colon crypts was calculated for each patient at baseline and 6-months follow-up by summing all the densities from all analyzed crypts from the biopsy specimens and dividing by the number of crypts analyzed. Measures of the within-crypt distributions of the apoptotic markers (e.g., the ratio of expression in the upper 40% to the lower 60% of the crypts) were calculated for each patient by taking the mean of the biomarker densities in the upper 40% of crypts, or in the lower 60% of crypts, and constructing ratios of expression in the upper 40% to the lower 60% of crypts. We decided a priori to use as measures of the within-crypt distributions of the apoptotic markers the ratio of expression in the upper 40% (differentiation zone) to the lower 60% (proliferation zone) of the crypts, and the ratio of expression in the upper 20% (closest to colon lumen contents) to the lower 20% (furthest colon lumen contents) of the crypts because they represent the ratios of well recognized functional or exposure zones. We transformed biomarker expression density data by dividing each individual's measurement by the staining batch’s mean density to adjust for possible batch effects, and then mean transformed biomarker densities were calculated for each treatment group for the baseline and 6-months follow-up visits.

Treatment effects were evaluated by assessing the differences in the transformed densities from baseline to the 6-months follow-up visit between patients in each active treatment group and the placebo group. The differences in the transformed densities from baseline to six months between each active treatment group and controls were tested with two-sided Wilcoxon exact non-parametric tests. The magnitude of the treatment effects on the biomarker staining densities and distributions were expressed as relative effects, defined as: [treatment group follow-up mean/treatment group baseline mean]/[placebo follow-up mean/placebo baseline mean]. The interpretation of the relative effect is somewhat analogous to that of an odds ratio (e.g., a relative effect of 2.0 would mean that the relative proportional change in the treatment group was twice as great as that in the placebo group). Primary analyses were based on randomization treatment assignment regardless of adherence status (intent-to-treat analysis).

The distributions of Bax and Bcl-2 batch-standardized staining densities were plotted along the colorectal crypts by normalizing each crypt to 50 sections, averaging within each section across all crypts separately for each patient, and then for each treatment group.

In sensitivity analyses, we also analyzed data without batch standardization by including batch as a covariate, and using different transformations; the results from these analyses did not differ materially from those reported.

Statistical analyses were done using SAS System software (version 9.1; SAS Institute, Inc., NC). A cutoff level of $P \leq 0.05$ (2-sided) was used for assessing statistical significance.

Results

Study Participants

Treatment groups did not differ significantly on characteristics measured at baseline (**Table 3.1**) or at final follow-up (data not shown). The mean age of participants was 61 years, 64% were men, 71% were white, and 20% had a family history of colorectal cancer in a first degree relative. Most participants were non-smokers, college graduates, and overweight.

Adherence to visit attendance averaged 92% and did not differ significantly among the four treatment groups. On average, at least 80% of pills were taken by 93% of participants at the first follow-up visit and 84% at the final follow-up visit. There were no adverse events attributed to study procedures or treatments. Seven participants (8%) were lost to follow-up due to perceived drug intolerance (n=2), unwillingness to continue participation (n=3), physician's advice (n=1), and death (n=1). Dropouts included one person from the vitamin D supplementation group, and two persons from each of other three groups.

At baseline, there were no significant differences between the four study groups in plasma 25-OH- or 1,25-$(OH)_2$-vitamin D levels (**Table 3.2**). By study end, plasma 25-OH-vitamin D levels had statistically significantly increased in the vitamin D and calcium plus vitamin D groups, and appeared to have slightly, non-significantly decreased in the placebo and calcium groups (**Table 3.2**). As expected, plasma levels of

1,25-$(OH)_2$-vitamin D at the end of follow-up did not differ significantly between treatment groups (**Table 3.2**).

Graphical Assessment of Changes over Six Months in the Distributions of Bax and Bcl-2 Expression along Normal Colorectal Crypts

The distributions of Bax and Bcl-2 staining densities (“expression”) along the colorectal crypts at the baseline and 6-months follow-up visits are shown in **Figures 3.2** and **3.3**, respectively. Bax and Bcl-2 staining densities were batch-standardized and multiplied by 100 for graphical presentation. In the placebo group, Bax expression appeared to decrease, especially in the crypt bases, while Bcl-2 expression appeared unchanged from baseline to follow-up (**Figures 3.2.A** and **3.3.A**). In the calcium group, Bax expression did not appear to change from baseline to follow-up, whereas Bcl-2 expression tended to be lower in the crypt bases (**Figures 3.2.B** and **3.3.B**). In the vitamin D group, there were no apparent changes in Bcl-2 expression; however, Bax expression appeared to increase, especially in the crypt opening onto the colon lumen (“crypt mouth”) (**Figures 3.2.C** and **3.3.C**). In the calcium plus vitamin D group, there appeared to be slight decreases in Bcl-2 and Bax expression in the crypt bases and a slight increase in Bax expression in the crypt mouth (**Figures 3.2.D** and **3.3.D**).

Effects of Calcium and/or Vitamin D on the Separate and Relative Expressions of Bax and Bcl-2 in Normal Colorectal Crypts

After six months treatment, Bax expression along the full lengths of crypts increased proportionately by 56% (p=0.02) in the vitamin D group and 33% in both the calcium (p=0.31) and calcium plus vitamin D (p=0.36) groups relative to the placebo group (**Table 3.2, A)**. The vitamin D treatment effect on Bax expression was more pronounced (80%, p=0.01) in the canonical differentiation zone, or upper 40%, of crypts (**Table 3.2, B**). Also, Bax expression in the upper 40% relative to the lower 60% of the crypts increased by 26% (p=0.06) in the vitamin D group relative to the placebo group (**Table 3.2, C**). There were no statistically significant treatment effects on Bcl-2 expression along the entire crypt, in the upper 40% of crypts, or in the ratios representing the biomarker's distribution in the crypts; however, there was a suggestion of some decrease in Bcl-2 expression in the calcium and calcium plus vitamin D groups (**Table 3.2, A)**. The estimated relative treatment effects on the Bax/Bcl-2 ratio along the entire crypt in the calcium, vitamin D, and calcium plus vitamin D groups were increases of 62% (p=0.52), 47% (p=0.37), and 71% (p=0.08), respectively (**Table 3.2, A)**. For the vitamin D group relative to the placebo group, the proportional increase in the Bax/Bcl-2 ratio in the upper 20% relative to the lower 20% of crypts was 464% (p=0.04).

Discussion

This study had intertwined missions of further clarification and/or development of calcium and vitamin D as chemopreventive agents against colorectal cancer in humans, understanding the mechanisms by which these agents reduce risk for the disease in humans, and the development of treatable biomarkers of risk for colorectal cancer. It addressed these missions by exploring the efficacy of two plausible and evidentially well-supported dietary agents, calcium and vitamin D_3 (alone and in combination), on modulating a plausible set of molecular phenotypic biomarkers of risk for colorectal neoplasia. In this preliminary trial we found strong evidence for an increase in Bax expression in normal colon crypts of sporadic colorectal adenoma patients in response to six months of vitamin D supplementation, a finding that is consistent with the hypothesis that a higher intake of vitamin D may increase pro-apoptotic stimuli in the normal human colon mucosa and reduce risk for colorectal cancer. Our findings also indicated that the strongest treatment effect on Bax expression was in the upper sections of colorectal crypts, suggesting that vitamin D increases apoptosis in the parts of colorectal crypts most exposed to bowel lumen carcinogens. Although not statistically significant, our data also suggested that calcium and calcium plus vitamin D supplementation may reduce Bcl-2 expression, that treatment effects of calcium and/or vitamin D on Bax may be more pronounced relative to Bcl-2 expression (emphasizing the importance of evaluating both apoptotic markers together), and that there may be stronger treatment effects of vitamin D when combined with calcium than for vitamin D alone.

This trial emphasizes the importance of randomization to treatment assignment and a placebo-control group in cancer chemoprevention trials. In the placebo group we observed a decrease in Bax expression and in the Bax/Bcl-2 ratio after six months of follow-up. The cause(s) for the time-related influence(s) producing these decreases in the placebo group is unknown, but may have been due to the Hawthorne effect (participants in clinical trials change their health-related behaviors while under observation), some participants may have been developing recurrent polyps, laboratory drift, and/or chance. In clinical trials such extraneous temporal influences are presumed to occur equally across all treatment groups; therefore, change in the placebo group is “subtracted” from any change in an active treatment group to yield the true treatment effect. Thus, in this trial, without a placebo-control group the treatment effect on apoptotic markers would have been underestimated.

Although we hypothesized that the effects of calcium plus vitamin D on the apoptotic markers would be greater than from either agent alone, we found that Bax expression went up more in the vitamin D group than in the vitamin D plus calcium group. There are several possible explanations for this finding, including chance—especially considering the small sample size—and that the two agents may have attenuated the effects of either alone. In at least one 2x2 factorial experiment of calcium and vitamin D in rodents substantial treatment effects in proliferation markers were found for the individual agents but not for the combination (246). In a Women's Health Initiative randomized clinical trial there was no evidence for an overall treatment effect from the combination of supplemental calcium (1,000 mg daily) and vitamin D (400 IU daily) on colorectal cancer incidence (27); however, these overall results are difficult to interpret because of the low doses and high rates of intervention agent drop in and drop out. On the other hand, most animal studies that investigated the combination of calcium and vitamin D reported that supplemental vitamin D has stronger anti-neoplastic effects in animals given relatively high-calcium diets (243-245); in two large cohort studies (29, 30), there was clear evidence of a positive interaction between the two nutrients; and in the Calcium Polyp Prevention adenoma recurrence trial, there were strong indications that vitamin D enhanced the chemopreventive effect of calcium (237).

There is substantial evidence that markers of apoptosis, including Bax and Bcl-2 expression, are plausible candidates for treatable biomarkers of risk for colorectal neoplasms. Failure to delete cells with accumulated genetic and epigenetic changes via apoptosis is an important step in colon carcinogenesis that may lead to adenoma development, and thus colorectal cancer (63, 65). Inadequate rates of apoptosis may be a consequence of the separate and combined influences of multiple genetic, epigenetic, and environmental factors. Pro- or anti-apoptotic tendencies in the normal colon mucosa are reflected by the expression of Bax and Bcl-2 proteins, respectively. Thus, measuring both of these proteins in colon crypts provides a good indicator of apoptosis.

There is substantial evidence from *in vitro* (254, 261, 265, 266) and animal studies (227, 273) that calcium and vitamin D enhance apoptosis in colonocytes. Possible mechanisms include direct effects on apoptotic proteins, mediated in part by the VDR and the CaSR (11, 293), induction of differentiation with subsequent promotion of apoptosis (254), indirect effects on apoptosis as a result of decreased inflammation (64), and others. Our data are consistent with the hypothesis that vitamin D promotes apoptosis in the normal human colon mucosa. We found an increase in the expression of pro-apoptotic Bax in the entire and upper 40% of colorectal crypts. We also observed statistically non-significant increases in Bax and decreases in Bcl-2 expression in the calcium and calcium plus vitamin D groups relative to the placebo group. The Bax/Bcl-2 ratio increased in the entire colorectal crypt after calcium and vitamin D supplementation, indicating a shift toward more apoptosis. The ratio of the upper 40% to the lower 60% of crypts represents the ratio of two functionally distinct zones, differentiation and proliferation. Vitamin D supplementation increased Bax and the balance of Bax to Bcl-2 in ratios of the upper 40% to the lower 60% of crypts, which can mostly be explained by an increase of Bax in the upper 40% of crypts. Another ratio that we constructed was of the upper 20% to the lower 20% of crypts, which represented the contrast between the areas of the crypts most proximal to or distant from damaging colon lumen contents. Again, in the ratio of the upper 20% to the lower 20% of crypts, we observed a significant increase in the balance of Bax to Bcl-2, some decrease in Bcl-2, and some increase in Bax in the vitamin D and calcium plus vitamin D groups. No effects of calcium supplementation on the apoptosis markers in the upper 20% relative to the lower 20% of crypts were observed.

There are few reported human studies of effects of supplemental calcium and vitamin D, including no large randomized trials of their combined effects, on apoptotic markers in the normal colon mucosa. One small, pilot cross-over study (n = 40) of calcium supplementation or low-fat dairy foods and biomarkers of apoptosis reported no change in epithelial cell apoptosis (measured by the terminal nucleotidyl transferase-mediated nick-end-labeling method (TUNEL)) or the expression of the pro-apoptotic gene product Bak in the normal-appearing colon mucosa (44). Another small study (n = 21) found no effects of calcium and vitamin D supplementation over six months on apoptosis by the TUNEL method or on Bcl-2 expression in either normal rectal mucosa or in situ polyps (45), but did find an increase in the frequency of Bak immunostained cells in the polyps (45). In a cross-sectional study weak, non-statistically significant direct associations between dietary calcium and an apoptosis score were found in patients both with and without adenomas (n = 498), and an inverse non-statistically significant association between serum vitamin D levels and an apoptosis score was found in adenoma patients (n = 92) (294).

This study has several limitations and strengths. Treatment effects could not be examined in parts of the colon other than the rectum; however, several studies suggest that patterns or levels of apoptosis across levels of the colon may be highly correlated (390, 391). Apoptotic markers are not proven biomarkers of risk for colon cancer;

however, substantial basic science literature supports an important role for apoptosis in colon carcinogenesis. This study cannot prove that because vitamin D or calcium can increase apoptosis in normal colon tissue, they can reduce risk for colon cancer. On the other hand, this study is the only randomized, double-blind, placebo-controlled trial to have assessed the independent and combined effects of supplemental calcium and vitamin D on apoptosis markers in the normal colorectal epithelium, there was high protocol adherence by study participants, immunostaining was automated, and, via the use of novel quantitative image analysis procedures, biopsy analysis reliability was high.

Overall, these results from this pilot clinical trial suggest that 1) vitamin D and calcium, individually or together, may enhance apoptosis in the normal human colorectal epithelium; 2) that they do so via upregulating Bax expression alone or relative to Bcl-2 expression; 3) that the strongest treatment effects may be vitamin D related and in the upper sections of the colorectal crypts; and 4) that Bax expression alone or in combination with Bcl-2 expression, may be a treatable biomarker of risk for colorectal neoplasms.

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Tables and Figures

Table 3.1. Selected baseline characteristics of the study participants[*] (n = 92).

	Treatment Group				
Characteristics	**Placebo** (n = 23)	**Calcium** (n = 23)	**Vitamin D** (n = 23)	**Calcium + vitamin D** (n = 23)	***P***[**]
Demographics					
Age, years	58.5 (8.2)	61.9 (8.2)	60.2 (8.1)	62.1 (7.5)	0.39
Men (%)	70	70	70	70	1.00
White (%)	74	83	65	61	0.39
College graduate (%)	65	61	57	44	0.53
Medical history					
History of colorectal cancer in 1° relative (%)	17	30	17	13	0.60
Take NSAID[***] regularly[§] (%)	22	13	9	22	0.60
Take aspirin regularly[§] (%)	22	52	30	56	0.05
If woman (n = 28), taking estrogens (%)	4	9	4	4	1.00
Habits					
Current smoker (%)	9	4	0	0	0.61
Take multivitamin (%)	30	30	26	39	0.86
Mean dietary intakes					
Total energy intake, kcal/d	1,596 (528)	1,788 (691)	1,848 (821)	1,845 (752)	0.59
Total calcium[§§], mg/d	618 (308)	746 (335)	843 (526)	824 (714)	0.41
Total vitamin D[§§], IU/d	277 (230)	336 (202)	360 (317)	415 (316)	0.40
Total fat, gm/d	67 (32)	72 (35)	70 (32)	74 (28)	0.59
Dietary fiber, gm/d	15 (7)	17 (9)	18 (9)	17 (11)	0.97
Alcohol, gm/d	9 (14)	11 (15)	14 (18)	10 (20)	0.84
Anthropometrics					
Body mass index (BMI), kg/m^2	30.6 (7.2)	29.4 (5.5)	28.9 (5.56)	31.6 (6.0)	0.44
Waist-to-hip ratio	0.9 (0.1)	0.9 (0.1)	0.9 (0.1)	1.0 (0.1)	0.17

(Table continues)

Table 3.1 (continued).

Characteristics	Placebo (n = 23)	Calcium (n = 23)	Vitamin D (n = 23)	Calcium + vitamin D (n = 23)	*P*[**]
	Treatment Group				
VDR BsmI genotype (%)					
bb	35	39	48	30	0.25
Bb	35	57	43	52	
BB	30	4	9	17	
Plasma vitamin D					
25-OH-vitamin D, ng/mL	20.4 (7.6)	25.7 (7.6)	21.0 (8.3)	20.9 (9.7)	0.12
1,25-$(OH)_2$-vitamin D, pg/mL	39.2 (12.2)	45.4 (35.3)	44.5 (22.6)	37.9 (12.5)	0.60

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

[**] By Fisher's exact χ^2 test for categorical variables, and by ANOVA for continuous variables.

[***] Nonsteroidal anti-inflammatory drug.

[§] At least once a week.

[§§] Diet plus supplements.

Table 3.2. Plasma 25-OH-vitamin D and 1,25-$(OH)_2$-vitamin D, Bax and Bcl-2 expression in colorectal crypts at baseline and 6-months follow-up Shown as batch-standardized[¶] optical density of staining from the immunohistochemically-detected biomarkers.

	Baseline				6-Months Follow-up				Absolute Difference**				Relative Effect [§]
	n	**Mean**	**SD**	**P***	**n**	**Mean**	**SD**	**P***	**n**	**Mean**	**SD**	**P***	
Plasma Vitamin D													
25-OH-vitamin D[¥]													
Calcium + vit. D	23	20.93	9.65	0.84	21	28.51	7.94	<0.0001	21	7.59	6.19	<0.0001	1.56
Vitamin D	23	21.04	8.33	0.81	22	29.48	7.23	<0.0001	22	8.44	6.05	<0.0001	1.60
Calcium	23	25.67	7.59	0.05	21	23.20	8.88	0.03	21	-2.46	4.45	0.88	1.03
Placebo	23	20.44	7.55	N/A	21	17.89	6.93	N/A	21	-2.55	6.00	N/A	1.00
1,25-$(OH)_2$-vitamin D[¥]													
Calcium + vit. D	23	37.89	12.54	0.85	21	36.71	28.84	0.95	21	-1.18	32.81	0.97	1.02
Vitamin D	23	44.48	22.58	0.44	22	47.40	27.97	0.13	22	2.92	35.74	0.57	1.12
Calcium	23	45.37	35.31	0.36	21	31.66	12.44	0.42	21	-13.71	32.49	0.21	0.74
Placebo	23	39.17	12.19	N/A	21	37.13	10.70	N/A	21	-2.04	9.76	N/A	1.00
Biomarker Expression in Colorectal Crypts													
A. Entire crypts													
Bax													
Calcium + vit. D	23	0.97	0.50	0.59	21	1.01	0.31	0.43	21	0.01	0.60	0.36	1.33
Vitamin D	23	0.82	0.44	0.05	22	1.00	0.28	0.56	22	0.16	0.54	0.02	1.56
Calcium	23	1.00	0.36	0.70	21	1.04	0.42	0.43	21	0.03	0.59	0.31	1.33
Placebo	23	1.21	0.87	N/A	21	0.94	0.32	N/A	21	-0.30	0.83	N/A	1.00
Bcl-2													
Calcium + vit. D	23	1.15	0.56	0.19	21	1.03	0.43	0.62	21	-0.12	0.66	0.57	0.84
Vitamin D	23	0.87	0.47	0.58	22	1.06	0.40	0.57	22	0.17	0.58	0.25	1.14
Calcium	23	1.06	0.62	0.66	21	0.91	0.50	0.44	21	-0.17	0.86	0.80	0.80
Placebo	23	0.93	0.46	N/A	21	0.99	0.40	N/A	21	0.07	0.60	N/A	1.00

(Table continues)

Table 3.2 (continued).

	Baseline				6-Months Follow-up				Absolute Difference**				Relative Effect §
	n	Mean	SD	*P**	n	Mean	SD	*P**	n	Mean	SD	*P**	
Bax/Bcl-2 ratio													
Calcium + vit. D	23	0.89	0.37	0.01	21	1.06	0.30	0.62	21	0.15	0.49	0.08	1.71
Vitamin D	23	1.03	0.46	0.05	22	1.06	0.43	0.91	22	0.02	0.43	0.37	1.47
Calcium	23	1.37	1.23	0.38	21	1.55	1.55	0.43	21	0.15	2.09	0.52	1.62
Placebo	23	1.54	1.55	N/A	21	1.08	0.50	N/A	21	-0.51	1.63	N/A	1.00
<u>B. Upper 40% of crypts</u>													
Bax													
Calcium + vit. D	21	0.014	0.01	0.29	21	0.018	0.01	0.40	21	0.004	0.01	0.13	1.52
Vitamin D	22	0.011	0.01	0.03	22	0.017	0.00	0.27	22	0.006	0.01	0.01	1.80
Calcium	21	0.016	0.01	0.63	21	0.017	0.01	0.52	21	0.002	0.01	0.60	1.25
Placebo	21	0.018	0.01	N/A	21	0.016	0.01	N/A	21	-0.003	0.01	N/A	1.00
Bcl-2													
Calcium + vit. D	21	0.001	0.00	0.07	21	0.002	0.00	0.78	21	0.001	0.00	0.27	0.52
Vitamin D	22	0.001	0.00	0.76	22	0.002	0.00	0.90	22	0.001	0.00	0.66	0.71
Calcium	21	0.001	0.00	1.00	21	0.002	0.00	0.38	21	0.001	0.00	0.50	0.84
Placebo	21	0.001	0.00	N/A	21	0.002	0.00	N/A	21	0.002	0.00	N/A	1.00
Bax/Bcl-2 ratio													
Calcium + vit. D	21	24.17	37.46	0.03	21	15.37	10.64	0.40	21	-10.43	33.49	0.11	0.97
Vitamin D	22	22.33	17.83	0.17	22	23.17	30.69	0.46	22	0.17	23.40	0.14	1.59
Calcium	21	36.67	32.32	0.85	21	29.01	45.97	0.33	21	-7.60	56.13	0.88	1.21
Placebo	21	45.61	57.42	N/A	21	29.80	66.10	N/A	21	-16.78	94.47	N/A	1.00

(Table continues)

Table 3.2 (continued).

