Distribution Agreement

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

_____ _____

Thomas U. Ahearn Date

The investigation and development of dietary and life-style modifiable, pre-neoplastic biomarkers of risk for colorectal neoplasms

By
Thomas U. Ahearn
M.P.H, Emory University, 2006
B.S. State University of New York at Albany, 2003

Graduate Division of Biological and Biomedical Science
Nutrition and Health Sciences

_____

Roberd M. Bostick, M.D., M.P.H.
Advisor

_____

W. Dana Flanders, DS.c, M.P.H.
Committee Member

_____

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

_____

Marjorie McCullough, Sc.D
Committee Member

_____

Victoria L. Stevens, Ph.D.
Committee Member

Accepted:

_____ _____

Lisa A. Tedesco, PhD Date
Dean of the Graduate School

The investigation and development of dietary and life-style modifiable, pre-neoplastic biomarkers of risk for colorectal neoplasms

By
Thomas U. Ahearn
M.P.H, Emory University, 2006
B.S. State University of New York at Albany, 2003

Advisor:
Roberd 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
Doctor of Philosophy
in Graduate Division of Biological and Biomedical Sciences
Nutrition and Health Sciences
2012

Abstract

The investigation and development of dietary and life-style modifiable, pre-neoplastic biomarkers of risk for colorectal neoplasms

By Thomas U. Ahearn

Colorectal cancer (CRC) is the second leading cause of cancer deaths in the United States. Despite advances in screening and treatment, mortality due to CRC has declined only modestly in recent years, highlighting the need for treatable, pre-neoplastic biomarkers of risk for the disease. The antineoplastic effects of calcium and vitamin D against CRC may, in part, depend on modifying markers of their metabolism and the APC/β-catenin pathway. In this dissertation I report the results of investigations of markers of these pathways (APC, β-catenin, and E-cadherin, calcium receptor (CaR), vitamin D receptor (VDR), CYP27B1, and CYP24A1) as treatable, pre-neoplastic biomarkers of risk for colorectal neoplasms.

In a pilot colonoscopy-based case-control study of incident, sporadic colorectal adenoma we found the ratio of the proportion of APC expression in the upper 40% of crypts (Φh APC) with total β-catenin expression (APC/β-catenin score) to be significantly greater in controls than in cases. The APC/β-catenin score was positively associated with protective risk factors against colorectal neoplasms. Controls had greater Φh APC and E-cadherin expression and lower β-catenin expression, but none of these differences were statistically significant.

In a randomized, double-blind, placebo-controlled clinical trial evaluating the effects of 6-months of supplemental calcium (2.0 g/d) and vitamin D_3 (800 IU/d), alone and in combination, we found increased Φh APC and E-cadherin expression, an increased APC/β-catenin score, and decreased β-catenin expression in the active treatment groups. We also found that supplemental calcium and vitamin D_3 modulated markers of their metabolism with increased CaR, VDR, and CYP27B1 expression in the active treatment groups, and increased

CYP24A1 expression in the vitamin D_3 treatment groups and decreased CYP24A1 expression in the calcium treatment group.

Taken together, these results suggest that 1) APC, β-catenin, CaR, VDR, CYP27B1, and CYP24A1 expression may be treatable, pre-neoplastic biomarkers of risk for colorectal neoplasms; and 2) the antineoplastic effects of calcium and vitamin D may, in part, depend on modifying markers of the APC/β-catenin pathway and markers of calcium and vitamin D metabolism. The results of this dissertation support further investigation of these markers as potential treatable, pre-neoplastic biomarkers of risk for colorectal neoplasms.

The investigation and development of dietary and life-style modifiable, pre-neoplastic biomarkers of risk for colorectal neoplasms

By
Thomas U. Ahearn
M.P.H, Emory University, 2006
B.S. State University of New York at Albany, 2003

Advisor:
Roberd M. Bostick, M.D., M.P.H.

A dissertation submitted to the Faculty of the Graduate School of Emory University in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in Graduate Division of Biological and Biomedical Sciences
Nutrition and Health Sciences
2012

Acknowledgments

I would like to thank my dissertation committee for all of their guidance, expertise, time, and encouragement. I am especially grateful to my advisor Dr. Robin Bostick for his many years of wonderful mentorship and being a constant source of inspiration and encouragement.

To my awesome parents, thank you so much for your support and encouragement. I would like to thank my best friend and wife Amparo for her love, patience, and always encouraging me to strive for my best.

I am very grateful to my friends, colleagues, and professors at Emory University for all their help and contributions to my dissertation research and graduate education.

Table of Contents

List of Tables

List of Figures

Chapter 1. Background and Significance

In the United States colorectal cancer is the fourth most common incident cancer and the second most common cause of cancer deaths [1]. Incidence of colorectal cancer varies 10-fold worldwide, with the highest rates found in Australia, New Zealand, Europe, and North America, and the lowest rates in Africa and South-Central Asia [2]. Over the past 20 years incidence rates have stabilized or modestly declined in most developed countries, but in regions of the world undergoing economic transitions (Eastern Europe and much of Asia) incidence rates have increased [3]. In the United States, colorectal cancer rates have been declining among adults 50 years and older; however, colorectal cancer rates have been increasing among adults younger than 50 [4-6]. International differences and migration studies strongly suggests that the etiology of CRC is highly attributable to environmental and lifestyle factors. Generally, within one generation the incidence rates of colorectal cancer among the descendants of immigrants converge to reflect the rates of their host country [7, 8]. These reports provide compelling evidence that dietary and lifestyle behaviors can modify the risk for sporadic colorectal cancer.

The colorectal adenoma is a well-established precursor for colorectal cancer. Approximately 96% of colorectal cancers arise from adenomas [9]. Having an adenoma markedly increases risk for colorectal cancer by a factor of 2 to 4 compared to not having an adenoma; however, the vast majority of adenomas do not progress to cancer [10]. The risk of developing advanced adenomas or cancer varies depending on the histopathological characteristics of the index adenomas, with greater risk associated with multiple adenomas (≥ 3 adenomas), increased size (≥ 1 cm), villous features, and high-grade dysplasia [11, 12]. Colorectal cancer is typically diagnosed 10 – 15 years after the detection of an adenoma [13, 14].

Colonoscopy is the current gold standard for screening for colorectal neoplasms; however, colonoscopies have limitations and risks (**Table 1.1**) [15-17]. Colonoscopies allow for the direct examination of the colon and rectum for neoplastic growths, and when necessary, biopsy or polypectomy. Some of the limitations and risk of colonoscopies include that they are invasive, screening quality is operator dependent, and there is a risk of bleeding and perforation. The miss rate of total adenomas by colonoscopy screening is reported to vary from 17 – 24% [16, 18, 19], and for adenomas ≥1 cm 6% [16, 18]. For average risk individuals colonoscopy screening guidelines recommend screening once every 10 years starting at 50 years of age [20]. If a polyp is discovered then depending on the histopathological features of the polyp the recommended duration until the following screenings may be reduced to 3 – 5 years. Individuals with a predisposition to colorectal cancer are recommended to initiate screening at an earlier age than average risk individuals [20]. Removal of adenomas markedly reduces the risk developing colorectal cancer and mortality due to colorectal cancer [11, 21].

Table 1.1. **Barriers and Limitations of Colonoscopy**

- Requires full bowel preparation
- Can miss some adenomas and cancer
- Sedation may be required
- Operator skill dependent
- Risk of bleeding and perforation

Despite advances in screening and treatment, mortality due to colorectal cancer has declined only modestly over the past 50 years, the decline probably most attributable to increased screening and polypectomy [6]. The observed decreased trend in deaths attributable to heart disease is, at least in part, due to the development of treatable biomarkers of risk, such as blood pressure and lipid profiles [22]. Currently, there are no validated treatable, pre-neoplastic biomarkers of risk for colorectal neoplasms. The development of such biomarkers of risk for colorectal neoplasms will enhance clinical risk identification, stratification, and monitoring, and facilitate the development of chemopreventive agents against the disease.

Potentially, with the aid of treatable, pre-neoplastic biomarkers of risk for colorectal neoplasms, we will see a precipitous decrease in colorectal cancer incidence and mortality

Colorectal morphology

The colon and rectum are lined by a single layer of epithelial cells. To increase the absorptive surface area, the colorectal epithelium is organized into crypts (**Figure 1.1**). Colorectal crypts are the proliferative source of the colorectal epithelium. Located at the base of each crypt are postulated to be multi-potent stem cells [23, 24]. The stem cells replicate to produce a population of transit amplifying daughter cells that undergo rapid proliferation and migrate towards the crypt apex. As cells approach the crypt apex they become a population of non-proliferative, differentiated daughter cells that eventually undergo apoptosis and are exfoliated into the colorectal lumen. The Wnt/β-catenin signaling pathway is responsible for maintaining the continuous self-renewal of the colorectal mucosa and crypt homeostasis. Wnt signaling promotes β-catenin translocation into the nucleus to activate Wnt target genes that promote cellular proliferation [23, 25]. In murine models blocking the Wnt/β-catenin signaling pathway results in the loss of stemness and the undifferentiated state, resulting in eventual crypt disappearance [26].