	Baseline				6-Months Follow-up				Absolute Difference**				Relative
	n	**Mean**	**SD**	***P****	**n**	**Mean**	**SD**	***P****	**n**	**Mean**	**SD**	***P****	**Effect §**
<u>C. Ratio of upper 40% to lower 60% of crypts</u>													
Bax													
Calcium + vit. D	21	0.70	0.27	0.27	21	0.95	0.19	0.15	21	0.24	0.36	0.25	1.19
Vitamin D	22	0.62	0.32	0.07	22	0.89	0.16	0.97	22	0.27	0.34	0.06	1.26
Calcium	21	0.75	0.36	0.85	21	0.85	0.22	0.53	21	0.12	0.43	0.80	1.00
Placebo	21	0.77	0.25	N/A	21	0.87	0.14	N/A	21	0.09	0.33	N/A	1.00
Bcl-2													
Calcium + vit. D	21	0.05	0.03	0.23	21	0.07	0.05	0.86	21	0.02	0.05	0.69	0.70
Vitamin D	22	0.06	0.13	0.95	22	0.06	0.04	0.67	22	0.00	0.12	0.61	0.47
Calcium	21	0.03	0.03	0.54	21	0.08	0.09	0.62	21	0.05	0.09	0.96	1.09
Placebo	21	0.04	0.03	N/A	21	0.08	0.06	N/A	21	0.04	0.07	N/A	1.00
Bax/Bcl-2 ratio													
Calcium + vit. D	21	32.68	53.91	0.08	21	20.04	13.26	0.47	21	-14.29	47.55	0.28	0.91
Vitamin D	22	28.45	22.26	0.45	22	31.73	44.18	0.64	22	2.57	36.41	0.19	1.66
Calcium	21	41.29	35.05	0.97	21	19.66	11.34	0.73	21	-20.97	33.31	0.90	0.71
Placebo	21	41.23	46.16	N/A	21	27.75	43.67	N/A	21	-14.28	68.02	N/A	1.00
<u>D. Ratio of upper 20% to lower 20% of crypts</u>													
Bax													
Calcium + vit. D	21	0.55	0.32	0.21	21	0.84	0.27	0.20	21	0.28	0.42	0.22	1.31
Vitamin D	22	0.52	0.40	0.08	22	0.77	0.18	0.78	22	0.24	0.44	0.14	1.27
Calcium	21	0.63	0.40	0.97	21	0.74	0.29	0.57	21	0.11	0.50	0.92	0.99
Placebo	21	0.63	0.28	N/A	21	0.74	0.19	N/A	21	0.09	0.39	N/A	1.00

(Table continues)

Table 3.2 (continued).

	Baseline				6-Months Follow-up				Absolute Difference**				Relative Effect [§]
	n	**Mean**	**SD**	***P****	**n**	**Mean**	**SD**	***P****	**n**	**Mean**	**SD**	***P****	
Bcl-2													
Calcium + vit. D	21	0.01	0.01	0.15	21	0.02	0.02	0.71	21	0.01	0.03	0.49	0.56
Vitamin D	22	0.01	0.03	0.60	22	0.02	0.02	0.43	22	0.001	0.02	0.12	0.32
Calcium	21	0.01	0.01	0.70	21	0.03	0.04	0.90	21	0.02	0.04	0.78	0.92
Placebo	21	0.01	0.01	N/A	21	0.02	0.02	N/A	21	0.02	0.03	N/A	1.00
Bax/Bcl-2 ratio													
Calcium + vit. D	21	174.48	310.09	0.08	21	101.47	108.63	0.57	21	-86.77	247.09	0.19	1.56
Vitamin D	22	175.65	211.44	0.16	22	303.18	682.54	0.43	22	120.26	659.71	0.04	4.64
Calcium	21	212.88	235.39	0.54	21	65.10	44.07	1.00	21	-157.37	233.21	0.81	0.82
Placebo	21	225.01	215.08	N/A	21	83.73	90.47	N/A	21	-151.99	251.61	N/A	1.00

[¶] Batch standardization for each biomarker was performed by dividing each individual measurement by the staining batch’s average density.

* Exact two-sided non-parametric test p-value for difference between each active treatment group and placebo group.

** Absolute Difference = [treatment group follow-up - treatment group baseline] or [placebo group follow-up - placebo group baseline].

[§] Relative effect = [(treatment group follow-up/treatment group baseline)/(placebo follow-up/placebo baseline)].

Figure 3.1. Flow diagram of a trial of supplemental calcium and vitamin D_3, alone and in combination vs. placebo over six months on markers of apoptosis in the normal colorectal mucosa.

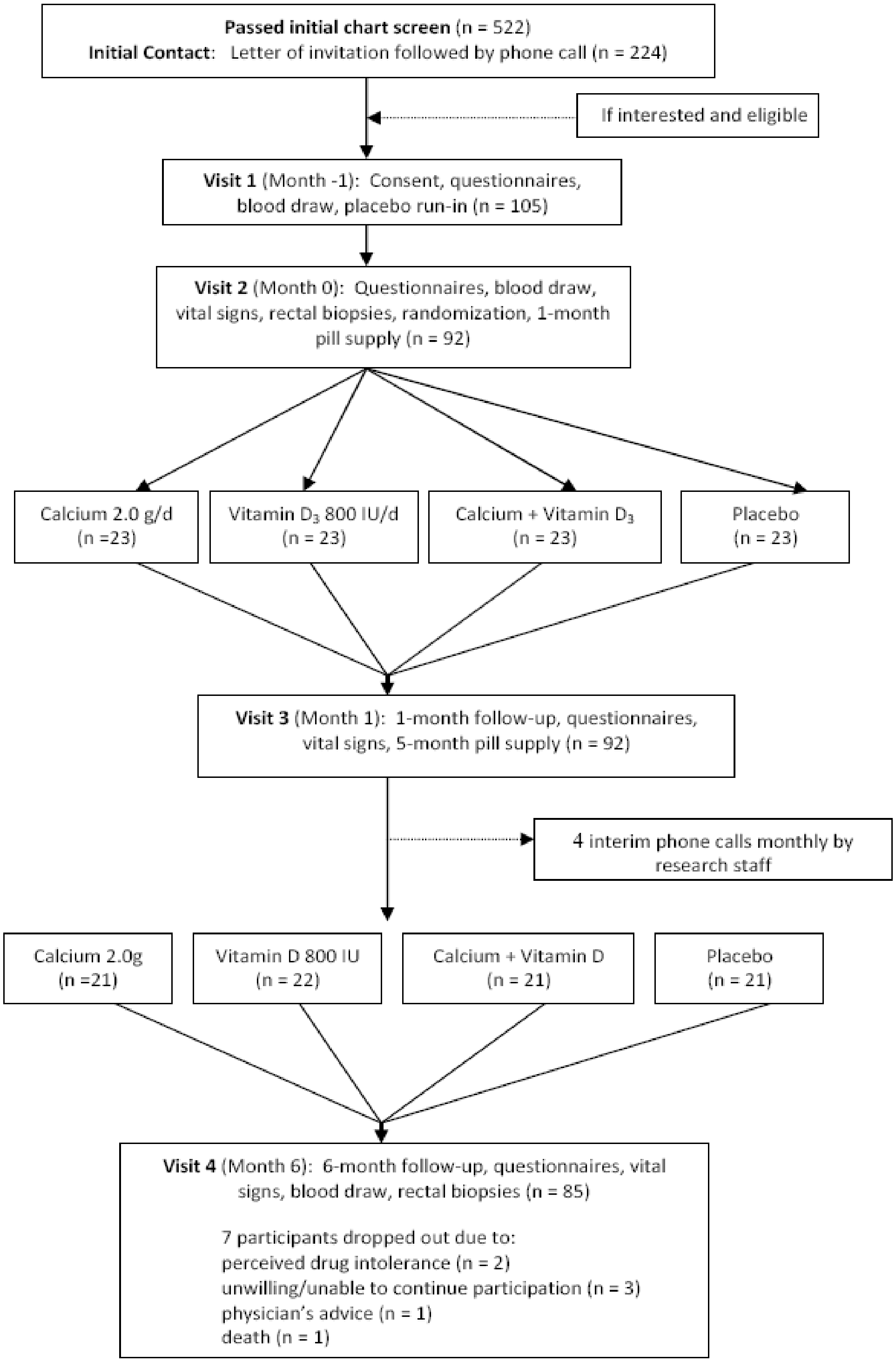
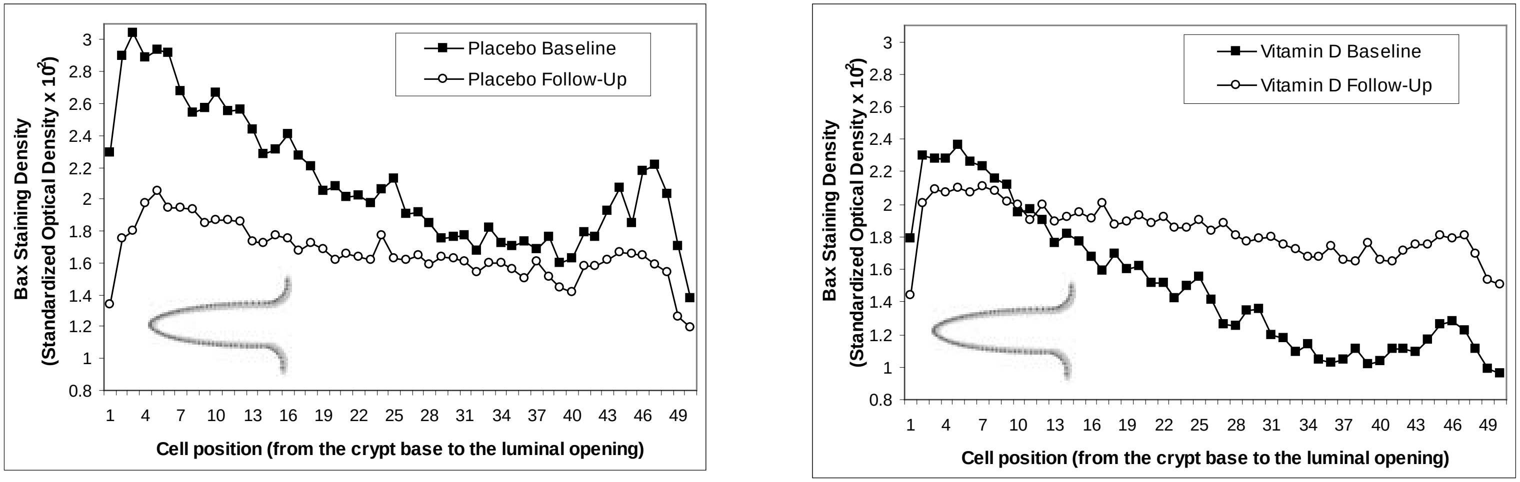
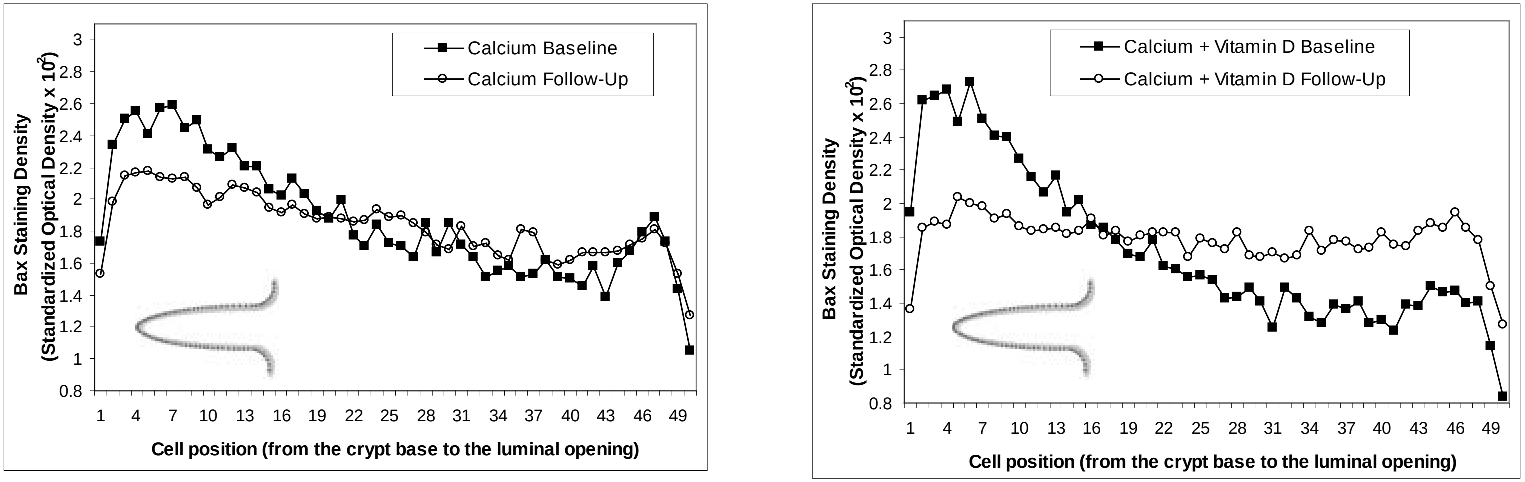


Figure 3.2. Distribution of Bax staining densities along normal colorectal crypts by treatment group at baseline and follow-up. A, Placebo Group. B, Calcium Group. C, Vitamin D Group. D, Calcium + Vitamin D Group.



A. Placebo Group

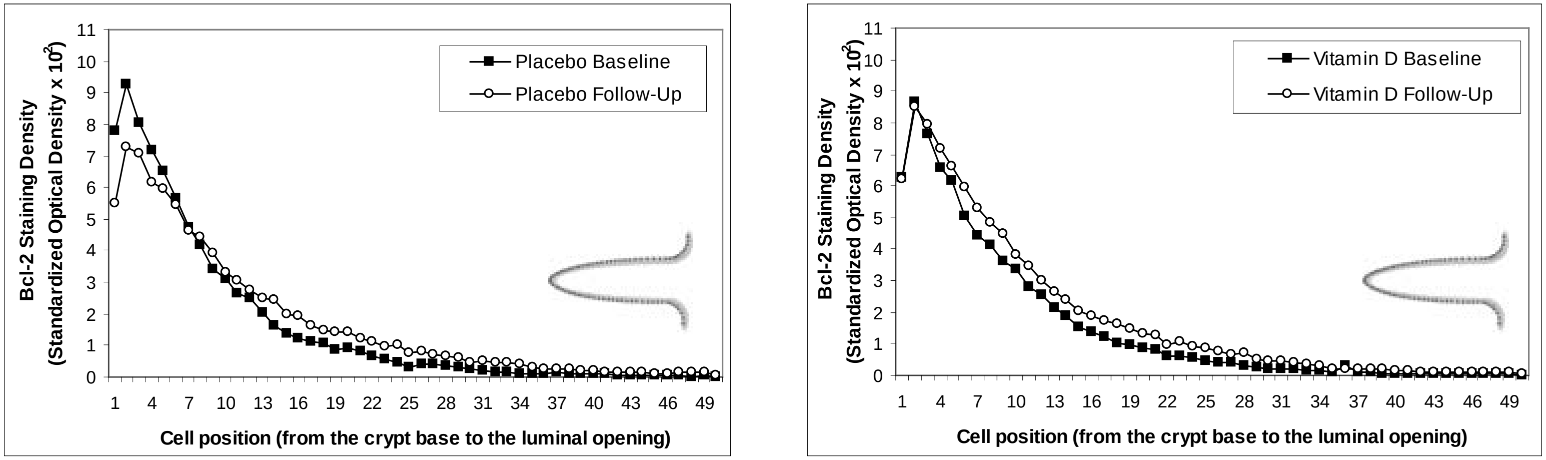
C. Vitamin D Group



B. Calcium Group

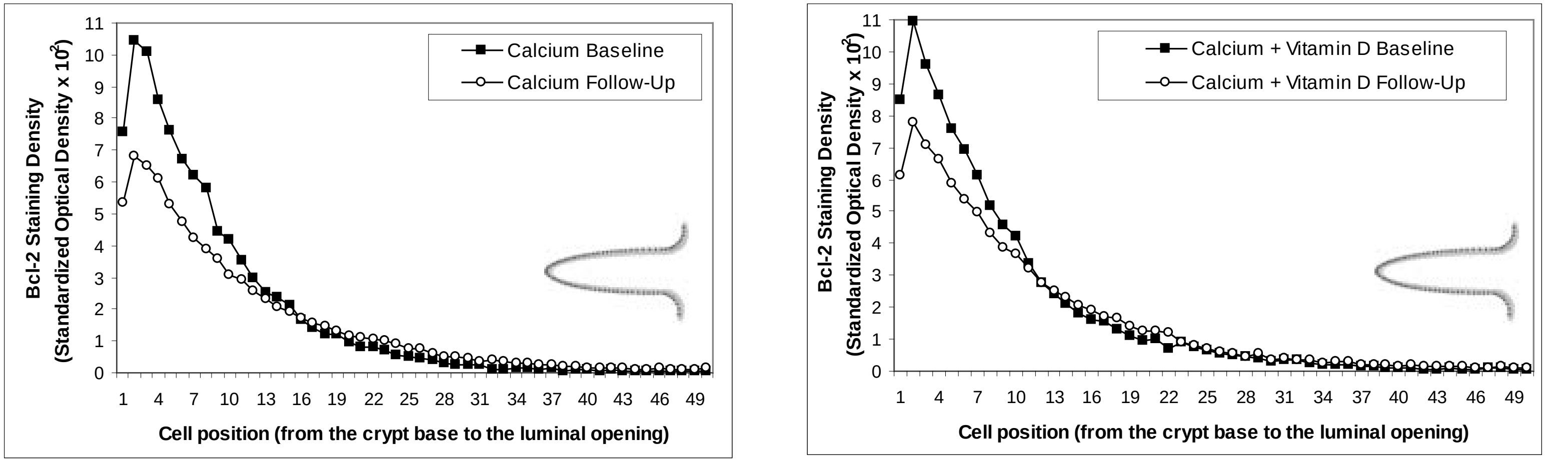
D. Calcium + Vitamin D Group

Figure 3.3. Distribution of Bcl-2 staining densities along normal colorectal crypts by treatment group at baseline and follow-up. A, Placebo Group. B, Calcium Group. C, Vitamin D Group. D, Calcium + Vitamin D Group.



A. Placebo Group

C. Vitamin D Group



B. Calcium Group

D. Calcium + Vitamin D Group

CHAPTER 4. EFFECTS OF VITAMIN D AND CALCIUM SUPPLEMENTATION ON MARKERS OF PROLIFERATION AND DIFFERENTIATION IN NORMAL COLON MUCOSA: A RANDOMIZED, DOUBLE-BLIND, PLACEBO-CONTROLLED CLINICAL TRIAL

Veronika Fedirko[1,2], Roberd M. Bostick[1,2], W. Dana Flanders[1,2,3], Qi Long[2,3], Eduard Sidelnikov[1,2], Aasma Shaukat[4], Carrie R. Daniel[5], Robin E. Rutherford[6], Jill Joelle Woodard[1,2]

[1]Department of Epidemiology, Rollins School of Public Health, Emory University, Atlanta, GA 30322
[2]Winship Cancer Institute, Emory University, Atlanta, GA 30322
[3]Department of Biostatistics and Bioinformatics, Rollins School of Public Health, Emory University, Atlanta, GA 30322
[4]Department of Medicine, GI Division, University of Minnesota, Minneapolis, MN 55455
[5]Nutritional Epidemiology Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, Department of Health and Human Services, Bethesda, MD 20852
[6]Emory University School of Medicine, Division of Digestive Diseases, Atlanta, GA 30322

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Abstract

To investigate the potential efficacy of calcium and vitamin D in reducing risk for colorectal neoplasms and to develop ‘treatable’ phenotypic biomarkers of risk for colorectal neoplasms, we conducted a pilot, randomized, double-blind, placebo-controlled, 2x2 factorial clinical trial to test the effects of these agents on cell cycle markers in the normal colorectal mucosa.

Ninety-two men and women with at least one pathology-confirmed colorectal adenoma were treated with calcium 2 g/day and/or vitamin D_3 800 IU/day vs. placebo over six months. Overall expression and distributions of $p21^{waf1/cip1}$ (marker of differentiation), MIB-1 (marker of short-term proliferation), and hTERT (marker of long-term proliferation) in colorectal crypts in the normal-appearing rectal mucosa were detected by automated immunohistochemistry and quantified by image analysis.

In the calcium, vitamin D, and calcium plus vitamin D groups relative to the placebo, p21 expression increased by 201% (P=0.03), 242% (P=0.005), and 25% (P=0.47), respectively, along the full lengths of colorectal crypts after six months of treatment. There were no statistically significant changes in the expression of either MIB-1 or hTERT in the crypts overall; however, the proportion of hTERT, but not MIB-1, expression that extended into the upper 40% of the crypts was reduced by 15% (P=0.02) in the vitamin D plus calcium group relative to the placebo.

These results indicate that calcium and vitamin D promote colorectal epithelial cell differentiation and may “normalize” the colorectal crypt proliferative zone in sporadic adenoma patients, and support further investigation of calcium and vitamin D as chemopreventive agents against colorectal neoplasms.

Introduction

Colorectal cancer is one of the leading causes of cancer death in the United States (1). Despite advances in treatment, screening, and prevention, mortality due to colorectal cancer has declined only modestly in recent years. This prompts the need for the discovery of treatable preneoplastic biomarkers of risk for colorectal neoplasms in humans that could be used to monitor the efficacy of preventive interventions, and to develop chemopreventive agents against colorectal cancer.

Calcium and vitamin D are two plausible and evidentially well supported dietary potential anti-neoplastic agents. Proposed, likely complementary, mechanisms of calcium against colorectal cancer include protection of colonocytes against bile acids and fatty acids (5), direct effects on cell proliferation, and modulation of the APC colon carcinogenesis pathway (7). Proposed, likely complementary, mechanisms for vitamin D include effects on regulating cell cycle events; promoting bile acid degradation; influencing growth factor signaling, cell adhesion, inflammation, and immune function; and modulating more than 200 responsive genes (7, 226). Also, epidemiologic studies found that higher total calcium intakes have been consistently associated with reduced risk for colorectal neoplasms (33, 221), calcium supplementation reduced adenoma recurrence (41), and higher blood 25-OH-vitamin D levels have been associated with reduced risk for colorectal adenoma (221, 238).

Almost all carcinomas of the colon and rectum develop from adenomatous polyps, which are thought to arise from the “susceptible” colorectal epithelium characterized by hyperproliferation, impaired apoptosis, and reduced differentiation. Removal of the polyps does not eliminate risk for adenoma recurrence, suggesting that the normal-appearing colorectal epithelium possesses molecular phenotypic changes that put a person at risk for developing a neoplasm. Reduced differentiation and altered cell cycle control occur during the early stages of colon tumorigenesis, therefore markers of cell proliferation and differentiation in the colorectal epithelium may serve as phenotypic biomarkers of risk for colorectal neoplasia and may be modifiable by chemopreventive agents.

As described herein, we tested the effects of calcium and vitamin D on a marker of cell differentiation ($p21^{waf1/cip1}$), and two markers of cell proliferation (MIB-1/Ki-67 and hTERT). p21 is a cyclin-dependent kinase inhibitor that plays an important role in cell differentiation, cell cycle control, apoptosis modulation, and tumorigenesis (298). In colorectal crypts, p21 is expressed only in fully differentiated cells (302), whereas telomerase (as indicated by detection of hTERT, the catalytic subunit of the telomerase) is expressed only in proliferative cells of colon crypts (392); and the proliferation-associated marker MIB-1/Ki-67 is expressed in all cells not in G_0 phase of the cell cycle (274). We used telomerase expression as indicated by hTERT in colon crypt cells as a marker of long term proliferative activity, and the S-phase marker MIB-1 as a “snapshot” or short-term proliferative indicator.

Few published human studies tested the effect of calcium and vitamin D supplementation on colorectal epithelial cell proliferation and differentiation (44, 45, 278, 279), which may serve as pre-neoplastic modifiable biomarkers of risk for colorectal neoplasms. To address this, we conducted a pilot, randomized, double-blind, placebo-controlled, 2 x 2 factorial chemoprevention clinical trial of supplemental calcium and vitamin D_3, alone and in combination vs. placebo over six months, to estimate the efficacy of these agents on markers of differentiation and proliferation in the normal colorectal mucosa. We hypothesized that calcium and vitamin D_3, alone and in combination, increase colonocyte differentiation, decrease the overall rate of proliferation, and normalize the distribution of proliferating cells in crypts within the normal-appearing colorectal epithelium.

Patients and Methods

This study was approved by the Emory University IRB; written informed consent was obtained from each study participant.

Participant Population

The detailed protocol of study recruitment and procedures with detailed specific exclusions was published previously (377). Briefly, eligible patients, 30-75 years of age, in general good health, with a history of at least one pathology-confirmed adenomatous colorectal polyp within the past 36 months, and no contraindications to calcium or vitamin D supplementation or rectal biopsy procedures and no medical conditions, habits, or medication usage that would otherwise interfere with the study treatment or procedures, were recruited from the patient population attending the Digestive Diseases Clinic at the Emory Clinic, Emory University.

Clinical Trial Protocol

Between April 2005 and January 2006, 522 eligible patients were identified after initial screening of electronic medical records, and 224 (43%) patients were sent an introductory letter and contacted by telephone to see if they would be interested and eligible to participate in the study. Potential participants (n=105; 47%) attended the eligibility visit, during which they were interviewed, signed a consent form, completed questionnaires, and provided a blood sample (377). Diet was assessed with a semiquantitative food frequency questionnaire (382). Medical and pathology records were reviewed. After a 30-day placebo run-in trial, 92 (88%) participants without significant perceived side effects and who had taken at least 80% of their tablets had their vital signs taken, underwent a baseline rectal biopsy and, if still willing to participate, were randomly assigned to the following four treatment groups (n=23/treatment group) for six months (duration to ensure 25-OH-vitamin D steady state): a placebo control group, a 2.0 g elemental calcium (as calcium carbonate in equal doses twice daily) supplementation group, an 800 IU vitamin D_3 supplementation group (400 IU twice daily), and a calcium plus vitamin D supplementation group taking 2.0 g elemental calcium plus 800 IU of vitamin D_3 daily.

Study tablets were custom manufactured by Tishcon Corporation, NY, USA. The supplement and placebo pills were identical in size, appearance, and taste. The placebo was free of calcium, magnesium, vitamin D, and chelating agents. Additional details on the rationale for the doses and forms of calcium and vitamin D supplementation forms were previously published (377).

Participants attended follow-up visits at 2 and 6 months after randomization and were contacted by telephone at monthly intervals between the second and final follow-up visits. Pill-taking adherence was assessed by questionnaire, interview, and pill count. Participants were instructed to remain on their usual diet and not take any nutritional supplements not being taken on entry into the study. At each of the follow-up visits participants were interviewed, filled out questionnaires, and had their vital signs taken. At the last visit all participants underwent venipuncture and a rectal biopsy procedure.

All visits for a given participant were scheduled at the same time of day to control for possible circadian variability in the outcome measures. Factors hypothesized to be related to the expression of biomarkers in normal colon mucosa (*e.g.*, diet, age) were assessed at baseline and at the final follow-up visit. Participants did not have to be fasting for their visits and did not take a bowel cleansing preparation or enema.

Six sextant 1-mm-thick biopsy specimens were taken from the rectal mucosa 10 cm proximal to the external anal aperture through a rigid sigmoidocsope with a jumbo cup flexible endoscopic forceps mounted on a semiflexible rod. The biopsies were then immediately placed in phosphate buffered saline, reoriented under a dissecting microscope and transferred to 10% normal buffered formalin followed by transfer to 70% ethanol 24 hours after initial placement in formalin. Then, within a week the biopsies were processed and embedded in paraffin blocks (three biopsies per block).

Immunohistochemistry Protocol

Five slides with four 3.0 μm-thick section levels each taken 40 microns apart were prepared for each antigen. Heat-mediated antigen retrieval was accomplished by placing the slides in a preheated Pretreatment Module (Lab Vision Corp., CA) with 100x Citrate Buffer pH 6.0 (DAKO S1699, DAKO Corp., Carpinteria, CA) and steaming them for 40 minutes. After antigen retrieval, slides were placed in a DAKO Automated Immunostainer and immunohistochemically processed using a labeled streptavidin-biotin method for p21, hTERT, and MIB-1 as summarized in **Figure 4.1**. The slides were not counterstained. After staining, the slides were coverslipped with glass coverslips with a Leica CV5000 Coverslipper (Leica Microsystems, Inc., IL). In each staining batch of slides, positive and negative control slides were included. Tonsil was used as a control tissue for all biomarkers. The negative and the positive control slides were treated identically to the patients' slides except that antibody diluent was used rather than primary antibody on the negative control slide.

Protocol for Quantifying Staining Density of Immunohistochemically Detected Biomarkers in Normal Colon Crypts ("Scoring")

A quantitative image analysis method ("scoring") was used to evaluate the expression of the biomarkers in the colon crypts, as described previously (377) and demonstrated in **Figure 4.2, A**. Briefly, a "scorable" crypt was defined as an intact crypt extending from the muscularis mucosa to the colon lumen (381). Before analysis, negative and positive control slides were checked for staining adequacy. Standardized settings were used on all equipment and software for the image analysis procedures: Olympus BX40 light microscope (*Olympus* America, Inc., PA), Polaroid DMC digital light microscope camera (Polaroid Corporation, MN), computer, digital drawing board, ImagePro Plus image analysis software (Media Cybernetics, Inc., MD), our custom plug-in software for colorectal crypt analysis, and Microsoft Access (Microsoft Corporation, WA). The technician reviewed slides under the light microscope and selected two of three biopsies with 8 to 10 "scorable" crypts per biopsy, then, created a background correction image for each slide, captured the 16-bit grayscale 1,600 x 1,200 pixel image of the crypt at 200x magnification, and traced the borders of the "hemicrypt" (one half of the crypt). The program then divided the tracing into equally spaced intervals to yield segments with the average widths of normal colonocytes, measured the optical density of the labeling across the entire hemicrypt as well as within each segment, and saved the resulting data into the database. Then, the technician moved to the next hemicrypt and repeated all the previously described analysis steps.

One slide reader analyzed all of the stained slides throughout the study with high intra-reader reliability – 0.95 for MIB-1, 0.98 for hTERT, and 0.96 for p21.

Protocol for Measuring Plasma 25-OH- and 1,25-$(OH)_2$-Vitamin D Levels

All laboratory assays for plasma 25-OH- and 1,25-$(OH)_2$-vitamin D were performed by Dr. Bruce Hollis at the Medical University of South Carolina in a blinded manner using a radioimmunoassay method as previously described (388). The average intra-assay coefficient of variation for 25-OH-vitamin D was 2.3%, and for 1,25-$(OH)_2$-vitamin D, 6.2%.

Statistical Analysis

Treatment groups were assessed for comparability of characteristics at baseline and at final follow-up by the Fisher's exact test for categorical variables and analysis of variance (ANOVA) for continuous variables. Slide "scoring" reliability was analyzed using intraclass correlation coefficients.

Several outcome variables were defined to estimate the expression of the markers in the crypts overall as well as how they were distributed within the crypts. The mean optical density of staining for MIB-1, hTERT, and p21 in normal colon crypts was calculated for each patient at baseline and 6-months follow-up by summing all the densities from all analyzed crypts from the biopsy specimens and dividing by the number of crypts analyzed (this measure indicates the overall rate of proliferation or differentiation of rectal crypt epithelial cells and is further referred to as LI, labeling index (381)). The crypt differentiation compartment was defined *a priori* as the upper 40% of the crypts, and the crypt proliferation compartment as the bottom 60% of the crypts (**Figure 4.1**) (43, 45, 381) . Measures of the within-crypt distributions of the proliferation markers (*i.e.*, the ratio of expression in the upper 40% to that in the entire crypts, φ_h) were calculated for each patient by taking the mean of the biomarker densities in the upper 40% of crypts and dividing it by the biomarker densities in the entire crypt. For the proliferation markers, we decided *a priori* to use the φ_h because it is an indicator of an upward extension of the canonical proliferative zone of the colon crypt and was found previously to be modified by calcium and/or calcium plus vitamin D supplementation (42, 45, 275).

Primary analyses were based on assigned treatment at the time of randomization, regardless of adherence status (intent-to-treat analysis). The three biomarkers were analyzed separately. We transformed biomarker expression density data by dividing each individual measurement by the staining batch's average density to adjust for possible batch effects (batch standardization). At baseline batch-specific mean staining densities were calculated using the measurements from all treatment groups, whereas for the follow-up visit, only measurements from the placebo group were used. Absolute treatment effects were calculated as the differences in the batch-standardized densities from baseline to the 6-months follow-up visit between patients in each active treatment group and the placebo group using a MIXED effects model. Since optical density is measured in arbitrary units, to provide perspective on the magnitude of the treatment effects we also calculated relative effects (377, 381), defined as: [treatment group follow-up mean/treatment group baseline mean]/[placebo follow-up mean/placebo baseline mean]. The relative effect provides an estimate of the proportional change in the treatment group relative to that in the placebo group, and its interpretation is somewhat analogous to that of an odds ratio (*e.g.*, a relative effect of 2.0 would mean that the relative proportional change in the treatment group was two times as great as that in the placebo group). Since the treatment groups were balanced on risk factors at baseline, no adjustment was made for other covariates in the primary intent-to-treat analyses.

Spearman's rank and partial Spearman's rank correlation coefficients were used to compare cell proliferation marker values at baseline and follow-up, respectively.

The distributions of the biomarkers' staining densities were graphically evaluated using the LOESS procedure with smoothing parameter 0.5 and local quadratic fitting. First, the number of sections within a hemicrypt was standardized to 50. Then, the average for each section across all crypts was predicted by the LOESS model separately for each patient, and then for each treatment group by follow-up visit. The results were plotted in the graphs along with smoothing lines.

In sensitivity analyses, we also analyzed data without standardization for batch, as well as by including batch as a covariate and using different transformations. The results from these analyses did not differ materially from those reported. Statistical analyses were done using SAS System software (version 9.1.3; SAS Institute, Inc., NC). A cutoff level of $P \leq 0.05$ (2-sided) was used for assessing statistical significance.

Results

Characteristics of Study Participants

The treatment groups did not differ significantly on participant characteristics measured at baseline (**Table 1**) or at the end of the study (data not shown). The mean age of the participants was 61 years, 64% were men, 71% were white, and 20% had a family history of colorectal cancer in a first degree relative. Most participants were non-smokers, college graduates, and overweight. Biopsy specimens that were “scorable” were obtained for 87, 90, and 90 participants at baseline, and for 83, 85, and 84 participants at 6-month follow-up for the hTERT, MIB-1, and p21 markers, respectively.

Adherence to visit attendance averaged 92% and did not differ significantly among the four treatment groups. On average, at least 80% of pills were taken by 93% of participants at the first follow-up visit and 84% at the final follow-up visit. There were no treatment or biopsy complications. Seven people (8%) were lost to follow-up due to perceived drug intolerance (n=2), unwillingness to continue participation (n=3), physician’s advice (n=1), and death (n=1). Dropouts included one person from the vitamin D supplementation group, and two persons from each of the other three groups.

At baseline, there were no significant differences between the four study groups in plasma 25-OH - or 1,25-$(OH)_2$-vitamin D levels. At the study end, the vitamin D and calcium plus vitamin D groups had significantly higher levels of plasma 25-OH-vitamin D ($P<0.001$), whereas the placebo and calcium groups had slight non-significant decreases in 25-OH-vitamin D levels (**Table 4.2, A**). As expected, plasma levels of 1,25-$(OH)_2$-vitamin D at the end of follow-up period did not differ significantly between study groups (data not shown, see (377)).

Effects of Calcium and/or Vitamin D on p21 Expression in Normal Colorectal Crypts

After six months treatment, p21 expression along the full lengths of crypts increased statistically significantly by 201% ($P=0.03$), 242% ($P=0.005$), and 25% ($P=0.41$) in the calcium, vitamin D, and calcium plus vitamin D groups, respectively, relative to the placebo group (**Table 4.2, B**). The graphical assessment of changes over six months in the distribution of p21 expression along crypts demonstrated that the largest post-supplemental increases in p21 were in the upper 40% of the crypts (**Figure 4.2, B** and **C**), and the numerical findings limited to the upper 40% of the crypts were essentially the same as for the entire crypt (**Table 4.2, B**).

Effects of Calcium and/or Vitamin D on MIB-1 and hTERT Expression in Normal Colorectal Crypts

There were no statistically significant treatment effects on the expression of MIB-1 in the crypts overall or in the proportion of its overall expression that extended into the upper 40% of the crypts (φ_h) in any active treatment group relative to placebo (**Table 4.2, C**). Also, there were no statistically significant changes in the expression of hTERT in the entire crypt at the end of follow-up; however, the hTERT labeling index φ_h decreased by 10% (*P*=0.13), 3% (*P*=0.61), and 15% (*P*=0.02) in the calcium, vitamin D and calcium plus vitamin D groups relative to the placebo, respectively (**Table 4.2, D**).

Graphical assessments of changes in the distributions of MIB-1 and hTERT, and separate analyses of changes in the expression of these biomarkers in the upper 40% and lower 60% of the crypts over six months treatment indicated that the decrease in the φ_h observed in each active treatment group relative to the placebo at the end of the follow-up, while related to decreases in biomarker expression in the upper 40% of the crypts, was also related, in part, to slight increases in expression in the bottoms of the crypts (data not shown).

A statistically significant positive correlation was found between the baseline expression of MIB-1 and hTERT with Spearman's rank correlation coefficients being 0.35 (*P*=0.001) and 0.28 (*P*=0.009) for the LI and φ_h, respectively. At the end of follow-up, the MIB-1 and hTERT labeling indices were positively correlated ($\rho_{partial}$=0.35, P=0.001), but not the MIB-1 φ_h with the hTERT φ_h ($\rho_{partial}$=0.13, P=0.24). A weak statistically non-significant correlation was noted between the LI and φ_h for each of the cell proliferation biomarkers at both study visits ($\rho<|0.15|$, $P>0.31$).

We also investigated whether VDR genotype, change in 25-OH-vitamin D levels, adherence to treatment, sex, family history of colorectal cancer, and NSAID use modified the observed associations; however, the sample size was too small for these results to be reliable (data not shown).

Discussion

These data provide evidence for a substantial increase in cell differentiation, as indicated by increased expression of p21, in the normal colorectal epithelium of sporadic adenoma patients in response to vitamin D_3 or calcium supplementation and, thus, are consistent with the hypothesis that increased levels of circulating vitamin D or a higher intake of calcium may reduce risk for colorectal neoplasms. Our data also suggest that vitamin D_3 may have a slightly greater effect than calcium on p21 expression, and vitamin D combined with calcium may have a lesser treatment effect than either calcium or vitamin D alone on p21. Furthermore, the data provide no evidence that the overall colorectal epithelial cell proliferation rate, as indicated by the expression of short- and long-term markers of proliferation in the entire colorectal crypt, can be reduced by calcium and vitamin D, alone or in combination. However, our data suggested that calcium combined with vitamin D may shift downwards (“normalize”) the distribution of proliferating cells in the colorectal crypts as indicated by the expression of a long-, but not short-term marker of cell proliferation.

p21$^{waf1/cip1}$, a cyclin-dependent kinase inhibitor used in this study as a marker of differentiation, is a potent inducer of differentiation in intestinal colonocytes (302), and its expression is known to be downregulated during the early stages of colon tumorigenesis (302, 303). p21 was also reported to participate in cell cycle regulation (298) and control of DNA methylation (301), and to interact with regulatory proteins, among which is calmodulin (300). As was found in colon cancer cells *in vitro* (201, 202, 261, 305, 306), we hypothesized that vitamin D and calcium would increase p21 expression in the normal human colorectal epithelium *in vivo*. The plausibility of this hypothesis is supported by the fact that the p21 gene is a primary 1,25-$(OH)_2$-vitamin D_3-responsive gene with at least three vitamin D response element (VDRE)-containing regions within its promoter (323); and that calcium, through the calcium-sensing receptor (CaSR), promotes differentiation in colorectal epithelial cells (201, 202). However, there is little literature regarding direct regulation of p21 by calcium, but there is some evidence that extracellular calcium activates protein kinase C, which is associated with the differential induction of p21 in the intestinal epithelium (7). Also, an intracellular calcium gradient along the colon crypt that coincides with the differentiation compartment may modulate differentiation of the colonocytes, thus, regulating p21 expression (203). As hypothesized, we observed the largest increase in p21 expression in the vitamin D group, and to a lesser extent in the calcium group; however, we have found a relatively small increase in the calcium plus vitamin D group. There are several possible explanations for the latter finding, including the possibility that the observed treatment effect in the calcium plus vitamin D group may have been due to chance, or that the two agents may have attenuated the effects of either alone. One animal study

(246) found that calcium and vitamin D separately are more potent inhibitors of colon tumorigenesis than when combined. However, several other animal studies that investigated the combined effect of calcium and vitamin D reported stronger effects with vitamin D and calcium combined (243, 244); and the results of a large adenoma recurrence trial suggested that vitamin D enhanced the chemopreventive effect of calcium (237). Thus, the combined effect of calcium and vitamin D on colon crypt epithelial cell differentiation as indicated by p21 expression is not clear and a larger more definitive study is needed to clarify it.