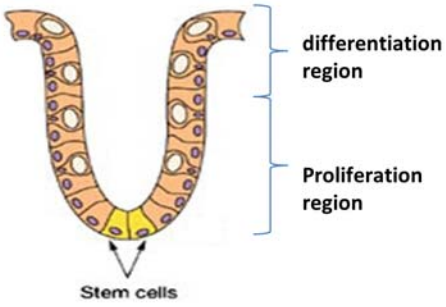


Figure 1.1. Proliferation in normal colorectal crypts occurs in the lower half of crypts and as cells migrate towards the crypt apex they become differentiated and eventually undergo apoptosis. Modified from: Nat Clin Pract Gastroenterol Hepatol. 2006;3(5):267-274.

Colorectal cancer pathogenesis

The pathogenesis of colorectal cancer involves the accumulation of genetic alterations that promote uncontrolled growth, replication, and evasion of apoptosis [27-29]. The majority of colorectal cancer cases are sporadic cases, meaning that they cannot be attributed to a

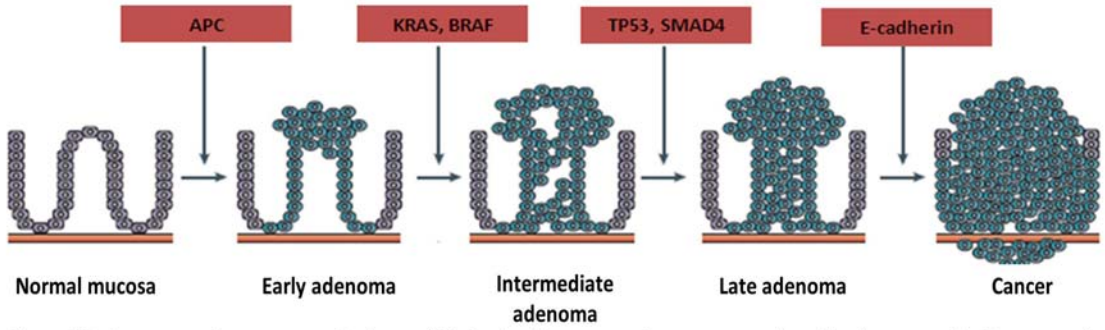


Figure 1.2. A summary of common genetic abnormalities in the adenoma–carcinoma sequence that drive the progression from normal colorectal mucosa to cancer. Modified from: Walther, A., et al., *Genetic prognostic and predictive markers in colorectal cancer.* Nat Rev Cancer, 2009. **9**(7): p. 489-99.

known familial or inherited risk [30]. Hereditary conditions contribute to approximately 5% of colorectal cancers cases, of which 1 – 4% are cases of hereditary nonpolyposis colorectal cancer (HNPCC or Lynch Syndrome) and <1% are cases of familial adenomatous polyposis (FAP) [4, 31-33].

The adenoma-carcinoma sequence is the dominant carcinogenic pathway, describing the neoplastic transition of small adenomas into cancer (**Figure 1.2**) [27, 34, 35]. The adenoma-carcinoma sequence may best characterize sporadic neoplasms that arise from the APC/β-catenin pathway. Mutations in the *APC* gene promote inappropriate activation of the Wnt/β-catenin pathway and increased cellular proliferation. Mutated APC is reported in approximately 70 – 80% of sporadic colorectal cancers (see the "APC, β-catenin, and E-cadherin" section below for more detail) [36-38].

The "Mismatch Repair Pathway" (MMR) is responsible for approximately 15% of sporadic colorectal neoplasms. MMR is a postreplicative DNA repair mechanism that maintains genomic integrity by repairing mismatched base pairs that occur during cellular replication [36, 37]. The mismatched base pairs most often occur in microsatellites (DNA tandem repeats) that result in microsatellite instability (MSI). MMR proteins, such as MLH1 and MSH2 are responsible for repairing MSI. Silencing of the *MLH1* and *MSH2* genes, often by hypermethylation, is

associated with sporadic MSI colorectal cancers, which may develop in a distinct fashion from the classic adenoma-carcinoma pathway [36, 37].

Colorectal carcinogenesis is heterogeneous, involving multiple, often overlapping, signaling pathways resulting in neoplasms that are characterized by various molecular features [38]. For example, basic science, animal models, and investigations of inflammatory bowels disease (IBD) suggest that the intestinal microflora, the immune system, and inflammation may play a role in colorectal carcinogenesis that overlaps with the APC/β-catenin pathway [39, 40]. In the presence of wild type APC, the inflammatory marker cyclooxygenase (COX)-2 may promote aberrant β-catenin signaling leading to increased cell proliferation and inhibition of cell differentiation and apoptosis [41].