No previous human studies tested the effect of calcium and/or vitamin D supplementation on p21 expression in the normal colorectal mucosa, but three small studies (43-45) investigated the effects of these agents or low fat dairy foods on other markers of differentiation (acidic mucins and/or cytokeratin AE1) in the normal colorectal mucosa with inconsistent results. Two small studies found no changes in the normal rectal crypt differentiation markers after supplementation with calcium and vitamin D_3 (45), or with calcium or low fat dairy foods (44); but a third, larger (N=70), randomized, placebo-controlled trial reported significant changes in differentiation markers after supplementation with low fat dairy foods, which are rich in calcium and vitamin D, but contain other components that may also exert prodifferentiative effects (43). Taken altogether, the results of the present and past studies combined with the biological evidence suggest that calcium and vitamin D induce differentiation in the normal human colorectal mucosa, and that expression of p21 may be a more suitable biomarker of differentiation than other currently investigated markers.

Unlike other studies, we used two different markers of proliferation, hTERT and MIB-1, detected by immunohistochemical methods. MIB-1/Ki-67 is expressed in all cells not in G_0 phase of the cell cycle (274); and hTERT protein, a catalytic subunit of telomerase, which functions to regenerate telomeres on the ends of chromosomes, is expressed in almost all human cancers and some normal proliferative epithelial cells such as in the colorectal crypt base (392-394). We hypothesized that hTERT expression in colorectal crypts better reflects average, long term proliferative activity than do “snapshot” proliferative indicators, such as the S-phase markers PCNA (proliferating cell nuclear antigen) and MIB-1, which demonstrate rapid, large responses to short term physiologic stimuli. Biological evidence supports the growth-restraining actions of calcium and vitamin D on colorectal epithelial cells (7), however few human studies tested the effect of vitamin D and calcium on cell proliferation in the colon.

There have been two large clinical trials of calcium and colorectal epithelial cell proliferation (278, 279) as well as several smaller trials (reviewed in (275), also (43, 44, 280-282)). One of these trials (N=193) found no evidence for a reduction in the labeling index (LI), but a marked, statistically significant proportional decrease in the φ_h (278), but the second trial (N=333) (279), with more methodological problems (275), found no effect on either measure of cell proliferation. The findings from several smaller controlled studies were inconsistent, with some suggesting decreases in the LI and/or φ_h, and other studies indicating no change or statistically non-significant increases in the LI and/or φ_h. The results of the present study for the LI are consistent with those from the previously conducted large clinical trials (278, 279); and for the φ_h with one large clinical trial (278) and several smaller clinical trials (reviewed in (275), also (281, 282)).

However, it must be emphasized that the present study was a pilot study with limited statistical power; thus, our findings may have been due to chance. Other possible explanations for our findings may have been the use of an antibody that may have low specificity detecting hTERT (392); that the MIB-1 and/or hTERT markers may not be good biomarkers of cell proliferation in normal colorectal crypts; or that calcium may indeed have no substantial effect on colorectal cell proliferation in sporadic adenoma patients.

No published human studies tested the effect of vitamin D alone or combined with calcium on the hTERT or MIB-1 markers of proliferation, but one small randomized clinical trial (N=21) found a significantly decreased MIB-1 labeling index, but not the φ_h, in flat mucosa and resected polypoid tissue after 6-months supplementation with calcium (1,500 mg/day) plus vitamin D_3 (400 IU/day) (45). Contrary to the results of one study (45), we did not find evidence for an effect of vitamin D alone or in combination with calcium on overall MIB-1 or hTERT labeling, but we did find a significant downward shift in hTERT expression in the calcium plus vitamin D group. However, as pointed out above, these findings may be due to chance, non-specific detection methods, or an insufficient vitamin D_3 dose or duration.

Previous studies (395) and our study found that the LI and φ_h are statistically independent variables, and other controlled trials testing calcium or other agents on cell proliferation rates found statistically significant reductions in the φ_h, but not the LI (278, 284, 396, 397). Therefore, the LI and φ_h may represent different biological aspects of colon tumorigenesis, and serve as independent markers of risk for colorectal neoplasia.

The present study was conducted to test the joint and separate effects of calcium and vitamin D on the individual components and aggregate profile of a molecular phenotype panel of biomarkers of risk for colorectal cancer, which includes biomarkers of APC and mismatch repair pathways, cell cycle events, and others. We previously reported a statistically significant effect of vitamin D on the pro-apoptotic marker Bax(377), and analyses for other biomarkers in the panel are currently underway. Taken all together, the present and previously published data (377) suggest that calcium and vitamin D may have stronger effects on cell differentiation and apoptosis than on proliferation; and that vitamin D may have greater effects on colorectal epithelial cell differentiation and apoptosis than does calcium alone or in combination with vitamin D. However, larger, more definitive clinical studies are needed to confirm these results.

This study has several limitations. The most obvious limitation is the small sample size resulting in an increased role for chance in detecting or not detecting a treatment effect. The small size also did not allow us to conduct additional subgroup analyses. Another limitation is that, although human studies have found that cell proliferation rates observed in the rectal mucosa are correlated with those found throughout the colon (398, 399), animal studies found that calcium affects cell proliferation throughout the colon (400, 401), and one intervention trial found that calcium decreases the LI and φ_h in the rectum and sigmoid colon, but not in the descending colon (284), there are insufficient data to assume that the effect of calcium is the same in the distal and proximal parts of the colon in humans. Furthermore, the effects of vitamin D alone or in combination with calcium on proliferation and differentiation in different parts of the colon (other than the rectum) are not clear, as there were no such studies in humans. Also, it is unknown whether vitamin D and/or calcium may affect human normal colon, adenoma, and cancer tissue differently. Another potential limitation of this study is that proliferation and differentiation markers are evidentially well-supported, but not proven biomarkers of risk for colorectal neoplasms. Therefore, this study cannot prove that because calcium and vitamin D substantially increase p21 expression and may shrink the proliferative zone in the colorectal crypts, they can reduce risk for colorectal neoplasms. The findings of this study may not be generalizable to other populations. Finally, there may be more specific methods and antibodies to detect telomerase expression in colorectal crypts (392), and MIB-1 and hTERT may not adequately reflect cell proliferation rates in normal-appearing colorectal crypts.

The strengths of this study are that it is, to our knowledge, the first clinical trial of the effects of calcium and vitamin D_3, alone and in combination on colorectal epithelial proliferation and differentiation in sporadic adenoma patients; the randomized, double-blind, placebo-controlled trial design; evaluation of both long- and short-term proliferation markers; high protocol adherence by study participants; automated biopsy processing and immunostaining procedures; the use of quantitative image analysis; and the strict quality control and consequent high scoring reliability of rectal biopsies.

In summary, these preliminary results from this pilot clinical trial indicate that calcium and vitamin D increase colorectal epithelial cell differentiation and may have relatively little, if any, effect on overall proliferation rates in the colorectal mucosa, but do not rule out a potential normalization of the proliferative zone in the colorectal crypts. This study suggests that p21 expression may be a treatable biomarker of risk for colorectal neoplasms and supports further investigation of calcium and vitamin D_3 as chemopreventive agents against colorectal neoplasms.

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Notes

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Tables and Figures

Table 4.1. Selected baseline characteristics of the study participants[*] (n=92).

	Treatment Group				
Characteristics	**Placebo** (n=23)	**Calcium** (n=23)	**Vitamin D** (n=23)	**Calcium + Vit. D** (n=23)	***P***[**]
Demographics, medical history, habits, anthropometrics					
Age, years	58.5 (8.2)	61.9 (8.2)	60.2 (8.1)	62.1 (7.5)	0.39
Men (%)	70	70	70	70	1.00
White (%)	74	83	65	61	0.39
College graduate (%)	65	61	57	44	0.53
History of colorectal cancer in 1° relative (%)	17	30	17	13	0.60
Take NSAID[***] regularly[§] (%)	22	13	9	22	0.60
Take aspirin regularly[§] (%)	22	52	30	56	0.05
If woman (n = 28), taking estrogens (%)	4	9	4	4	1.00
Current smoker (%)	9	4	0	0	0.61
Take multivitamin (%)	30	30	26	39	0.86
Body mass index (BMI), kg/m^2	30.6 (7.2)	29.4 (5.5)	28.9 (5.56)	31.6 (6.0)	0.44
Mean dietary intakes					
Total energy intake, kcal/d	1,596 (528)	1,788 (691)	1,848 (821)	1,845 (752)	0.59
Total[§§] calcium, mg/d	618 (308)	746 (335)	843 (526)	824 (714)	0.41
Total[§§] vitamin D, IU/d	277 (230)	336 (202)	360 (317)	415 (316)	0.40
Total fat, gm/d	67 (32)	72 (35)	70 (32)	74 (28)	0.59
Dietary fiber, gm/d	15 (7)	17 (9)	18 (9)	17 (11)	0.97
Alcohol, gm/d	9 (14)	11 (15)	14 (18)	10 (20)	0.84

(Table continues)

Table 4.1 (continued).

	Treatment Group				
	Placebo	**Calcium**	**Vitamin D**	**Calcium + Vit. D**	***P***[**]
Characteristics	(n=23)	(n=23)	(n=23)	(n=23)	
Adenoma characteristic					
Multiple adenomas[¤] (%)	17	22	39	26	0.45
Large adenoma[£] (%)	19	32	17	9	0.32
Villous/tubulovillous adenoma[££] (%)	4	9	9	4	1.00
Mild dysplasia (%)	100	96	100	100	1.00

* Data are given as means (SD) unless otherwise specified.

** By Fisher's exact χ^2 test for categorical variables, and ANOVA for continuous variables.

*** Nonsteroidal anti-inflammatory drug.

§ At least once a week.

§§ Diet plus supplements.

¤ At least two adenomas.

£ At least one large (≥ 1cm) adenoma.

£ At least one villous or tubulovillous adenoma.

Table 4.2. Plasma 25-OH-vitamin D and colorectal expression of p21, MIB-1, and hTERT during the clinical trial.

	Baseline				6-Months Follow-up				Absolute Rx Effect**				Relative Effect §
	N	Mean	SE	P*	N	Mean	SE	P*	N	Mean	SE	P*	
A. Plasma 25-OH-vitamin D, ng/mL													
Placebo	23	20.44	1.57		21	17.89	1.51		21	0			1.00
Calcium	23	25.67	1.58	0.05	21	23.20	1.94	0.03	21	0.20	1.77	0.88	1.03
Vitamin D	23	21.04	1.74	0.81	22	29.48	1.54	<0.0001	22	10.90	1.75	<0.0001	1.60
Ca + Vit. D	23	20.93	2.01	0.84	21	28.51	1.73	<0.0001	21	10.50	1.77	<0.0001	1.56
B. P21$^{¥}$ Expression in Colorectal Crypts													
Entire crypts (LI)													
Placebo	22	1.23	0.17		21	1.00	0.18		20	0			1.00
Calcium	23	0.85	0.17	0.11	21	1.37	0.18	0.14	21	0.78	0.33	0.03	2.01
Vitamin D	22	0.81	0.17	0.08	21	1.58	0.18	0.02	20	0.98	0.34	0.005	2.42
Ca + Vit. D	23	1.12	0.17	0.62	21	1.13	0.18	0.60	21	0.23	0.33	0.47	1.25
Upper 40% of crypts (LI$_{40}$)													
Placebo	22	1.10	0.15		21	0.91	0.16		20	0			1.00
Calcium	23	0.86	0.15	0.26	21	1.43	0.16	0.02	21	0.77	0.31	0.02	2.02
Vitamin D	22	0.77	0.15	0.13	21	1.54	0.16	0.01	20	0.96	0.31	0.003	2.44
Ca + Vit. D	23	1.02	0.15	0.70	21	1.09	0.16	0.43	21	0.26	0.31	0.41	1.29

(Table continues)

Table 4.2 (continued).

	Baseline				6-Months Follow-up				Absolute Rx Effect**				Relative Effect [§]
	N	Mean	SE	*P**	N	Mean	SE	*P**	N	Mean	SE	*P**	
C. MIB-1[¥] Expression in Colorectal Crypts													
Entire crypts (LI)													
Placebo	22	1.01	0.10		21	1.00	0.10		20	0			1.00
Calcium	23	0.90	0.09	0.42	21	1.09	0.10	0.50	21	0.18	0.19	0.30	1.23
Vitamin D	22	0.83	0.10	0.18	22	1.08	0.10	0.58	22	0.25	0.19	0.18	1.32
Ca + Vit. D	23	1.25	0.09	0.09	21	1.10	0.10	0.49	21	-0.13	0.19	0.50	0.89
Ratio of upper 40% to entire crypts (φ_h)													
Placebo	22	0.070	0.012		21	0.064	0.013		20	0			1.00
Calcium	23	0.085	0.012	0.40	21	0.073	0.013	0.64	21	-0.006	0.03	0.80	0.94
Vitamin D	22	0.081	0.012	0.56	22	0.071	0.012	0.71	22	-0.003	0.03	0.89	0.97
Ca + Vit. D	23	0.077	0.012	0.72	21	0.068	0.013	0.84	21	-0.003	0.03	0.92	0.97
D. hTERT[¥] Expression in Colorectal Crypts													
Entire crypts (LI)													
Placebo	21	1.08	0.10		20	1.00	0.10		19	0			1.00
Calcium	22	1.01	0.10	0.63	20	1.00	0.10	0.99	19	0.07	0.21	0.73	1.07
Vitamin D	22	0.83	0.10	0.08	22	0.97	0.10	0.85	21	0.25	0.21	0.27	1.27
Ca + Vit. D	22	1.08	0.10	0.98	21	1.06	0.10	0.70	20	0.14	0.21	0.80	1.05

(Table continues)

Table 4.2 (continued).

	Baseline				6-Months Follow-up				Absolute Rx Effect**				Relative Effect §
	N	**Mean**	**SE**	***P****	**N**	**Mean**	**SE**	***P****	**N**	**Mean**	**SE**	***P****	
Ratio of upper 40% to entire crypts (φ_h)													
Placebo	21	0.366	0.014		20	0.417	0.014		19	0			1.00
Calcium	22	0.385	0.013	0.33	20	0.394	0.014	0.24	19	-0.04	0.03	0.13	0.90
Vitamin D	22	0.371	0.013	0.81	22	0.407	0.013	0.63	21	-0.01	0.03	0.61	0.97
Ca + Vit. D	22	0.388	0.013	0.25	21	0.374	0.014	0.03	20	-0.07	0.03	0.02	0.85

* *P*-value for difference between each active treatment group and placebo group from Mixed model.

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

§ Relative effect = [(treatment group follow-up/treatment group baseline)/(placebo follow-up/placebo baseline)]; interpretation similar to that for an odds ratio (*e.g.*, a relative effect of 1.8 would indicate a proportional increase of 80% in the treatment group relative to that in the placebo group).

[¥] Biomarkers detected immunohistochemically and then their labeling densities were quantified by image analysis; all biomarkers values shown as batch-standardized optical densities. Batch standardization for each biomarker was performed by dividing each individual measurement by the staining batch's average optical density.

Figure 4.1. Summary of biomarker immunohistochemical protocols and images (at 200x magnification) of colon crypts immunohistochemically processed for: **A**. p21, differentiation marker; **B.** MIB-1/Ki-67, marker of short term proliferative activity; **C.** hTERT, marker of long term proliferative activity.

Antibody	Clone	Manufacturer	Catalog No.	Dilution	Detection Kit*
MIB-1/Ki-67	MIB-1	DAKO Corp., Carpinteria, CA	M7240	1:350	LSAB2
hTERT	Y182	Epitomics, Inc., Burlingame, CA	1531-1	1:50	LSAB2
p21$^{waf1/cip1}$	SX118	DAKO Corp., Carpinteria, CA	M7202	1:40	LSAB2

* DAKO Corp., Carpinteria, CA

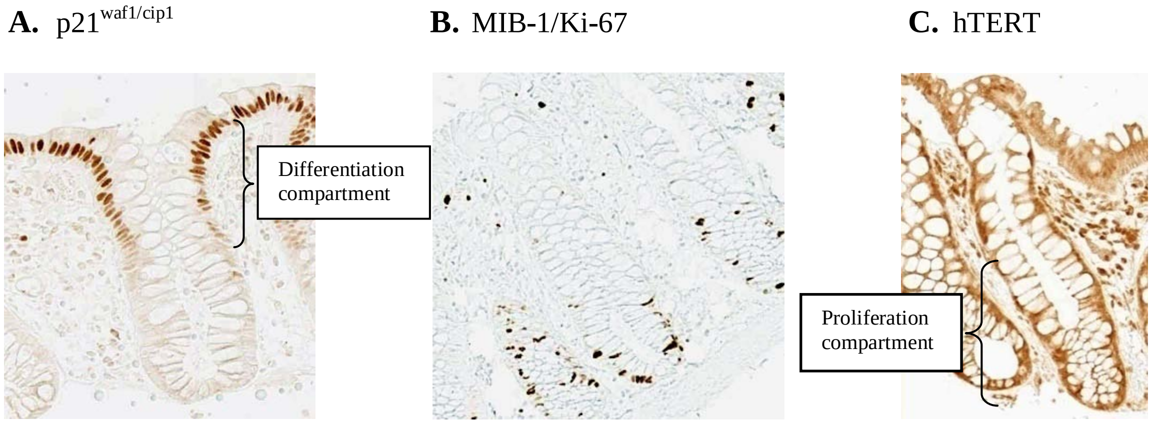
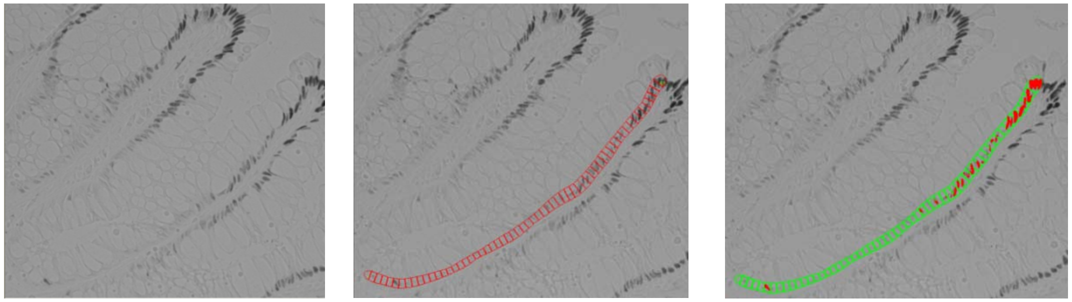


Figure 4.2. A quantitative image analysis (**A**) with an example of resulting distributions of p21 marker expression (staining optical densities) along the normal-appearing colorectal crypts in the calcium (**B**) and vitamin D (**C**) groups at baseline and follow-up visits.

A. Quantitative image analysis of p21 staining optical densities along the normal colorectal crypts consists of several steps: i) finding “scorable” crypts (refer to text for details); ii) manually tracing half of the crypt (“hemicrypt”), followed by automated division of the outline into segments representing the width of an average colonocyte; iii) automated background-corrected densitometry of the overall and segment-specific labeling of the biomarker and entry of the resulting data into the database.



i) Finding “scorable” crypt (hemicrypt = half of the crypt)

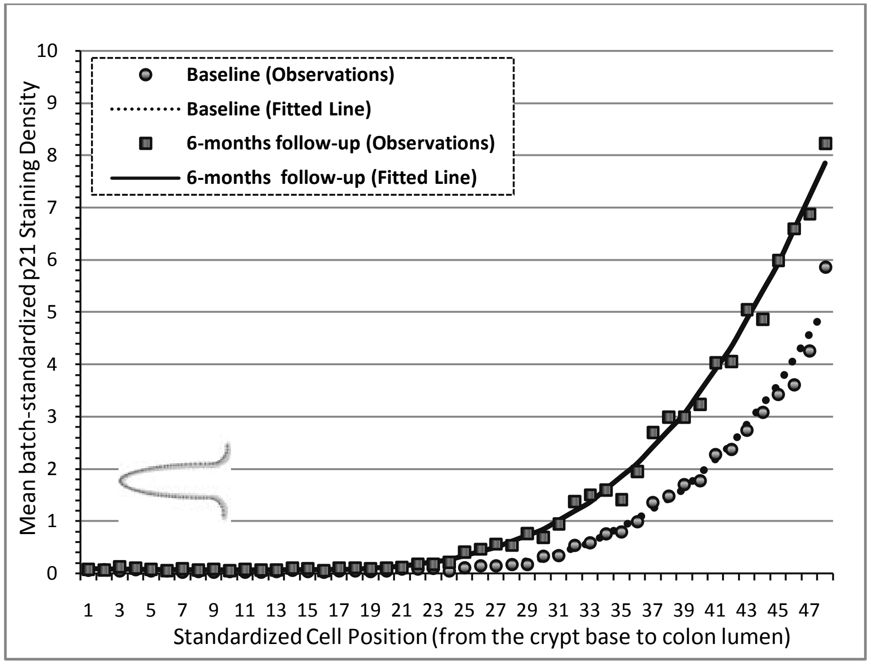
ii) Tracing the hemicrypt and segmenting the outline

iii) Detecting p21 staining optical density and storing the data

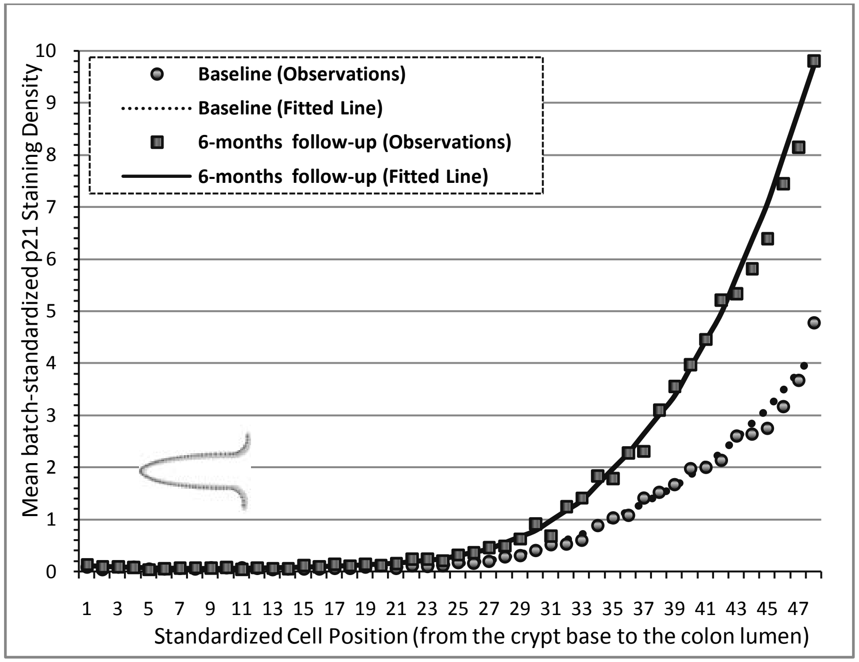
(Figure continues)

Figure 4.2 (continued).