The heterogeneous nature of colorectal carcinogenesis highlights the need to develop treatable, pre-neoplastic, biomarkers of risk for the disease. Pre-neoplastic biomarkers will enable better risk stratification, provide better insight into disease progression, and help guide intervention strategies targeted at disease prevention. This dissertation focuses on markers from the APC/β-catenin pathway (APC, β-catenin, and E-cadherin) and of calcium and vitamin D metabolism (the calcium receptor, the vitamin D receptor, CYP27B1, and CYP24A1) as potential treatable, pre-neoplastic biomarkers of risk for colorectal neoplasms.

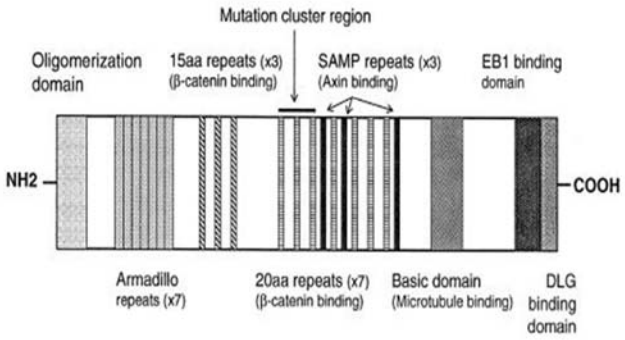


Figure 1.3. Functional domains of the APC protein. From: Gryfe, R., *The Chromosomal-Instability Pathway and APC Gene Mutation in Colorectal Cancer*. Genetics of Colorectal Cancer, ed. J.D. Potter and N.M. Lindor2009, New York: Springer Science and Business Media.

APC, β-catenin, and E-cadherin

APC mutations are present in most (approximately 70 – 80%) sporadic colorectal cancers, and are often attributed with causing inappropriate β-

catenin nuclear accumulation and subsequent cellular proliferation. Most APC mutations are truncating mutations in the "mutation cluster region" (codons 1286 to 1513) [42]. The Wnt/β-catenin signaling pathway is negatively regulated by the "β-catenin destruction complex", which is formed by APC, GSK3β, axin, and CK1α/ε. The APC protein contains a β-catenin binding region composed of three 15-amino-acid repeats and seven 20-amino-acid repeats, and an axin protein binding region composed of the three SAMP repeats (**Figure 1.3**). In the absence of Wnt signaling the "β-catenin destruction complex" phosphorylates cytoplasmic β-catenin, leading to β-catenin proteasomal degradation. However, in the presence of Wnt signaling axin is sequestered and degraded at the plasma membrane, inhibiting the formation of the "β-catenin destruction complex", and permitting nuclear accumulation of β-catenin (**Figure 1.4**). The precise function of APC in the destruction complex is not entirely clear, but proposed functions include facilitating β-catenin phosphorylation by GSK3β[43], shielding phosphorylated β-catenin from de-phosphorylation [44], and facilitating the ubiquination of phosphorylatied β-catenin [45]

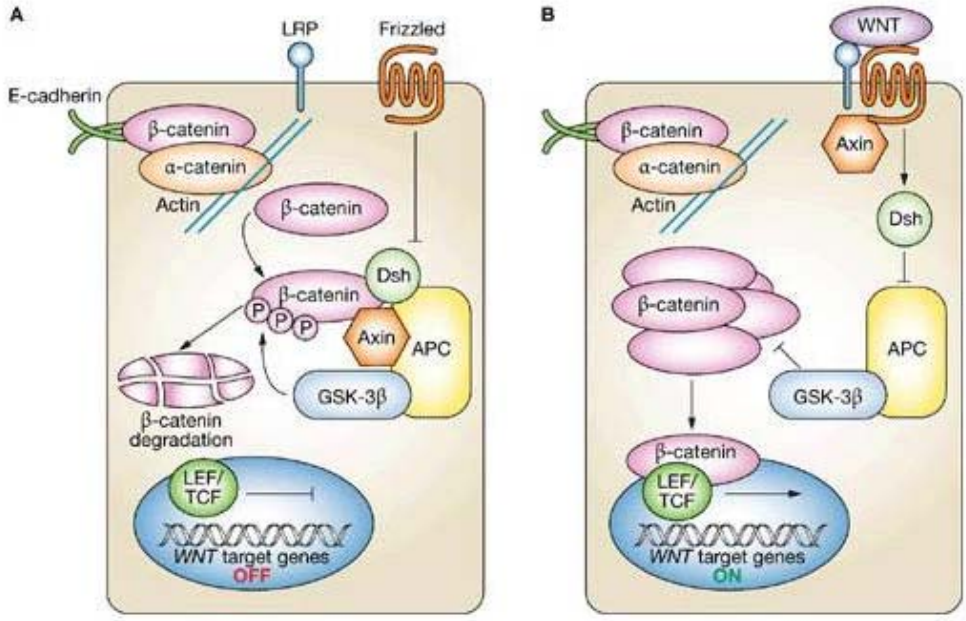


Figure 1.4. The Wnt/β-catenin signaling pathway. (A) In the absence of WNT signaling β-catenin is degraded by the "β-catenin destruction complex" and (B) in the presence of Wnt signaling the "β-catenin destruction complex" is inhibited and β-catenin may translocate to the nucleus. Modified from: Nat Clin Pract Gastroenterol Hepatol. 2006;3(5):267-274.

Aberrant and constitutively active Wnt/β-catenin signaling is an early event in colorectal carcinogenesis [28, 34, 46]. In normal colorectal mucosa APC and β-catenin are strongly expressed; however during the adenoma-carcinoma sequence APC expression markedly decreases and β-catenin expression markedly increases [47]. Malfunctioning of APC alone may not directly lead to β-catenin nuclear localization. Additional oncogenic events such as activating mutations of KRAS and BRAF, or inhibition of the vitamin D receptor may also be required for β-catenin nuclear translocation [48-51]. Less often in sporadic colorectal cancer (approximately 5% of cases), activating β-catenin mutations lead to nuclear β-catenin expression, but these mutations tend to be associated with HNPCC [52, 53].

APC also has important functions in maintaining normal cell migration, polarity, and division [54-56]. Located at the C-terminus of the APC protein are three cytoskeletal binding domains for microtubule (MT), microtubule end-binding protein (EB1), and discs large gene (DLG) [57-59] (**Figure 1.3**). Lack of APC may inhibit cellular protrusions and decrease cell migration, which can be reversed by APC overexpression [56]. APC loss may also produce spindle defects and malfunctioning mitotic checkpoints that result in tetraploid cells and decreased apoptosis [54]. Transfecting truncated APC into the APC wild-type HCT116 human colorectal-cancer line resulted in a 2.5 – 5-fold increase in chromosomal instability [60]. Furthermore, because the HCT116 colon-cancer cell line harbor an oncogenic β-catenin mutation, but normal APC, these results suggest that APC's role in chromosomal stability are independent of the Wnt/β-catenin pathway [60].

Hypermethylation of the APC promoter is a potential mechanism for silencing APC expression/function; however, the role of *APC* hypermethylation in carcinogenesis is unclear. The *APC* gene has two promoter regions, 1A and 1B, of which promoter 1A is most commonly active [42]. One small study that compared normal appearing colonic mucosa (n=24), adenomas

(n=10), and carcinomas (n=18), reported no difference in *APC* promoter 1A methylation between normal mucosa and adenomas, but carcinomas had significantly greater methylation [61]. However, of 108 colorectal cancer cases, 48 adenoma cases, and 28 normal appearing tissue samples, the *APC* promoter 1A was reported to be hypermethylated in 18% of colorectal cancer cases and 18% of adenoma cases, while no hypermethylation was found in the adjacent normal tissue [62]. Bai et al. reported that of 47 colorectal cancer cases, 36 adenoma cases, and 34 neoplasm-free controls, the *APC* promoter 1A was hypermethylated in approximately 50% of colorectal cancers, 60% of adenoma cases, and 3% of controls [63]. In the reviewed literature there was no evidence of epigenetic modification of the *APC* promoter 1B.

E-cadherin is a calcium dependent, homophilic, transmembrane glycoprotein that is the primary mediator of epithelial cell-cell adhesion [64]. In the presence of calcium, the extracellular domain of E-cadherin binds to the extracellular domain of another E-cadherin molecule on an adjacent cell, essentially creating a "molecular zipper". E-cadherin mediated cellular adhesion requires α- and β-catenin to bind to the cytoplasmic domain of E-cadherin. The cadherin-catenin complex binds to the cellular cytoskeleton creating a physical link between cells [64, 65].

Loss of E-cadherin expression can lead to increased proliferation, decreased differentiation, and increased metastatic potential. In normal colorectal mucosa E-cadherin is strongly expressed, but during the neoplastic transition E-cadherin expression is decreased, particularly during the later stages of the neoplastic transition [47, 66, 67]. The ability of E-cadherin to bind and sequester β-catenin at the cell membrane suggests that decreased E-cadherin expression may results in increase nuclear β-catenin expression. Indeed, *in vitro* studies have reported that the loss of E-cadherin expression promotes β-catenin/TCF

transcriptional activity, and restoring E-cadherin expression promotes increased membrane β-catenin expression and decreased nuclear β-catenin expression [68-70].