B. Mean batch-standardized optical density of p21 staining along normal colorectal crypts in the calcium group at baseline and six months follow-up.



C. Mean batch-standardized optical density of p21 staining along normal colorectal crypts in the vitamin D group at baseline and six months follow-up.



CHAPTER 5. EFFECTS OF VITAMIN D AND CALCIUM SUPPLEMENTATION ON MARKERS OF DNA DAMAGE IN NORMAL COLON MUCOSA: A RANDOMIZED, DOUBLE-BLIND, PLACEBO-CONTROLLED CLINICAL TRIAL

Veronika Fedirko[1,2], Roberd M. Bostick[1,2], Qi Long[2,3], W. Dana Flanders[1,2,3], Marjorie L. McCullough[4], Eduard Sidelnikov[1,2], Carrie R. Daniel[5], Robin E. Rutherford[6], Aasma Shaukat[7]

[1]Department of Epidemiology, Rollins School of Public Health, Emory University, Atlanta, GA 30322

[2]Winship Cancer Institute, Emory University, Atlanta, GA 30322

[3]Department of Biostatistics and Bioinformatics, Rollins School of Public Health, Emory University, Atlanta, GA 30322

[4]Epidemiology and Surveillance Research Department, American Cancer Society, Atlanta, GA 30303

[5]Nutritional Epidemiology Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, Department of Health and Human Services, Bethesda, MD 20852

[6]Emory University School of Medicine, Division of Digestive Diseases, Atlanta, GA 30322

[7]Department of Medicine, GI Division, University of Minnesota, Minneapolis, MN 55455

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Abstract

The exact anti-neoplastic effects of calcium and vitamin D_3 in the human colon are unclear. Animal and *in vitro* studies demonstrated that these two agents reduce oxidative stress, but these findings have never been investigated in humans. To address this, we conducted a pilot, randomized, double-blind, placebo-controlled, 2x2 factorial clinical trial to test the effects of calcium and vitamin D_3 on a marker of oxidative DNA damage, *8*-hydroxy-2'-deoxyguanosine (8-OH-dG), in the normal colorectal mucosa.

Patients (n=92) with at least one pathology-confirmed colorectal adenoma were treated with calcium 2 g/day and/or vitamin D_3 800 IU/day *vs.* placebo over six months. Overall labeling and colorectal crypt distribution of 8-OH-dG in biopsies of normal-appearing rectal mucosa were detected by standardized automated immunohistochemistry and quantified by image analysis.

After six months treatment, 8-OH-dG labeling along the full lengths of colorectal crypts decreased by 22% (*P*=0.15) and 25% (*P*=0.10) in the calcium and vitamin D groups, respectively, but not in the calcium plus vitamin D group. The estimated treatment effects were strongest among participants with higher baseline colon crypt vitamin D receptor (VDR) expression (*P*=0.05).

Overall, these preliminary results indicate that calcium and vitamin D may decrease oxidative DNA damage in the normal human colorectal mucosa; support the hypothesis that 8-OH-dG labeling in colorectal crypts is a treatable oxidative DNA damage biomarker of risk for colorectal neoplasms; and provide support for further investigation of calcium and vitamin D as chemopreventive agents against colorectal neoplasms.

Introduction

Colorectal cancer, the second leading cause of cancer death in the U.S. (1), is a disease highly correlated with the Western-style diet, which is characterized by relatively low calcium consumption, and with low vitamin D exposure (4). Twenty-fold variations in international colon cancer rates, and migration studies showing acquired high risk within a generation, emphasize the importance of environmental exposures, especially diet and physical activity, in the etiology of colorectal cancer (4), and thus to its preventability. Currently, there is no complete agreement as to what dietary factors protect against or promote the development of colorectal cancer, nor any accepted pre-neoplastic biomarkers of risk. Further investigation of potential mechanisms whereby dietary agents lead to clinically relevant changes in normal colon tissue, and the development of biomarkers of risk derived from such mechanistic understanding, are urgently needed.

There is strong biological plausibility and animal experimental evidence for protection against colorectal cancer by calcium and vitamin D (402). Moreover, in epidemiologic studies, higher total calcium intakes have been consistently associated with reduced risk for colorectal neoplasms (33, 221, 378-380), and calcium supplementation reduced adenoma recurrence (41). Also, higher circulating 25-OH-vitamin D levels have been associated with reduced risk for colorectal neoplasms (221, 238). However, the anti-neoplastic effects of calcium and vitamin D on the normal colorectal epithelium remain unclear.

Proposed mechanisms of calcium against colorectal cancer include protection of colonocytes against free bile and fatty acids (5), direct effects on the cell cycle, and modulation of the APC colon carcinogenesis pathway (7). Beyond calcium homeostasis, vitamin D regulates cell cycle events; promotes bile acid degradation; influences growth factor signaling, cell adhesion, and DNA repair; and modulates more than 200 genes (7, 226). Recent evidence also indicates that vitamin D and the VDR (vitamin D receptor) are involved in protection against oxidative damage (228, 230, 331).

Despite the basic science evidence, there are no published human trials of the effects of vitamin D and/or calcium supplementation on markers of oxidative DNA damage, such as *8*-hydroxy-2'-deoxyguanosine (8-OH-dG), in the normal-appearing colorectal mucosa. To address this, we conducted a pilot, randomized, double-blind, placebo-controlled, 2 x 2 factorial chemoprevention clinical trial of supplemental calcium and vitamin D_3, alone and in combination *vs*. placebo over six months, to estimate the efficacy of these agents on a panel of biomarkers (including 8-OH-dG) in the normal colorectal mucosa. We hypothesized that calcium and vitamin D_3, alone and in combination, decrease colorectal epithelial oxidative DNA damage.

Patients and Methods

Participant Population

The detailed protocol of study recruitment and procedures was published previously (377). Briefly, eligible patients, 30-75 years of age, in general good health, capable of informed consent, with a history of at least one pathology-confirmed adenomatous colorectal polyp within the past 36 months, and no contraindications to calcium or vitamin D supplementation or rectal biopsy procedures and no medical conditions, habits, or medication usage that would otherwise interfere with the study were recruited from the patient population attending the Digestive Diseases Clinic at the

Emory Clinic, Emory University. Detailed specific study exclusion criteria were presented elsewhere (377). This study was approved by the Emory University IRB. Written informed consent was obtained from each study participant.

Clinical Trial Protocol

All age-eligible patients who had been diagnosed with at least one pathology-confirmed adenomatous colorectal polyp within the past 36 months were identified as potential study participants. Between April 2005 and January 2006, 522 patients passed initial chart screening for eligibility, and 224 (43%) patients were sent an introductory letter followed by a telephone interview. A total of 105 (47%) potential participants attended an eligibility visit during which there were interviewed, signed a consent form, completed questionnaires, provided a blood sample, and started a one-month placebo run-in period. Diet was assessed with a semiquantitative food frequency questionnaire (382). Medical and pathology records were reviewed. After a 30-day placebo run-in trial, 92 (88%) participants without significant perceived side effects and who had taken at least 80% of their tablets were eligible for randomized assignment. Adherence for the run-in trial was assessed by questionnaire, interview, and pill count. Eligible participants then had their vital signs taken, underwent a baseline rectal biopsy and, if still willing to participate, were randomly assigned to the following four treatment groups: a placebo control group, a 2.0 g elemental calcium (as calcium carbonate in equal doses twice daily) supplementation group, an 800 IU vitamin D_3 supplementation group (400 IU twice daily), and a calcium plus vitamin D supplementation group taking 2.0 g elemental calcium plus 800 IU of vitamin D_3 daily.

All study tablets were custom manufactured by Tishcon Corporation, NY, USA. The corresponding supplement and placebo pills were identical in size, appearance, and taste. The placebo was free of vitamin D, calcium, magnesium, and chelating agents. Additional details on the rationale for the doses and forms of calcium and vitamin D supplementation forms were previously described (377).

The treatment period was six months, and participants attended follow-up visits at 2 and 6 months after randomization and were contacted by telephone between the second and final follow-up visits. Pill-taking adherence was assessed by questionnaire, interview, and pill count. Participants were instructed to remain on their usual diet and not take any nutritional supplements not in use on entry into the study. At each of the follow-up visits participants were interviewed, filled out questionnaires, and had their vital signs taken. At the last visit all participants underwent venipuncture and a rectal biopsy procedure. All participants were asked to abstain from aspirin use for seven days prior to each biopsy visit. All visits for a given participant were scheduled at the same time of day to control for possible circadian variability in the outcome measures. Factors hypothesized to be related to 8-OH-dG levels in the normal colon mucosa (*e.g.*, antioxidant micronutrient intakes) were assessed at baseline and at the final follow-up visit. Participants did not have to be fasting for their visits and did not take a bowel cleansing preparation or enema.

Adverse events were monitored by interview at each study visit and interim telephone call and two weeks after the last visit, questionnaire (included questions about hospitalizations, medical visits and diagnoses, medication changes, and symptoms) at each study visit, and by participant-initiated telephone calls, and graded according to NIH

Common Toxicity Criteria and the likelihood that they were study-related. Two adverse events unrelated to study treatments (cardiovascular disease death and hospitalization) were documented during the course of the trial.

Tissue Collection and Processing

Six sextant 1.0 mm-thick biopsy specimens were taken from the rectal mucosa 10 cm proximal to the external anal aperture through a rigid sigmoidocsope with a jumbo cup flexible endoscopic forceps mounted on a semiflexible rod. The biopsies were then immediately placed in phosphate buffered saline, oriented under a dissecting microscope and placed in 10% normal buffered formalin, and then transferred to 70% ethanol 24 hours after initial placement in formalin. Within a week, the biopsies were processed and embedded in paraffin blocks with three biopsies per block.

Laboratory Methods

The paraffin blocks were cut into 3.0 μm-thick sections, with each level 40 μm apart. Five slides with four section levels per patient per biomarker were prepared for immunostaining. To break the protein cross-links formed by formalin and uncover the epitope, heat-mediated antigen retrieval was used: slides were placed in a preheated Pretreatment Module (Lab Vision Corp., CA) with 100x Citrate Buffer pH 6.0 (DAKO S1699, DAKO Corp., Carpinteria, CA) and steamed for 40 minutes. Then, slides were placed in a DAKO Automated Immunostainer and immunohistochemically processed using a labeled streptavidin-biotin method for 8-OH-dG (mouse monoclonal antibody to 8-Hydroxy-2'-deoxyguanosine manufactured by Abcam Inc., MA, clone number N45.1, at a concentration of 1:100). For each participant, baseline and follow-up biopsy slides were stained in the same batch, and each staining batch included a balance of participants from each treatment group. The slides were not counterstained. After staining, the slides were coverslipped with glass coverslips with a Leica CV5000 Coverslipper (Leica Microsystems, Inc., IL). In each staining batch of slides, positive and negative control slides were included. Colon adenocarcinoma was used as a control tissue. The negative and the positive control slides were treated identically to the patients' slides except that antibody diluent was used rather than primary antibody on the negative control slide. For vitamin D receptor (VDR), slides were processed as previously described, but using mouse monoclonal antibody to VDR (SC-13133, Santa Cruz Biotechnology, Inc., CA) at a concentration of 1:7,500.

Image Analysis of Immunohistochemically Detected Biomarkers in Normal Colon Crypts

A quantitative image analysis method ("scoring") was used to evaluate detected levels of the biomarkers in colon crypts, as depicted in **Figure 5.1**. The major equipment and software for the image analysis procedures were: ScanScope CS digital scanner (Aperio Technologies, Inc., CA), computer, digital drawing board, Matlab software (MathWorks, Inc., MA), CellularEyes Image Analysis Suite (DivEyes LLC, GA), and MySQL (Sun Microsystems Inc., CA). First, slides were scanned with the Aperio ScanScope CS digital scanner, then, electronic images were reviewed in the CellularEyes program to identify colon crypts acceptable for analysis. A "scorable" crypt was defined as an intact crypt extending from the muscularis mucosa to the colon lumen (377, 381). Before analysis, images of negative and positive control slides were checked for staining adequacy. Standardized settings were used on all equipment throughout the scoring procedures. The technician reviewed slides in the CellularEyes program and selected two of three biopsies with 16 to 20 “scorable” hemicrypts per biopsy. Using the digital drawing board the borders of each selected hemicrypt were traced. The program then divided the outline into the equally spaced segments with the average widths of normal colonocytes. Finally, the program measured the background corrected optical density of the biomarker labeling across the entire hemicrypt as well as within each segment. All resulting data were automatically transferred into the MySQL database. Then, the technician moved to the next identified hemicrypt and repeated all the previously described analysis steps. A reliability control sample previously analyzed by the reader was re-analyzed during the course of the trial to determine intra-reader “scoring” reliability by intraclass correlation coefficient, which was 0.94 for 8-OH-dG.

Protocol for Measuring Plasma Vitamin D Levels

All laboratory assays for plasma 25-OH-vitamin D and 1,25-$(OH)_2$-vitamin D were performed by Dr. Bruce Hollis at the Medical University of South Carolina using a radioimmunoassay method as previously described (388). Plasma samples for baseline and follow-up visits for all subjects were assayed together, ordered randomly, and labeled to mask treatment group, follow-up visit, and quality control replicates. The average intra-assay coefficient of variation for plasma 25-OH-vitamin D was 2.3 %, and for 1,25-$(OH)_2$-vitamin D, 6.2 %.

Statistical Analysis

We assessed treatment groups for comparability of characteristics at baseline and at final follow-up by the Fisher’s exact test for categorical variables and analysis of variance (ANOVA) for continuous variables.

Several outcome variables were defined to estimate the overall labeling and within-crypt distributions of 8-OH-dG in the crypts. The mean optical density of 8-OH-dG labeling in the crypts was calculated for each patient at baseline and 6-months follow-up by summing all the densities from all analyzed crypts from the biopsy specimens and dividing by the number of crypts analyzed. Measures of the within-crypt distributions of the marker were calculated for each patient by taking the means of the biomarker densities in various zones of the crypt (*e.g.*, the upper 40%, lower 60%).

Primary analyses were based on assigned treatment at the time of randomization, regardless of adherence status (intent-to-treat analysis). Mean biomarker densities were calculated for each treatment group for the baseline and 6-months follow-up visits. Treatment effects were evaluated by assessing the differences in the densities from baseline to the 6-months follow-up visit between patients in each active treatment group and the placebo group by a repeated measures linear MIXED effects model. The model included the intercept, follow-up visit effects (baseline and follow-up), and interactions between treatment groups and the follow-up visit effect (the absolute treatment effect). Since optical density is measured in arbitrary units, to provide perspective on the magnitude of the treatment effects we also calculated relative effects, defined as: [treatment group follow-up mean/treatment group baseline mean]/[placebo follow-up mean/placebo baseline mean]. The relative effect provides an estimate of the proportional change in the treatment group relative to that in the placebo group. The interpretation of the relative effect is somewhat analogous to that of an odds ratio (*e.g.*, a relative effect of 2.0 would mean that the proportional change in the treatment group was twice as great as that in the placebo group). Since the treatment groups were balanced on risk factors at baseline, no adjustment was made for other covariates in the primary intent-to-treat analyses.

The distributions of 8-OH-dG staining density were graphically evaluated using the LOESS procedure with smoothing parameter 0.5 and local quadratic fitting. First, the number of sections within a hemicrypt was standardized to 50. Then, the average for each section across all crypts was predicted by the LOESS model separately for each patient, and then for each treatment group by follow-up visit. The results were plotted in the graphs along with smoothing lines.

A questionnaire derived oxidative balance score (OBS) was calculated as described in (403, 404). Briefly, continuous variables that reflect pro-oxidant (saturated fat and total iron intake), and antioxidant (total tocopherol, carotenoid, vitamin C, lycopene, lutein/zeaxanthin, and β-cryptoxanthin intake) exposures were divided into high and low categories based on the median value among all participants at baseline. Participants with low (below median) exposure to a particular pro-oxidant were awarded 1 point, whereas those with high (above median) exposure to the same pro-oxidant were awarded 0 points. For antioxidant exposure, a point was awarded for each high-level (above median) exposure, and 0 points for each low-levels (below median) exposure. For dichotomous variables (“yes” vs. “no”), participants received one point for each antioxidant exposure (regular use of NSAIDs and/or aspirin, supplementation with selenium, and never smoker). Then the points assigned for each individual component of OBS were summed up to calculate the overall score. Lower OBS values indicate a higher prevalence of pro-oxidant exposures, whereas higher OBS values indicate a predominance of antioxidant exposures. The range of the baseline OBS in this study was between 3 and 10, and the median was 6. We dichotomized baseline OBS based on the median value, and assigned each participant to a high OBS (above median, “antioxidant”) or low OBS (below median, “pro-oxidant”) category. Similarly, continuous variables (*e.g.,* age and VDR expression) were dichotomized (into high/low categories) based on the median value in all study participants at baseline. Then, stratified analyses were conducted to explore differential treatment effects by baseline age (<60 and ≥60 years), VDR expression (high/low), 8-OH-dG labeling (high/low), OBS (≤6 and >6), first-degree family history of colorectal cancer (yes/no), sex (male/female), regular NSAID use (yes/no), plasma 25-OH-vitamin D levels (<22 and ≥22 ng/mL), and adherence to treatment (<80% or ≥80% treatment pills taken). Differences between categories were tested by including the category-intervention interaction term in the model.

Statistical analyses were done using SAS System software (version 9.1.3; SAS Institute, Inc., NC). A cutoff level of $P \leq 0.05$ (2-sided) was used for assessing statistical significance.

Results

Characteristics of Study Participants

Treatment groups did not differ significantly on participant characteristics measured at baseline (**Table 5.1**) or at the end of the study (data not shown). The mean age of participants was 61 years, 64% were men, 71% were White, and 20% had a family history of colorectal cancer in a first degree relative. Most participants were overweight, non-smokers, college graduates, and had a single small mildly dysplastic tubular adenoma (**Table 5.1**).

Adherence to visit attendance averaged 92% and did not differ significantly among the four treatment groups. On average, at least 80% of pills were taken by 93% of participants at the first follow-up visit and by 84% at the final follow-up visit. There were no treatment or biopsy complications. Seven people (8%) were lost to follow-up due to perceived drug intolerance (n=2), unwillingness to continue participation (n=3), physician's advice (n=1), and death (n=1). Dropouts included one person from the vitamin D supplementation group, and two persons from each of other three groups.

At baseline, there were no significant differences between the four study groups in plasma 25-OH- or 1,25-$(OH)_2$-vitamin D levels. At the study end, the vitamin D and calcium plus vitamin D groups had significantly higher levels of plasma 25-OH-vitamin D ($p<0.001$), whereas the placebo and calcium groups had slight non-significant decreases in 25-OH-vitamin D levels (**Table 5.2, A**). As expected, plasma levels of 1,25-$(OH)_2$-vitamin D at the end of follow-up period did not differ significantly between study groups (377).

Graphical Assessment of Changes over Six Months in the Distribution of 8-OH-dG Labeling along Normal Colorectal Crypts

The distribution of 8-OH-dG staining optical density ("labeling") along the colorectal crypts at the baseline and 6-months follow-up visits is shown in **Figure 5.2**. In each treatment group, 8-OH-dG labeling appeared to be the highest in the lower 20%-30% of the crypts, to decrease in the middle part of the crypts, and then to increase somewhat again toward the colon lumen. The baseline distribution of 8-OH-dG along the crypts in all four treatment groups appeared to be almost identical in shape and optical density range. In the placebo group, from baseline to follow-up 8-OH-dG labeling appeared to increase slightly in the middle part of the crypts (**Figures 5.2, A**). A large post-supplemental decrease in 8-OH-dG labeling along the full lengths of the crypt was noted in the calcium and vitamin D groups (**Figure 5.2, B & C**). In the vitamin D plus calcium group, similar to as in the placebo group, 8-OH-dG labeling slightly increased from baseline to follow-up (**Figure 5.2, D**).

Effects of Calcium and/or Vitamin D Supplementation on 8-OH-dG Labeling in Normal Colorectal Crypts

At baseline, there were no differences in 8-OH-dG labeling along the full lengths of crypts among the four treatment groups. Relative to placebo, 8-OH-dG labeling along the full lengths of the crypts decreased by 22% (*P*=0.14) in the calcium group and by 25% (*P*=0.10) in the vitamin D group, and increased by 6% (*P*=0.70) in the calcium plus vitamin D group (**Table 5.2, B**). The findings for the upper 40% and the lower 60% of the crypts (the differentiation and proliferation zones, respectively; **Table 5.2, B**), and for the upper and lower 20% (areas closest to and furthest from colon lumen exposures, respectively; data not shown) did not differ substantially from those for the entire crypts.