E-cadherin may also inhibit signaling involving receptor tyrosine kinase (RTK). E-cadherin may inhibit the ligand-dependent activation of RTK by decreasing receptor mobility and ligand-binding affinity [71]. Transfecting E-cadherin expression into carcinoma cell lines that endogenously express low E-cadherin levels resulted in down-regulated RTK signaling [71]. However, the relationship between E-cadherin and RTK is bi-directional, as RTK have been reported to inhibit E-cadherin expression during the epithelial-mesenchyme transition [72]. In human mammary carcinoma cell line, IGF-II binding to IGF-1R induced the release and translocation of β-catenin from E-cadherin leading to nuclear β-catenin expression and E-cadherin degradation [73].

Down-regulation of E-cadherin expression in colorectal neoplasms is rarely attributed to mutations in the E-cadherin gene (*CDH1*) [74-76]; although, epigenetic modifications of the *CDH1* promoter may inhibit E-cadherin expression. From a small study it was reported that the *CDH1* promoter was hypermethylated in 57% of ulcerative colitis associated colorectal cancer (n=14) and 36% of sporadic colorectal cancer cases (n=14) [75]. However, Kim et al reported that the *CDH1* promoter was hypermethylated in approximately 12%, 5%, and 0% of cases of adenoma (n=42), adenocarcinomas (n=77), and hepatic metastases (n=26), respectively [77].

Risk Factors for Colorectal Cancer

Hereditary Colorectal Cancer

Well-defined hereditary conditions such as FAP and Lynch Syndrome account for approximately 5% of all colorectal cancers [30-32]. The most common hereditary syndrome that confers increased risk for colorectal cancer is Lynch Syndrome, accounting for approximately 1 –

4% of cases [4, 30, 32, 33]. Lynch Syndrome is most commonly caused by germline mutations in the *MLH1*, *MSH2*, and *MSH6* genes, resulting in impaired DNA repair mechanisms [27]. The condition is autosomal dominant and conveys an approximate 50 – 80% lifetime risk for colorectal cancer [27, 30].

Familial adenomatous polyposis is a highly penetrant, autosomal dominant syndrome caused by germline mutations in one of the alleles of the *APC* genes [27, 32]. Adenomas develop following somatic mutations to the second *APC* allele [27, 32]. The characteristic feature of FAP is the development of hundreds to thousands of adenomas early in life, and if left untreated result in a nearly 100% risk of progressing to colorectal cancer. The prevalence of FAP is approximately 1 in 8,000 – 10,000 [27, 30, 32].

Family History

Individuals with a family history of colorectal cancer are at an increased risk for the disease. A recent meta-analysis of 20 case-control studies and 7 cohort studies reported an odds ratio (OR) of 2.25 (95% CI: 2.00-2.53) for individuals with a first-degree relative with colorectal cancer, and an OR of 4.25 (95% CI: 3.01-6.08) for individuals with more than one relative with colorectal cancer [78].

Demographic Risk Factors

The risk of colorectal neoplasms markedly increases with age [79-82]. Approximately 90% of colorectal cancer cases occur in individuals 50 years of age or older [4, 83, 84]. Colorectal cancer incidence and mortality rates tend to be slightly greater in males than females [4, 85]. A meta-analysis of 18 observational studies found a statistically significant increased risk for colorectal adenomas for males compared to females (relative risk (RR) 1.22; 95%CI 1.12-

1.32) [82]. Incidence and mortality rates are highest in black males and females [4, 86]. Between 1985 to 1987 and 2006 to 2008, mortality rates for all stages of colorectal cancer declined in both whites and blacks; however, the declines were smaller for every stage in blacks compared to whites [87]. Compared to whites, incidence and mortality rates are lower in Asian Americans/Pacific Islanders, American Indians/Alaska Natives, and Hispanics/Latinos [4].

Aspirin and Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)

The antineoplastic mechanisms of aspirin and NSAIDs are not entirely clear; however, they likely involve inhibition of chronic inflammatory signaling. Both aspirin and NSAIDs inhibit the COX-1 and COX-2 enzymes, resulting in the inhibition of inflammatory signaling, proliferation, and angiogenesis, and promotion of apoptosis [88-90]. A pooled analysis from four randomized cardiovascular disease prevention trials reported that treatment with aspirin (75 – 500 mg/d) over a median follow-up time of 18.3 years reduced the 20-year risk of colon cancer by 24% and colon cancer mortality by 35% [91]. Increased duration of aspirin use appeared to increase the protective benefits. Aspirin use for 5 or more years reduced overall incidence 32%, incidence of proximal colon cancer by 65%, and incidence of rectal cancer by 42% [91]. In a meta-analysis of four randomized adenoma prevention trials, any aspirin use (81 – 325 md/d) over a median follow-up time of 33 months, reduced the risk of any adenoma and advanced adenoma by 17% and 28%, respectively [92]. Three randomized controlled trials reported that selective COX-2 inhibitors (celecoxib and rofecoxib) reduced the risk of recurrent colorectal adenomas; however, all three studies also reported increased risk of serious cardiovascular events in the active treatment groups [93-95]. In the Adenoma Prevention with Celecoxib trial, 200 mg of celecoxib twice daily reduced the risk of any adenoma by 33% and advanced adenoma by 66% [95]. Overall, there is strong, consistent evidence that aspirin and

selective COX-2 inhibitors reduce the risk for colorectal neoplasms; however, their routine use by the general public is not recommended for the prevention of colorectal neoplasms due to the concern of associated adverse effects [96].

Smoking and Alcohol

A recent meta-analysis of 106 observational studies estimated that ever-smokers were 18% more likely to develop colorectal cancer compared to never-smokers [97]. Data from the Iowa Women's Health Study (IWHS) suggest that smoking may be disproportionately associated with colorectal cancer subtypes that are associated with epigenetic modifications. In the IWHS ever-smokers were at a moderately increased risk for colorectal cancer (RR=1.19; 95% CI: 1.05 – 1.35); however, current smokers were at an increased risk of MSI-high tumors (RR=1.99; 95% CI: 1.26 – 3.14), CpG island methylator phenotype-positive tumors (RR=1.88; 95% CI: 1.22 – 2.90), and *BRAF* mutation-positive tumors (RR=1.92; 95% CI: 1.22 – 3.02) [98]. A wide variety of carcinogens are found in tobacco smoke, including heterocyclic amines (HCA), polycyclic hydrocarbons, and nitrosamines [99].

There is consistent evidence, especially in men, suggesting that alcohol consumption increases the risk of colorectal cancer [100, 101]. A meta-analysis of 27 cohort and 34 case-control studies reported that moderate (2-3 drinks/d) and heavy (≥ 4 drinks/d) drinkers have a 21% and 52% increased risk for colorectal cancer, respectively [101]. The association for moderate drinkers was stronger in men (RR=1.24; 95% CI = 1.13-1.28) than women (RR = 1.08; 95% CI = 1.03-1.13) [101]. The mechanism by which alcohol may promote colorectal neoplasms may be due to microbial metabolism of ethanol to acetaldehyde. Acetaldehyde has been reported to damage colorectal mucosal integrity [102], increase degradation of folate [103], inhibit DNA repair [104], and stimulate cell proliferation [102, 105].

Body Composition and Physical Activity

The 2007 World Cancer Research Fund report (WCRF) found convincing evidence that obesity and lack of physical activity increased colorectal cancer risk [100]. The WCRF estimated that the risk for colorectal cancer increased 15% per 5 kg/m^2 in body mass index (BMI), and increased 5% per inch of waist circumference [100]. The positive association was more apparent for colon cancer than rectal cancer [100]. The mechanisms by which obesity promotes colorectal neoplasms are not fully understood; however, obesity is associated with chronic elevated inflammatory mediators such as TNF-α, IL-6, and CRP, and elevated hormones such as insulin, IGF-1, and leptin, which may contribute to the initiation and/or progression of colorectal neoplasms [100, 106, 107]. A meta-analysis of 10 prospective cohort that studies investigated the association between IGF-1 and colorectal cancer reported a RR of 1.07 (95% CI 1.01–1.14) for an increase corresponding to 1 standard deviation of the average IGF-1 distribution of all studies combined, and a RR of 1.13 (95% CI 0.97 – 1.32) when comparing the most extreme to the lowest category of IGF-1 [108].

Two recent meta-analyses estimated an approximately 20% lower risk for colon cancer when comparing the most *vs.* least active individuals; however, the anti-neoplastic effects of physical activity did not appear to apply to rectal cancer [109, 110]. The Nurses' Health Study (NHS) reported a similar 23% lower colon cancer risk when comparing the most to the least active individuals [111]. In the NHS study the highest activity levels corresponded to about 5-6 hours of brisk walking per week and the least activity corresponded to approximately 0.5 hours of brisk walking per week [111]. There are multiple proposed mechanisms by which physical activity may protect against colon cancer including increasing colonic mobility, improving insulin sensitivity, reducing chronic inflammation, reducing body fatness, and increasing vitamin D levels [106, 111, 112].

Dietary Risk Factors

Dietary behavior is well recognized to play an important role in the pathogenesis of colorectal cancer. Dietary patterns characterized by high concentrations of fruits and vegetables, fish and poultry and whole grains are generally associated with lower risk for colorectal neoplasms, while diets characterized by high concentrations of red and processed meats, refined grains, high fat foods, and alcohol consumption are generally associated with higher risk of colorectal neoplasms [113]. Presented below is a brief review of the association between selected dietary components and colorectal neoplasm.