Stratified Analyses

We investigated whether change in 25-OH-vitamin D levels, adherence to treatment, family history of colorectal cancer, sex, age, smoking, NSAID use, baseline oxidative balance score (OBS), and baseline batch-standardized VDR expression or 8-OH-dG labeling modified response to treatment; however, the sample size was too small for most of these results to be reliable. The effect of treatment on 8-OH-dG variables did not vary by age, smoking status, family history of colorectal cancer, NSAID use, or change in plasma 25-OH-vitamin D levels (data not shown). In women, 8-OH-dG labeling decreased only in the calcium group (-25%, *P*=0.43); however, in men, 8-OH-dG labeling decreased in all three active treatment groups (**Table 5.3**). In those with a high ("anti-oxidant") baseline OBS, 8-OH-dG labeling decreased in all three active treatment groups after 6-months treatment, whereas in those with low ("pro-oxidant") baseline OBS, 8-OH-dG decreased only in the vitamin D group (-19%, *P*=0.40; **Table 5.3**).

There were no substantial differences in the estimated treatment effects according to baseline levels of 8-OH-dG labeling (data not shown). Among those with high baseline colorectal crypt VDR expression, 8-OH-dG labeling decreased by 35% (*P*=0.09) in the calcium group, 54% (*P*=0.003) in the vitamin D group, and 17% (*P*=0.34) in the calcium plus vitamin D group relative to the placebo; whereas there were no decreases seen in those with low baseline VDR expression, and there was a 75% increase in 8-OH-dG labeling in the calcium plus vitamin D group relative to the placebo. The test for interaction for treatment effect by VDR status was statistically significant (*P*=0.05; **Table 5.3**).

Discussion

The results from this pilot, randomized, controlled clinical trial suggest that supplementation with calcium or vitamin D_3, but not with both agents combined, may decrease oxidative DNA damage, as indicated by decreased 8-OH-dG immunohistochemical labeling, in the normal-appearing colorectal epithelium of sporadic adenoma patients. These findings are consistent with the hypothesis that high intakes of calcium or vitamin D_3 may decrease oxidative stress and oxidative DNA damage in the colon, and, thus, reduce risk for colorectal neoplasms. Our findings also suggest that vitamin D_3 combined with calcium may have either a lesser or no treatment effect on 8-

OH-dG labeling than does either calcium or vitamin D alone. Consistent with existing animal data (331, 332), we found evidence that baseline VDR (vitamin D receptor) expression levels may modify treatment effects of calcium and vitamin D_3, such that those with higher colorectal crypt VDR expression may be more strongly responsive to treatment. Finally, the treatment effect of calcium and vitamin D_3 tended to be stronger in men and those with higher baseline anti-oxidant relative to pro-oxidant exposures.

Oxidative stress, a condition characterized by an imbalance of pro-oxidants to antioxidants which results in macromolecular damage and disruption of redox signaling and control (325), may play a role in colon carcinogenesis, inducing protein and DNA damage and lipid peroxidation, and impairing intracellular signaling. Under normal conditions, reactive oxygen species (ROS) have an important role as intracellular signaling molecules that regulate many genes (118). However, under inflammatory conditions, increased generation of ROS products leads to cell molecule damage such as oxidation of DNA (118). The most abundant product of oxidative DNA modifications by ROS is 8-hydroxy-2′-deoxyguanosine (8-OH-dG) (326). This oxidized base is a useful biomarker of oxidative stress that can be measured in urine, blood, and tissues (327, 328). Several studies demonstrated increased levels of oxidatively modified DNA in colorectal adenocarcinomas when compared to adenomas and adjacent normal epithelium (329, 330). This suggests that inhibition of oxidative stress in the normal colorectal epithelium may slow down or even prevent carcinogenesis, and prompts the development of chemopreventive agents, such as calcium and vitamin D, that target oxidative stress in the colon.

There are several lines of evidence to support our hypotheses that calcium and vitamin D may act as antioxidants and DNA damage reducing agents in the colon. Bile acids damage cell membranes, at least in part through an oxidative mechanism (204, 205), provoking an inflammatory response and causing DNA damage (206), and both calcium and vitamin D can reduce the free bile acid load in the colon lumen. Calcium directly binds bile acids, rendering them inert (6). Vitamin D activation of the ubiquitous vitamin D receptor (VDR) in the colon up-regulates CYP3A4, which in turn catabolizes the secondary bile acid, lithocholic acid (11, 227). Furthermore, high blood 25-(OH)-vitamin D levels provide a pool of vitamin D that is available for various tissues, such as the colorectal epithelium. In colonocytes, vitamin D increases expression of enzymes involved in antioxidant response, inhibits iron-dependent lipid peroxidation in liposomes, lowers glutathione reductase levels, induces glutathione peroxidase and manganese dependent superoxide dismutase activity, and elevates glutathione levels ((228, 229), also reviewed in (230)), thereby decreasing oxidative stress in the colorectal epithelium. The results of this study, combined with the biological evidence, support calcium and vitamin D_3 as oxidative DNA damage reducing agents.

Contrary to our original hypothesis and to what has been described in some epidemiologic and clinical studies (22, 29, 30, 237, 405), we did not observe a treatment effect in the calcium plus vitamin D group. We also previously reported that vitamin D combined with calcium may have a lesser treatment effect on colorectal epithelial apoptosis and differentiation than does calcium or vitamin D separately (377, 406). There are several possible explanations for this finding. Considering the study’s small sample size, the lack of treatment effect in the calcium and vitamin D group may have been due to chance. It is also possible that the two agents may have attenuated the effects of one another. 1,25-$(OH)_2$-vitamin D_3 regulates calcium homeostasis (407). As calcium concentration decreases, the production of 1,25-$(OH)_2$-vitamin D_3 increases, which in turn increases intestinal calcium absorption (407). Elevated calcium in the diet may suppress 1,25-$(OH)_2$-vitamin D_3 synthesis at the cellular level, which in turn may also attenuate activation of vitamin D-responsive detoxifying enzymes. One animal study (246) found that calcium and vitamin D were more potent inhibitors of colon tumorigenesis when given separately, but several other animal studies reported synergistic effects with calcium and vitamin D combined (243, 244). A large adenoma recurrence trial also supported an enhanced chemopreventive effect of vitamin D with calcium (237). Taken altogether, the combined effect of calcium and vitamin D on oxidative DNA damage in colorectal epithelium is unclear and will require clarification via larger studies.

In contrast to as in men, there was no evidence for a treatment effect of vitamin D alone and in combination with calcium on colorectal crypt 8-OH-dG labeling levels in women. There are several possible explanations for this finding, including a very low statistical power to detect treatment effects due to the small sample size. Another possible explanation may be that women in our study may have had decreased estrogen levels as the majority of them were postmenopausal and not taking estrogens. The Women's Health Initiative Hormone Replacement Therapy Trial (146) showed that endogenous estrogen plus progestin therapy reduced risk for colorectal cancer; but not estrogen alone therapy (408). However, one human study found that an estrogen intervention activated the VDR pathway, and downregulated inflammatory and immune signaling pathways in the rectal mucosa of postmenopausal women (251). So, the findings of our study are consistent with the hypothesis that low estrogen levels may interfere with VDR signaling in the colorectal mucosa, resulting in no changes in 8-OH-dG levels after supplementation with vitamin D; however, further studies are needed to clarify these issues.

Those with a high baseline OBS (higher balance of anti- to pro-oxidant exposures) had greater estimated calcium and calcium plus vitamin D treatment effects on 8-OH-dG labeling than those with a low OBS. A low OBS reflects low total intakes of antioxidants such as vitamin C and carotene, combined with high pro-oxidant exposures such as high fat or iron intakes. In the colon lumen, free calcium directly binds bile acids (6), thereby reducing pro-carcinogenic effects of bile acids on the colorectal epithelium. Persons with high fat intake have higher colonic lumen levels of deoxycholic and lithocholic bile acids (206), and may require more calcium to neutralize the DNA damaging bile acids than do persons on a low-fat diet. Antioxidant enzymes in humans function in combination with low weight antioxidant compounds such as vitamin C, α-tocopherol, and β-carotene (409). In the colorectal epithelium, vitamin D activates the expression of antioxidant enzymes (230), which may not function properly in the antioxidant-depleted environment. Therefore, it is possible that calcium and vitamin D effects on the oxidative DNA damage marker, 8-OH-dG, are modified by the presence or absence of various pro- or antioxidant exposures.

Since complete loss of the VDR significantly increased 8-OH-dG labeling in the mouse colon (331, 332), we hypothesized that different VDR expression levels in the normal-appearing colorectal mucosa modify vitamin D treatment effects. Consistent with this hypothesis, we observed substantial decreases in 8-OH-dG labeling in study participants with high, but not low, baseline VDR expression.

This study has several limitations. First, treatment effects of vitamin D and calcium on the oxidative DNA damage marker 8-OH-dG in parts of the colon other than the rectum are unclear, as we did not collect tissue biopsies from different parts of the colon and there are no published studies of 8-OH-dG labeling throughout the colon. Another potential limitation of this study is that it is not known whether oxidative stress markers are associated with risk for colon cancer in humans. However, substantial published literature supports the plausibility of an important role for increased oxidative DNA damage in colon carcinogenesis, especially for the transition from colorectal adenoma to carcinoma (329, 330). Persistent oxidative stress leads to protein and DNA damage and lipid peroxidation which can cause genetic and epigenetic alterations, and may facilitate the development of neoplasia from the normal colorectal mucosa (118). Therefore, 8-OH-dG in the normal colorectal mucosa may serve as a biomarker of risk for colorectal neoplasms. Finally, the most obvious limitation of the study is the small sample size, which may have increased the probability of chance findings in detecting or not detecting a treatment effect.

The strengths of this study include the randomized, double-blind, placebo-controlled trial design; high protocol adherence by study participants; examination of both the independent and combined effects of calcium and vitamin D_3 on an oxidative stress marker; automated standardized biopsy handling and immunostaining procedures; and the use of cutting edge technologies to conduct the quantitative image analyses. Another strength of this study is that we used immunohistochemical detection of 8-OH-dG in the colorectal epithelium as it was important to detect 8-OH-dG in colonocytes, but not in infiltrating lymphocytes or other intermingled cells. Such detection was made possible by the development of a specific monoclonal antibody against 8-OH-dG (328), and our novel image analysis methods. HPLC (high-performance liquid chromatography), an alternative method of measuring 8-OH-dG in colon tissue, may overestimate oxidative DNA damage in the colonocytes, especially in the presence of inflammation. Finally, this study is the first human study to test the effect of calcium and/or vitamin D_3 on an oxidative DNA damage marker in the normal-appearing colorectal mucosa.

Overall, these preliminary results from this pilot clinical trial suggest that calcium and vitamin D, given separately, may decrease oxidative DNA damage in the normal-appearing colorectal epithelium; the treatment effects of calcium and vitamin D on oxidative DNA damage marker 8-OH-dG may be strongest in those with higher vitamin D receptor expression in the colon; 8-OH-dG may be a modifiable biomarker of oxidative stress that can be used in colon cancer-related chemoprevention trials to assess treatment efficacy; and support further investigations of calcium and vitamin D as chemopreventive agents against colorectal neoplasms.

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Tables and Figures

Table 5.1. Selected baseline characteristics of the study participants[*] (n=92).

	Treatment Group				
Characteristics	**Placebo** (n=23)	**Calcium** (n=23)	**Vitamin D** (n=23)	**Calcium + Vit. D** (n=23)	***P***[**]
Demographics, medical history, habits, anthropometrics					
Age, years	58.5 (8.2)	61.9 (8.2)	60.2 (8.1)	62.1 (7.5)	0.39
Men (%)	70	70	70	70	1.00
White (%)	74	83	65	61	0.39
College graduate (%)	65	61	57	44	0.53
History of colorectal cancer in 1° relative (%)	17	30	17	13	0.60
Take NSAID[¥] regularly[§] (%)	22	13	9	22	0.60
If woman (n = 28), taking estrogens (%)	4	9	4	4	1.00
Current smoker (%)	9	4	0	0	0.61
Take multivitamin (%)	30	30	26	39	0.86
Body mass index (BMI), kg/m^2	30.6 (7.2)	29.4 (5.5)	28.9 (5.6)	31.6 (6.0)	0.44
Mean dietary intakes					
Total energy intake, kcal/d	1,596 (528)	1,788 (691)	1,848 (821)	1,845 (752)	0.59
Total[§§] calcium, mg/d	618 (308)	746 (335)	843 (526)	824 (714)	0.41
Total[§§] vitamin D, IU/d	277 (230)	336 (202)	360 (317)	415 (316)	0.40
Total fat, gm/d	67 (32)	72 (35)	70 (32)	74 (28)	0.59
Dietary fiber, gm/d	15 (7)	17 (9)	18 (9)	17 (11)	0.97
Alcohol, gm/d	9 (14)	11 (15)	14 (18)	10 (20)	0.84
Oxidative balance score (OBS)[¤]	6 (2)	7 (2)	7 (2)	7 (2)	0.46

(Table continues)

Table 5.1 (continued)

Characteristics	**Treatment Group** **Placebo** (n=23)	 **Calcium** (n=23)	 **Vitamin D** (n=23)	 **Calcium + Vit. D** (n=23)	P^{**}
Adenoma characteristic					
Multiple adenomas¤¤ (%)	17	22	39	26	0.45
Large adenoma £ (%)	19	32	17	9	0.32
Villous/tubulovillous adenoma££ (%)	4	9	9	4	1.00
Mild dysplasia (%)	100	96	100	100	1.00

* Data are given as means (SD) unless otherwise specified.

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

¥ Nonsteroidal anti-inflammatory drug.

§ At least once a week.

§§ Diet plus supplements.

¤ See the "Statistical Analysis" section for details.

¤¤ At least two adenomas.

£ At least one large (≥ 1 cm) adenoma.

££ At least one villous or tubulovillous adenoma.

Table 5.2. Plasma 25-OH-vitamin D, and optical density of immunohistochemically detected 8-OH-dG in colorectal crypts at baseline and 6-months follow-up.

	Baseline				**6-Months Follow-up**				**Absolute Rx Effect***				**Relative Effect[§]**
	n	**Mean**	**SE**	**P****	**n**	**Mean**	**SE**	**P****	**n**	**Mean**	**SE**	**P****	
A. Plasma vitamin D measurements													
25-OH-vitamin D, ng/mL													
Placebo	23	20.7	1.7		21	18.2	1.8		21	0			1.00
Calcium	23	25.7	1.7	0.05	21	23.4	1.7	0.03	21	0.2	1.8	0.92	1.03
Vitamin D	23	21.0	1.7	0.81	22	29.5	1.7	<0.0001	22	10.9	1.8	<0.0001	1.59
Calcium + Vit. D	23	20.9	1.7	0.84	21	28.9	1.7	<0.0001	21	10.5	1.8	<0.0001	1.57
B. 8-OH-dG[$] labeling optical density in colorectal crypts													
Entire crypts													
Placebo	23	2,360.8	193.2		21	2,509.0	202.1		21	0			1.00
Calcium	23	2,349.4	193.2	0.97	21	1,946.2	202.1	0.05	21	-551.5	374.4	0.14	0.78
Vitamin D	23	2,318.4	193.2	0.88	22	1,847.3	197.5	0.02	22	-619.3	372.0	0.10	0.75
Calcium + Vit. D	23	2,347.8	193.2	0.96	21	2,642.6	202.1	0.64	21	146.5	264.8	0.70	1.06

(Table continues)

Table 5.2 (continued).

	Baseline				6-Months Follow-up				Absolute Rx Effect*				Relative
	n	**Mean**	**SE**	***P*****	**n**	**Mean**	**SE**	***P*****	**n**	**Mean**	**SE**	***P*****	**Effect §**
Upper 40% of crypts													
Placebo	22	677.7	64.1		21	655.9	67.0		21	0			1.00
Calcium	23	704.7	64.1	0.77	21	525.1	67.0	0.17	21	-157.8	125.8	0.21	0.77
Vitamin D	23	684.4	64.1	0.94	22	505.9	65.5	0.11	22	-156.8	125.0	0.21	0.76
Calcium + Vit. D	23	655.0	64.1	0.80	21	741.5	67.0	0.37	21	108.3	125.8	0.39	1.17
Lower 60% of crypts													
Placebo	22	1,418.7	112.0		21	1,459.7	117.2		21	0			1.00
Calcium	23	1,431.8	112.0	0.93	21	1,201.5	117.2	0.12	21	-271.3	213.5	0.21	0.82
Vitamin D	23	1,450.7	112.0	0.84	22	1,145.1	114.5	0.06	22	-346.6	212.1	0.11	0.77
Calcium + Vit. D	23	1,390.0	112.0	0.86	21	1,556.8	117.2	0.56	21	125.8	213.5	0.56	1.09

* Absolute treatment effect = [treatment group follow-up - treatment group baseline] – [placebo group follow-up - placebo group baseline].

** P-value for difference between each active treatment group and placebo group from repeated measures Mixed model.

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

$ Biomarker detected immunohistochemically and then its labeling optical density quantified by image analysis (see text for details).

Table 5.3. 8-OH-dG labeling in colorectal crypts stratified by sex, baseline oxidative balance score (OBS), and baseline colorectal crypt VDR expression.

		Absolute Rx Effect*			Relative	
	n	Mean	SE	*P***	Effect[§]	*P*[§§]
Women						
Placebo	7	0			1.00	
Calcium	7	-577.4	714.7	0.43	0.75	
Vitamin D	6	253.3	729.4	0.73	1.11	
Calcium + Vit. D	7	508.3	714.7	0.48	1.23	
Men						
Placebo	14	0			1.00	
Calcium	14	-526.1	422.9	0.22	0.80	
Vitamin D	16	-959.0	414.3	0.02	0.62	
Calcium + Vit. D	14	-26.2	422.9	0.95	0.99	0.35
High oxidative balance score (OBS)[&]						
Placebo	8	0			1.00	
Calcium	13	-898.0	594.8	0.14	0.67	
Vitamin D	12	-879.0	599.7	0.15	0.67	
Calcium + Vit. D	11	-120.5	615.1	0.85	0.94	
Low oxidative balance score (OBS)[&]						
Placebo	13	0			1.00	
Calcium	8	25.1	509.7	0.96	1.02	
Vitamin D	9	-421.6	498.6	0.40	0.81	
Calcium + Vit. D	10	383.2	478.1	0.43	1.19	0.71

(Table continues)

Table 5.3 (continued).

		Absolute Rx Effect*			Relative	$P^{\S\S}$
	n	**Mean**	**SE**	***P*********	**Effect**§	
High baseline colorectal crypt VDR expression[&]						
Placebo	10	0			1.00	
Calcium	10	-827.0	477.0	0.09	0.68	
Vitamin D	8	-1,626.1	506.7	0.003	0.46	
Calcium + Vit. D	11	-443.6	462.5	0.34	0.83	
Low baseline colorectal crypt VDR expression[&]						
Placebo	8	0			1.00	
Calcium	9	264.6	532.4	0.62	1.11	
Vitamin D	13	63.5	498.8	0.90	1.00	
Calcium + Vit. D	8	1,420.4	551.5	0.02	1.75	0.05

* Absolute treatment effect = [treatment group follow-up - treatment group baseline] – [placebo group follow-up - placebo group baseline].

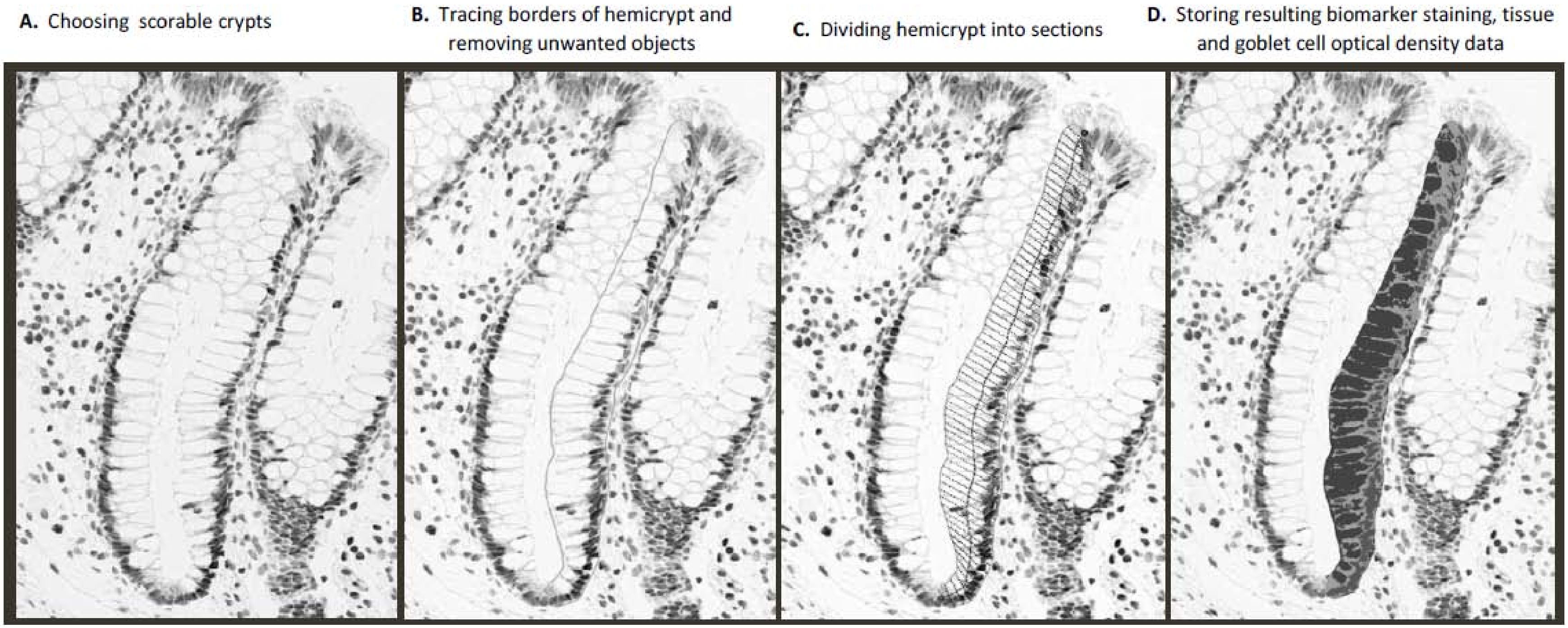
** P-value for difference between each active treatment group and placebo group from repeated measures Mixed model.

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

$$ P-value for the category-intervention interaction term.

& OBS and baseline colorectal crypt VDR expression were dichotomized into high/low categories based on the median value in all study participants at baseline. OBS was calculated as described in the 'Statistical Analysis' section. VDR detected immunohistochemically and then its labeling optical density was quantified by image analysis (see text for details).

Figure 5.1. Quantitative image analysis using Aperio Scanscope and CellularEyes software to measure 8-OH-dG labeling in normal-appearing colorectal crypts.



A. Choosing scorable crypts

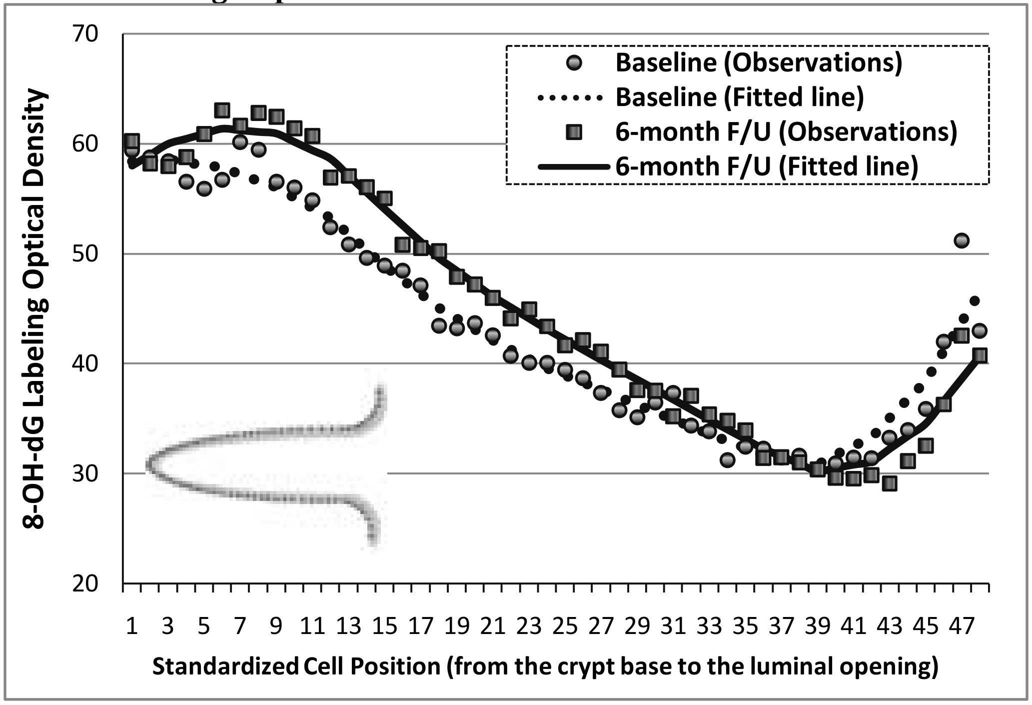
B. Tracing borders of hemicrypt and removing unwanted objects

C. Dividing hemicrypt into sections

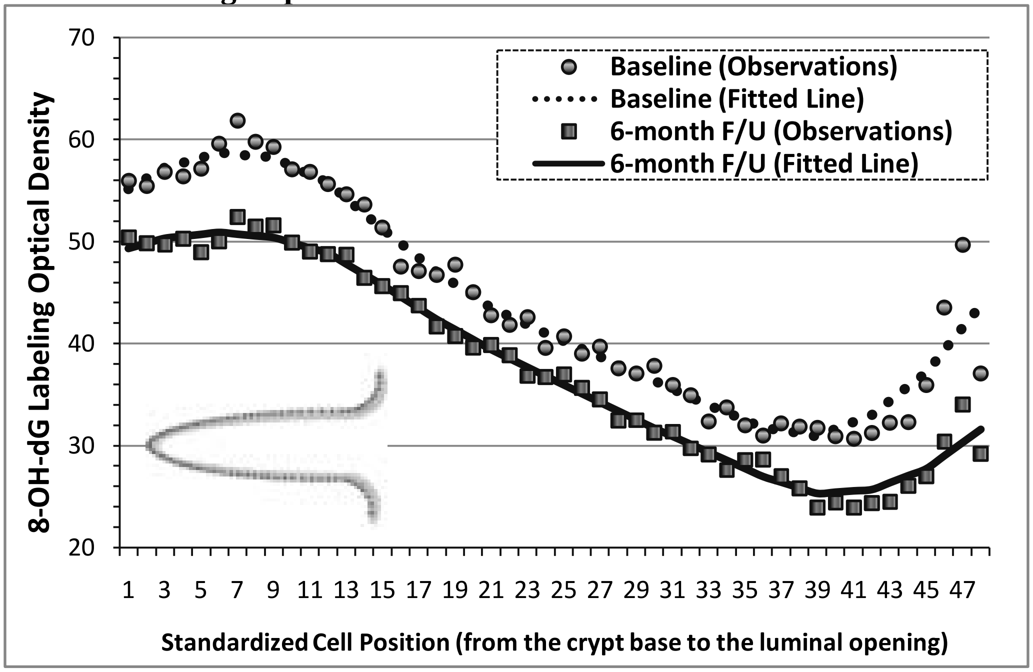
D. Storing resulting biomarker staining, tissue and goblet cell optical density data

Figure 5.2. Distribution of 8-OH-dG staining optical densities along normal colorectal crypts by treatment group at baseline and follow-up. **A**, Placebo group. **B**, Calcium Group. **C**, Vitamin D group. **D**, Vitamin D + Calcium group.

A. Placebo group



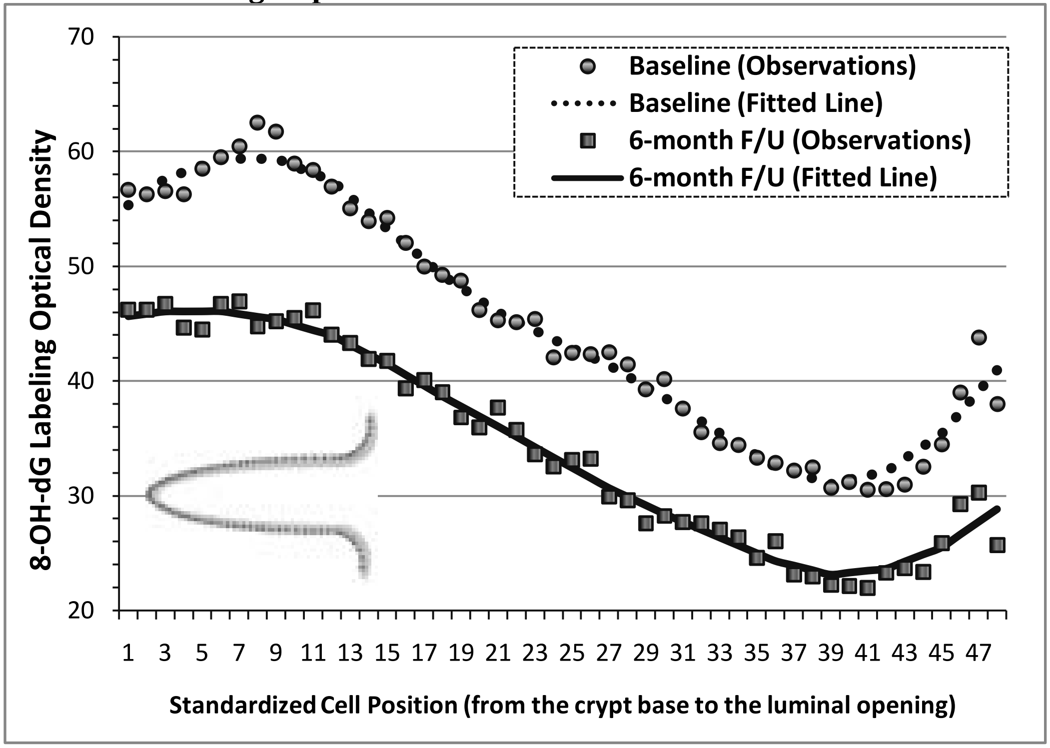
B. Calcium group



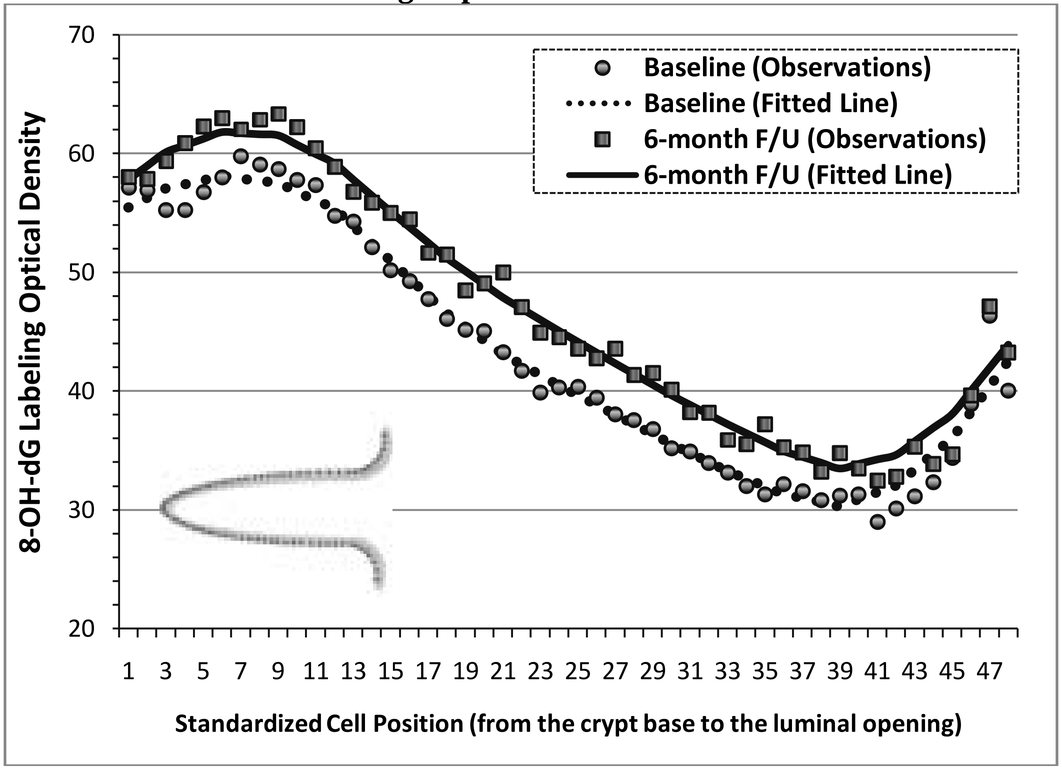
(Figure continues)

Figure 5.2 (continued).

C. Vitamin D group



D. Vitamin D + Calcium group



CONCLUSIONS AND PUBLIC HEALTH IMPLICATIONS

One investigation from a pooled colonoscopy based case-control study and three investigations from a pilot, randomized clinical trial were conducted to examine the potential of vitamin D_3 and calcium in reducing risk for colorectal neoplasms, and to develop modifiable pre-neoplastic biomarkers of risk for colorectal neoplasia.

In the first dissertation project, a pooled colonoscopy based case-control study, we found that higher circulating 25-(OH)-vitamin D_3 concentrations were associated with lower risk for incident, sporadic colorectal adenomas. Our data also suggested that higher circulating 25-(OH)-vitamin D_3 concentrations combined with anti-inflammatory agents (aspirin or other NSAIDs) may more markedly reduce risk for colorectal neoplasms (Study #1).

In the other three dissertation projects, based on data from a pilot randomized clinical trial, we found that supplementation with vitamin D_3 and calcium, individually or together, may enhance apoptosis in the normal human colorectal epithelium; that they do so via upregulating Bax (pro-apoptotic protein) expression alone or relative to Bcl-2 (anti-apoptotic protein) expression; and that the strongest treatment effects on apoptosis markers may be in the upper sections of the colorectal crypts, and vitamin D_3 related (Study #2). The data from these studies also suggested that calcium and vitamin D_3 supplementation increase colorectal epithelial cell differentiation and may have relatively little, if any, effect on overall proliferation rates in the colorectal mucosa, but do not rule out a potential normalization of the proliferative zone in the colorectal crypts (Study #3). Furthermore, we found that calcium and vitamin D_3, given separately, may decrease oxidative DNA damage in the normal-appearing colorectal epithelium, and that the treatment effects of calcium and vitamin D_3 on the oxidative DNA damage marker 8-OH-dG may be strongest in those with higher vitamin D receptor (VDR) expression in the colon (Study #4). In addition, our clinical trial data supported the use of Bax expression alone or in combination with Bcl-2 expression, $p21^{waf1/cip1}$ (cell differentiation marker) expression, and 8-OH-dG (oxidative DNA damage marker) labeling in the normal-appearing colorectal mucosa as modifiable biomarkers of risk for colorectal neoplasms that can be used in colon cancer-related chemoprevention trials to assess treatment efficacy (Study #2–4).

Overall, the results of this dissertation support hypotheses that higher circulating 25-(OH)-vitamin D_3 concentrations reduce risk for incident, sporadic colorectal adenomas; and that supplementation with vitamin D_3, alone or in combination with calcium, favorably modulates expression of proteins involved in colorectal carcinogenesis. It is estimated that at least a billion people worldwide have less than sufficient vitamin D exposure. From the public health perspective, this observation may have far-reaching implications because insufficient levels of 25-(OH)-vitamin D have been linked to a variety of health problems including well-known vitamin D-related illnesses such as rickets, osteomalacia, and osteoporosis, as well as number of chronic conditions such as autoimmune, infectious, and cardiovascular diseases, schizophrenia, depression, and cancer (410, 411). The current recommended intakes of vitamin D (400 IU/day for adults and 200 IU/day for children) are inadequate for maintaining sufficient blood 25-(OH)-vitamin D levels. Therefore, public health agencies should develop strategies to prevent, identify and treat vitamin D deficiency and insufficiency in the general population.

In summary, this dissertation supports further explorations of the mechanisms by which vitamin D and calcium, independently or synergistically, prevent colorectal neoplasms, further investigations of calcium and vitamin D as chemopreventive agents against colorectal neoplasms, and further development of modifiable pre-neoplastic biomarkers of risk for colorectal neoplasms.

FUTURE DIRECTIONS

In the first study of this dissertation we investigated several lifestyle and dietary risk factors as potential modifiers of the association between circulating 25-(OH)-vitamin D_3 and colorectal neoplasms. In addition to environmental factors, genetic risk factors such as variants in genes involved with vitamin D and calcium metabolism, physiology, and mechanisms may alter risk of colorectal adenomas. There are 27 genes in the vitamin D pathway that are involved in the transcriptional activation or repression of vitamin D-sensitive genes, four genes coding major enzymes that activate and metabolize vitamin D products (*CYP27B1*, *CYP24A1*, *CYP2R1* and *CYP3A4*); and one gene in the calcium signaling pathway coding for the calcium-sensing receptor (*CaSR*). In the few epidemiologic studies that investigated a few polymorphisms of the *VDR* gene (*BsmI, ApaI, Tru9I, CDX2, TaqI, Poly(A),* and *FokI*), no consistent association between the *VDR* genetic variants and colorectal neoplasms was found (21, 22, 208, 237, 348, 412-414). Also, no consistent association was found between *CaSR* (*A986S*, *R990G,* and *Q1011E*) and *CYP3A4* (seven non-coding in introns and within 2 kb of the mRNA) gene variants and colorectal adenomas or cancer (415, 416). Furthermore, there were no published epidemiologic studies of associations between the other genes in the vitamin D pathway (e.g., *RXRA*, *CYP2R1*, *GC*) and risk of colorectal adenomas. Moreover, in those few studies that investigated genetic variants, the candidate SNPs were identified based on either their location in the promoter region or their potential ability to change the functionality of the gene or resulting protein. None of the studies used a selection hypothesis based on evolutionary adaptations to the low UVB exposure in populations that reside in high latitudes to identify SNP candidates. The use of a new selection (or population differentiation) hypothesis to identify candidate SNPs may determine previously unexplored variants in vitamin D pathway genes that may be associated with risk for colorectal adenomas and/or different levels of circulating vitamin D. Therefore, I propose to investigate whether the vitamin D – colorectal adenoma risk association can be explained or modified by variations in the genes that code for components of the vitamin D and calcium pathway and were shown to have different frequencies in native Black African and Northern European populations.

In the first study of this dissertation we also investigated whether the relation of circulating 25-(OH)-vitamin D_3 to colorectal neoplasms differed in individuals with different dietary intakes of retinol, folate and soy products. The intakes of these hypothesized effect modifiers were estimated based on self-administered food frequency questionnaires; however, it is possible that blood levels of retinol (in serum), isoflavones (in plasma), and folate (in serum or whole-blood) may be better indicators of relevant exposures (417, 418). Furthermore, other circulating molecules, such as vitamin D-binding protein or TNFα may also modify the 25-(OH)-vitamin D_3–colorectal adenoma association. Further investigations will involve additional blood assays for the aforementioned nutrients and proteins, and examining the association between circulating 25-(OH)-vitamin D_3 levels and colorectal neoplasms in subgroups of participants (*e.g.*, among those with high serum retinol, or low plasma TNFα levels).

In the last dissertation study we tested the effect of calcium and vitamin D_3 supplementation on the marker of oxidative DNA damage (8-OH-dG) in the normal-appearing colorectal mucosa. Oxidative stress processes affect various macromolecules in the cell (*e.g.*, proteins and lipids) in addition to DNA, and are closely related to inflammation, and both chemopreventive agents, vitamin D and calcium, were found to be involved in protecting against oxidative damage and modulating inflammation, including regulating growth factor and cytokine synthesis and signaling (10, 14, 227, 230, 419-425). In animal models, vitamin D inhibited iron-dependent lipid peroxidation in liposomes, lowered glutathione reductase levels, and induced glutathione peroxidase and manganese dependent superoxide dismutase activity (230); down-regulated iNOS, which is synthesized in response to inflammatory stimuli (426); elevated glutathione levels by nearly 50%, which reduced the extent of lipid peroxidation (229); and inhibited the NF-κB pathway, which resulted in decreased levels of proinflammatory cytokines (427). Moreover, vitamin D can directly or indirectly regulate several genes involved in inflammatory responses: *IL-2, IL-12, TNFα, IL-4, IL-5,* and *IFNγ* (14, 15, 428). All these data indicate that vitamin D and calcium may modulate various functional molecules involved in the inflammatory and oxidative stress pathways in the colon. To clarify the role of vitamin D and calcium in these pathways, I propose to expand the current panel of tissue biomarkers by including other tissue markers of inflammation (*e.g.,* TNFα and IL-6) and oxidative stress (*e.g.,* HNE).

In the three clinical trial-based studies, we tested the effects of calcium and vitamin D_3 supplementation on the biomarkers in biopsies of normal-appearing rectal mucosa, which were obtained during a minimally invasive sigmoidoscopy procedure. However, supplementation with calcium and vitamin D_3 may also modulate the corresponding biomarkers in blood and/or urine. Since colorectal tissue cannot always be used for testing treatment effects of chemopreventive agents because its procurement is relatively invasive, expensive, and requires professional medical personnel, I propose to investigate systemic effects of calcium and vitamin D supplementation on circulating markers of inflammation (*e.g.*, IL-6, TNFα, and TGFβ_1), oxidative stress (*e.g.*, protein carbonyl, 8-OH-dG and F_2-isoprostanes), and apoptosis (*e.g.*, cytokeratin 18) in plasma or serum; and how tissue levels of these markers correlate with systemic levels of the corresponding markers within the same pathways. The local inflammatory processes in the colon contribute to systemic inflammation. TNFα, IL-1, and IL-6 are released by the gastrointestinal tract into the bloodstream (429). In turn, high circulating levels of TNFα may cause over-expression of COX-2 in tissues through an NFκB pathway. Transforming growth factor beta 1 (TGFβ_1), a pleiotropic cytokine with important functions for maintaining immune homeostasis that is expressed in the colon and found in the circulation has been implicated in the pathogenesis of chronic inflammatory diseases and cancer (430) and is directly or indirectly regulated by vitamin D (7, 431, 432). Also, tissue levels of oxidative stress markers may correlate with systemic levels of oxidative stress markers, as has been found in animal studies (433). Furthermore, there have been no reported human studies of correlations between blood and colorectal tissue levels for these biomarkers, and, as previously described, there is strong evidence to support that there are anti-inflammatory and anti-oxidative actions of calcium and vitamin D_3.

Based on the preliminary results of my dissertation, I propose to test the local and systemic effects of calcium and vitamin D_3 supplementation in a larger randomized clinical trial that will examine several doses of vitamin D (1,000, 2,000, and 4,000 IU/day), alone or in combination with several doses of calcium (1.0 and 2.0 g/day). This trial will allow us to estimate dose-response trends, to clarify the potential vitamin D–calcium interaction, and to validate the modifiable biomarkers of risk that were identified in the pilot clinical trial described in this dissertation project. Also, in a large clinical trial, we will be able to more definitively assess potential lifestyle, dietary and genetic modifiers of calcium and vitamin D treatment effects.

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APPENDIX. Laboratory Procedures

Protocol for Biopsy Specimen Processing and Immunohistochemical Staining

One millimeter thick biopsy specimens were taken from the mucosa of a valve or fold in the rectum 10 cm above the level of the external anal aperture. The biopsies were then immediately placed in normal saline and transferred to an on-site dissecting microscope where they were immediately examined and reoriented, if necessary, to ensure that they were not twisted or curled on the bibulous paper. The biopsies were then immediately placed in 10% normal buffered formalin, left undisturbed for at least six hours, and transferred to 70% ethanol 24 hours after being placed in formalin. The biopsy specimens were embedded in paraffin blocks within two weeks of the biopsy procedure, cut and stained within another four weeks, and analyzed within another four weeks. Five slides with four section levels each taken 40 microns apart were prepared for each antigen, yielding a total of 20 levels for each antigen. Heat-mediated antigen retrieval (AR) was used to break the protein cross-links formed by formalin to uncover the epitope. To accomplish this, slides were placed in a preheated Pretreatment (PT) Module (Lab Vision Corp., CA) with 100x Citrate Buffer pH 6.0 (Target Retrieval Buffer, DAKO S1699, DAKO Corp., Carpinteria, CA) and steamed for 40 minutes. After antigen retrieval, slides were placed in a DAKO Automated stainer (DAKO Corp., Carpinteria, CA) and rinsed with warm PT Module Buffer. Immunohistochemical (IHC) staining was done using a LSAB (Labeled Streptavidin Biotin) method on the DAKO Automated stainer. The Autostainer was programmed for each IHC run and the following reagents were used: antibody (bcl-2 antibody manufactured by Santa Cruz Biotechnology, Inc., CA, catalog no. sc-509, dilution 1:100; or bax antibody manufactured by DAKO Corp., Carpinteria, CA, catalog no. A3533, dilution 1:200; or 8-Hydroxy-2'-deoxyguanosine antibody [N45.1] manufactured by Abcam Inc., MA, catalog no. ab48508, dilution 1:100; or hTERT (telomerase) antibody manufactured by Epitomics, Inc., CA, catalog no. 1531-1; or MIB-1/Ki-67 antibody manufactured by DAKO Corp., catalog no. M7240, dilution 1:350; or p21 antibody manufactured by DAKO Corp., catalog no. M7202, dilution 1:40) diluted with Antibody Diluent (DAKO SS0809; DAKO Corp., Carpinteria, CA), LSAB2 Detection System (DAKO K0675, which consists of H_2O_2 , Link Antibody, and Streptavidin Peroxidase; DAKO Corp., Carpinteria, CA), DAB (diaminobenzidine) 2-component (DAKO K3466; DAKO Corp., Carpinteria, CA), and TBS buffer (DAKO S1968, DAKO Corp., Carpinteria, CA). The slides were not counterstained. After staining, the slides were automatically coverslipped with glass coverslips with a Leica CV5000 Coverslipper (Leica Microsystems, Inc., IL) and placed in opaque slide folders. In each staining batch of slides, positive and negative control slides were included. Tonsil was used as a control tissue for apoptosis (Bax and Bcl-2), differentiation (p21), and proliferation (MIB-1 and hTERT) biomarkers, and normal colon was used as a control tissue for 8-OH-dG. The control tissues were fixed, embedded, and cut in the same manner as the patient’s tissue. The negative and the positive control slides were treated identically to the patient’s slides except that antibody diluent was used rather than primary antibody on the negative control slide.

Protocol for Quantifying Staining Density of Immunohistochemically Detected Biomarkers in Normal Colon Crypts ("Scoring")

Colon crypts longitudinally sectioned from base to colon lumen were analyzed. A "scorable" crypt was defined as an intact crypt extending from the muscularis mucosa to the colon lumen. Crypts had to extend to the colon lumen but did not have to be fully open if the crypt column could still be clearly followed to the lumen. Crypts with artifactual cell loss (>2 cells) from handling or cutting were not used. Crypts did not have to be perfectly U-shaped or symmetrical for scoring. Before scoring, negative and positive control slides were checked for the adequacy of the staining procedures, and the patient's slides were scanned to assess the adequacy of the biopsy specimen (whether scorable crypts were present). If the staining procedure appeared successful and the specimen was adequate, scoring began.

a) Quantitative image analysis ("scoring") using ImagePro Plus software and our copyright-pending software to measure biomarkers in normal colon crypts.

The major equipment and software for the image analysis scoring procedures were: personal computer, light microscope with appropriate filters and attached digital light microscope camera, digital drawing board, ImagePro Plus image analysis software (Media Cybernetics, Inc., MD), our in-house developed plug-in software for colorectal crypt analysis, and Microsoft Access (Microsoft Corporation, WA). The following preparations were performed before starting the scoring program: 1) ensuring standardized settings on the microscope, digital camera, drawing board, and imaging software; and 2) cleaning and visually scanning the slides. After that, study name, participant ID number, scorer ID, visit number, antigen, and colon site, followed by the number of the first biopsy to be scored, whether it had scorable crypts, whether it was labeled, and if so, the section level number on the biopsy on which scoring was begun was recorded. Slides were oriented in a standardized fashion and the section levels on the slides were viewed in sequence using light microscopy. All scoring was conducted at 200x magnification. The unit of analysis was the "hemicrypt", defined as one complete side of a scorable crypt. Scoring began when the first complete hemicrypt was found. Then, the reader created a slide background correction image for the slide to be scored, and, focusing on the first hemicrypt, captured the image as a 16-bit per pixel grayscale image from the camera program to the image analysis/hemicrypt analysis program (Figure A.1, a). Next, the hemicrypt was analyzed by precisely tracing the borders of the hemicrypt using a digital drawing board, taking care not to include cells outside the crypt epithelium basement membrane or cells in the crypt lumen, or to exclude portions of the crypt epithelial cells (Figure A.1, b). The program then created a crypt length line precisely midway along the hemicrypt axis, and then drew equally spaced perpendicular lines to the crypt length line at intervals to yield segments with the average widths of normal crypt epithelial cells (Figure A.1, c).

Figure A. 1. Quantitative image analysis using ImagePro Plus software and our copyright-pending software to measure biomarkers in normal colon crypts.

a) Choosing crypt

b) Removing unwanted objects and tracing borders of hemicrypt

c) Dividing hemicrypt into sections

d) Storing resulting biomarker staining density data

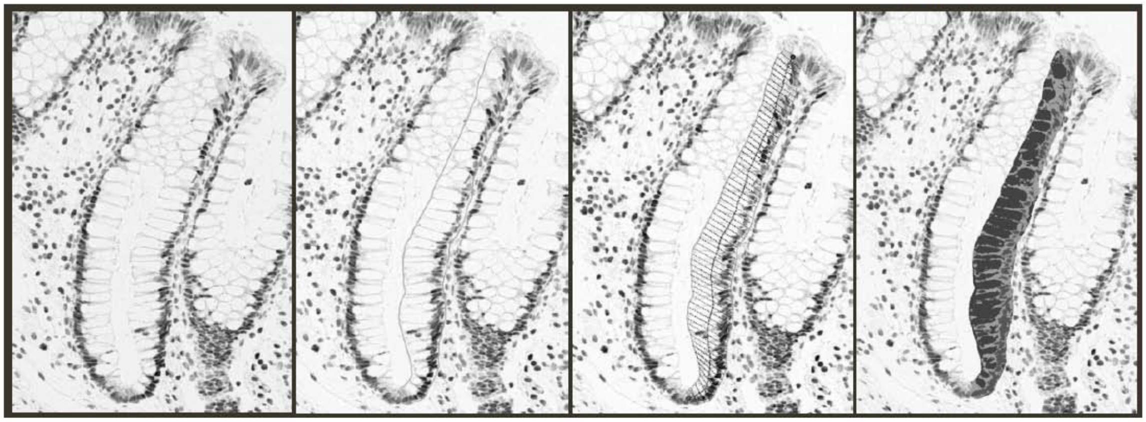
e) Plotting average biomarker signal intensities from crypt base to lumen for each subject/group of subjects

Finally, the program, adjusted for any background levels on the slide, and measured the optical density of the labeling across the entire hemicrypt as well as within each segment (Figure A.1, d). Via pre-set thresholds, clear areas (mostly corresponding to areas over the mucin in goblet cells) were excluded from the density measurements. All resulting data were entered into the database automatically. Then, the reader moved to the next hemicrypt on the same or next image, section level, biopsy, and/or slide and repeated all the previously described scoring steps. The goal was to score a minimum of 16 hemicrypts on each of two biopsies, for a total of 32 hemicrypts. A reliability control sample previously scored by the reader was re-scored during the course of the trial to determine intra-reader reliability.

b) Quantitative image analysis ("scoring") using Aperio Scanscope and CellularEyes software.

A quantitative image analysis method ("scoring") was used to evaluate detected levels of the biomarkers in colon crypts, as depicted in **Figure A.2**. The major equipment and software for the image analysis procedures were: Scanscope CS digital scanner (Aperio Technologies, Inc., CA), computer, digital drawing board, Matlab software (MathWorks, Inc., MA), CellularEyes Image Analysis Suite (DivEyes LLC, GA), and MySQL (Sun Microsystems Inc., CA). First, slides were scanned with the Aperio Scanscope CS digital scanner, then, electronic images were reviewed in the CellularEyes program to identify colon crypts acceptable for analysis. A "scorable" crypt was defined as an intact crypt extending from the muscularis mucosa to the colon lumen. Before analysis, images of negative and positive control slides were checked for staining adequacy. Standardized settings were used on all equipment throughout the scoring procedures. The technician reviewed slides in the CellularEyes program and selected two of three biopsies with 16 to 20 "scorable" hemicrypts per biopsy. Using the digital drawing board the borders of each selected hemicrypt were traced. The program then divided the outline into the equally spaced segments with the average widths of normal colonocytes. Finally, the program measured the background corrected optical density of the biomarker labeling across the entire hemicrypt as well as within each segment. All resulting data were automatically transferred into the MySQL database. Then, the technician moved to the next identified hemicrypt and repeated all the previously described analysis steps. A reliability control sample previously analyzed by the reader was re-analyzed during the course of the trial to determine intra-reader "scoring" reliability by intraclass correlation coefficient.

Figure A.2. Quantitative image analysis ("scoring") using Aperio Scanscope and CellularEyes software to measure 8-OH-dG labeling in normal colon crypts.



a) Choosing 8 scorable crypts/biopsy

b) Removing unwanted objects and tracing borders of hemicrypt

c) Dividing hemicrypt into sections

d) Storing resulting biomarker staining, tissue, and goblet cells density data

Protocol for Measuring Plasma 25-(OH)– and 1,25-(OH)$_2$– Vitamin D Levels (CaDvMAP Study)

All laboratory assays for plasma 25-(OH)-vitamin D and 1,25-(OH)$_2$-vitamin D were performed by Dr. Bruce Hollis at the Medical University of South Carolina using a radioimmunoassay method as previously described (388, 389). Plasma samples for baseline and follow-up visits for all subjects were assayed together, ordered randomly, and labeled to mask treatment group, follow-up visit, and quality control replicates. All laboratory personnel were blinded with regard to treatment group, follow-up visit, and quality control status. The average intra-assay coefficient of variation for plasma 25-(OH)-vitamin D was 2.3 %, and for 1,25-(OH)$_2$-vitamin D, 6.2 %.

Protocol for Genotyping of the VDR BsmI Polymorphism (CaDvMAP Study)

Genomic DNA was extracted from 700 µl of whole blood on a Qiagen BioRobot M48 workstation employing a magnetic bead separation technology (Qiagen, Valencia, CA) following the manufacturer’s instructions. DNA concentration was determined fluorescently using the Quant-iT PicoGreen dsDNA Kit (Invitrogen, Carlsbad, CA), on a SPECTRAmax Microplate Spectrofluorometer (Molecular Devices, Sunnyvale, CA). All DNA samples were normalized to 10 ng/µl using a Biomek FX Laboratory Automation Workstation (Beckman Coulter, Fullerton, CA).

Historically, the *VDR BsmI* polymorphism is defined by means of a restriction fragment length polymorphism (RFLP) (434, 435). The b allele is characterized by the presence of a *BsmI* site, whereas in the B allele this site is absent. The actual nucleotide sequence change corresponds to a T/C single nucleotide polymorphism (SNP) in the *BsmI* site (GATG<u>C</u>N, where the variable base C is underlined), as reported by Kikuchi et al. (436) This SNP corresponds to rs1544410 in the dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP).

A TaqMan allelic discrimination assay was employed for the *VDR* polymorphism SNP typing. The assay (Assay ID: C_8716062_10) was validated and inventoried by Applied Biosystems (Foster City, CA). Briefly, a forward primer and a reverse primer were designed to amplify the region surrounding the polymorphism. In addition, two fluorescent TaqMan probes labeled with a different colored dye represented the two alleles; i.e., VIC for the b allele (base C) and FAM for the B allele (base T). Each PCR reaction consisted of 12.5 μl of TaqMan Universal PCR Master Mix, 1.25 μl of 20X primer/probe mix, and 35 ng of genomic DNA, in a final volume of 25 μl. Thermocycling conditions were 2 mins. at 50°C, 10 mins. at 95°C, followed by 40 cycles of 15 secs. at 92°C and 1 min. at 60°C. Samples were amplified on a Peltier-based, 96-well block thermal cycling system (ABI GeneAmp 9700 PCR instrument) with standard optical 96-well reaction plates. Post PCR, the assay endpoint was read and genotypes distinguished by allelic discrimination on an ABI 7000 Sequence Detection System.

For quality assurance, two samples from each of the three genotypes (BB, Bb and bb) were randomly selected to be validated on a different platform, i.e., automated sequencing. PCR primers (forward: CCATCTCTCAGGCTCCAAAG; reverse: CCTCACTGCCCTTAGCTCTG) were designed to amplify a 209 bp DNA fragment flanking the SNP of interest. Sequencing was carried out for both the forward and reverse strands on an ABI 3100 Genetic Analyzer (Applied Biosystems). For all samples assayed (representing 6.2% of the total sample population), the concordance rate between the two platforms was 100%.

Protocol for 8-Hydroxy-2'-deoxyguanosine (8-OH-dG) Immunohistochemical (IHC) Staining

Preparation for Staining

Slides to be stained will be kept in the 100 count slide boxes labeled with the patient identification number in the Winship Cancer Iinstitute Lab until ready to be stained. Biopsy slides will be accompanied by their corresponding Forms E-210 and E-211.

Deparaffinizing and Antigen Unmasking Equipment and Supplies

1. Leica Automated H&E Stainer
2. Lab Vision Pretreatment Module(PT Module)
3. Labvision PT Module 100x Citrate Buffer pH 6.0

Deparaffinize and Antigen Retrieval

Remove lids from top row of reagents on Leica Automated H&E Stainer, and make sure all reagents are clean and at the fill line of each container. Place slides in gray slide rack, attach metal handle, and load rack into far right drawer. Select the “stain” option, enter the deparaffinization program (#2), and then press “load” to begin the program.

Deparaffinizing Protocol:

1.	Oven (Heat)	6 minutes
2.	Xylene	5 minutes
3.	Xylene	4 minutes
4.	Xylene	4 minutes

5. 100% Ethanol	3 minutes
6. 95% Ethanol	3 minutes
7. 95% Ethanol	3 minutes
8. 95% Ethanol	3 minutes
9. 95% Ethanol	3 minutes

Slides are unloaded from station 12 on the automated stainer, placed in DH_2O for 1 minute, and are then ready for antigen unmasking.

Antigen Retrieval Protocol:

1. Fill tanks with the desired Pretreatment Module buffer (15ml of 100x buffer to 1,485 ml of distilled water). The buffer must be changed every 3 sets of slides or every 3 to 4 days to maintain the integrity of the buffer.
2. Place previously cut and baked slides into Dako Autostainer racks and place racks into the tanks.
3. Close and lock lid with external lock.
4. Press the **Run** button for each tank to start the antigen retrieval run.
5. The CYCLE will show **WARMUP** (lid lock engages).
6. The Pretreatment Module will warm up to 98 degrees Fahrenheit and then start a 20 minute countdown for the retrieval cycle.
7. When the retrieval cycle is finished, CYCLE will show COOL
8. Slides may be removed by pressing the **PAUSE** buttons for both tanks to unlock the lid or when the COOL CYCLE is finished, CYCLE will show IDLE and lid will unlock.

9. Take slide racks out of the Pretreatment Module and place on the Dako Autostainer

Controls

A positive and negative control slide must be run with each batch of slides for immunohistochemistry. The control tissue for 8-OH-dG is colon adenocarcinoma. The control tissue must be fixed, embedded, and cut in the same manner as the patient's tissue. The negative and the positive control slides should be treated identically to the patient's slides except that antibody diluent is used rather than primary antibody on the negative control slide. Also, the negative control slide is not counterstained unless otherwise specified.

Automated Immunostaining Equipment and Supplies

1. DAKO Automated Stainer
2. Adjustable microliter pipettes
3. Refrigerator
4. Vortex
5. Pipette tips
6. Lab towels
7. Transfer pipettes
8. Antibody Diluent (DAKO S0809)
9. Signet Tris Buffered Saline + Tween 20 X (Signet 2380)
10. Cardboard slide trays

Pre-made Solutions

1. LSAB2 Detection System (DAKO K0675)
2. H_2O_2
3. Link Antibody
4. Streptavidin Peroxidase

Vortex all detection components and allow to sit until the bubbles dissolve before use.

Stock Solutions

Antibody

1. 8-Hydroxy-2'-deoxyguanosine antibody [N45.1] (ab48508, Abcam Inc., MA) at a concentration of 1:100
2. Antibody Diluent (DAKO S0809)

Vortex the solution and allow to sit until the bubbles dissolve before use; store at 4°C until ready to use.

DAB (Diaminobenzidine) 2-component (DAKO K3466)

1.	Tris Buffer	1,000 µl
2.	DAB	1 drop

Make fresh before each use. Mix well and let sit for 5 minutes before use. Do not reuse reagent. Because DAB is a carcinogen, gloves should be worn when handling DAB and all supplies and glassware used with it should be treated with bleach; store all reagents at 4°C until ready for use.

Working Buffer

1. Signet Tris Buffered Saline + Tween 20X (500 ml) 1 Bottle
2. 10 liters of distilled water

Make 10 liters of buffer. (Note: Stable at room temperature for 7 days or at 4°C for 10 days.)

Automated Staining Protocol

Set up the DAKO Automated Stainer according to the manufacturer's directions and stain using the DAKO LSAB2 detection protocol as follows:

1.	TBS Buffer Rinse	
2.	H_2O_2	5 minutes
3.	Blow	
4.	Primary Antibody	30 minutes
5.	TBS Buffer Rinse and Blow	
6.	Link Antibody	10 minutes
7.	TBS Buffer Rinse and Blow	
8.	Streptavidin Peroxidase	10 minutes
9.	TBS Buffer Rinse and Blow	
10.	DAB	5 minutes
11.	DH_2O Rinse	
12.	TBS Buffer Rinse	

Clearing and Coverslipping Equipment and Supplies

1. Leica H&E Stainer

2. Leica Coverslipper
3. Ethanol – 95% and 100%
4. Xylene
5. Mounting medium
6. Coverslips
7. Staining racks
8. Slide racks
9. Slide trays
10. Lab towels
11. Tap H_2O
12. DH_2O

Clearing and Coverslipping

Remove the slides from the DAKO staining racks and load them into a slide rack for staining on the Leica H&E stainer. Select the "stain" option, enter clearing program #3, and press "load" to begin the following staining program:

1.	Tap H_2O	20 seconds
2.	95% ethanol	20 seconds
3.	95% ethanol	20 seconds
4.	100% ethanol	30 seconds
5.	100% ethanol	30 seconds
6.	100% ethanol	30 seconds
7.	Xylene	30 seconds

8.	Xylene	1 minute
9.	Xylene	1 minute

The slides, still in the staining rack, will rest in the last xylene until the automated arm moves the slide into the xylene bath for the attached Leica coverslipper.

When you load the slides on the stainer for clearing program # 3, turn on the coverslipper and remove the lid from the coverslipper xylene bath metal lid. Prime the mounting media needle and then place the needle in the dispense position. Check to make certain that the needle cleaning attachment has sufficient xylene. The coverslip supply should also be checked. The coverslip holding container should be three-quarters full. After slides have been coverslipped remove the slides from the coverslipper and place slides in slide folders labeled with the date, antibody, and batch number.

Figure A.3. Images of the normal-appearing colorectal crypts immunohistochemically stained for the 8-OH-dG biomarker.