Red and Processed Meat

The WCRF concluded that there is substantial evidence supporting a positive association between red and processed meat consumption and risk of colorectal cancer. The WCRF meta-analysis estimated risk of colorectal cancer increased 15% per 50 g/d consumption of red meat, and 21% per 50 g/d consumption of processed meats [100]. A 2011 meta-analysis of prospective studies reported similar estimates, finding a 17% increased risk per 100 g/d consumption of red meat, and an 18% increased risk per 50 g/d consumption of processed meats [114].

Several biologically plausible mechanisms may explain the positive association between red and processed meats with colorectal neoplasms. Meat cooked at high temperatures is a major source of HCA [115]; however, chicken cooked at high temperatures is also a major source of HCA [116] and chicken consumption generally is not positively associated with colorectal neoplasms [117]. Also, the doses of HCAs that promote colorectal neoplasms in animal studies are 1,000 to 100,000 times greater than human exposure via cooked meet [118]. Red and processed meats, but not white meat and fish, are a source of nitrite and N-nitroso

compounds which may promote epigenetic modifications to DNA [118-120]. Heme iron may also promote carcinogenesis by promoting the formation of lipoperoxides in the intestines and catalyzing the endogenous formation of N-nitroso compounds [118].

Fats

Epidemiologic evidence does not provide consistent evidence for an association between total fat intake or subtypes of fat intake with colorectal neoplasms [121-127]. Furthermore, a meta-analysis of six prospective cohorts found no association between dietary animal fat and colorectal cancer [128].

Despite a lack of supporting epidemiologic evidence, basic science evidence suggests that fatty acids may modify colorectal carcinogenesis. High fat diets induce greater bile acid secretion from the gall bladder and subsequent passage of bile acids from the small intestine to the colon [129, 130]. The microflora of the colon synthesize primary bile acids to deoxycholic acid (DCA) and lithocholic acid (LCA). LCA may promote carcinogenesis through inhibiting DNA repair proteins and promoting of DNA strand breaks [131, 132]. The carcinogenic effects of DCA may be attributed to promoting cell proliferation through activation of protein kinase C / prostaglandin-E2 (PGE2) [133], promoting β-catenin nuclear expression [134], and increasing oxidative stress [135]. A balanced omega-6/omega-3 fatty acid ratio may reduce the risk of colorectal neoplasms. Omega-3 fatty acids may inhibit inflammatory COX-2-mediated PGE2 signaling, while omega-6 fatty acids may promote PGE2 synthesis [136].

Vegetables, Fruits, Folate, and Fiber

Basic science, animal experiments, and human feeding studies suggest that vegetables, fruits, folate, and fiber have antineoplastic effects against colorectal neoplasms [137-142]. Fruits

and vegetables contain large number of potentially anticarcinogenic bioactive compounds such as carotenoids, selenium, glucosinolates, indoles, ascorbate, chlorophyll, flavonoids, allylsulphides, flavonoids, and phytoestrogens [99, 100]. These compounds may have both complementary and overlapping mechanisms including antioxidant effects, promoting activation of detoxification enzymes, and inhibiting nitrosamine formation[138]. The WCRF found limited evidence that non-starchy vegetables and fruits protect against colorectal cancer [100]. A pooled analysis of 14 cohort studies found an estimated a modest lower risk for those in the highest relative to those in the lowest quintile of fruit and vegetable intake (RR=0.82; 95% CI 0.82-1.01) [143]. A recent meta-analysis suggested that the fruit and vegetable intake-colorectal cancer association may be non-linear, with most risk reduction occurring between 100 – 200 g/d consumption of fruits and vegetables, with little additional benefit from greater vegetable and fruit intake [144].

Foods high in fiber include fruits, vegetables, whole grains, and legumes. Fiber is fermented by the colonic microflora producing short-chain fatty acids (SCFA). The SCFA butyrate is an important fuel source for the colorectal epithelium and may promote apoptosis [145]. The synthesis of SCFA reduces the colorectal luminal pH, and possibly inhibits the conversion of primary bile acids to secondary bile acids (e.g., DCA and LCA) [138]. Fiber also increases stool bulk and reduces transit time [99]. A recent meta-analysis of prospective cohorts estimated a 10% lower risk of CRC per 10 g/d intake of total and cereal fiber, and a 17% lower risk per three servings of whole grain [146]. Inverse associations between risk of colorectal cancer and fiber intake from vegetables, fruits, and legumes was also reported; however, none of these associations were statistically significant [146]. Despite convincing basic science and observational evidence that fiber may be inversely associated with colorectal cancer, randomized controlled trials have not been supportive [147-149]. In the Polyp

Prevention Trial, a high-fiber (18 g/1,000 kcal), high-fruit and-vegetable (3.5 servings/1,000 kcal), and low-fat (20% of total energy) diet did not reduce adenoma recurrence compared to "regular diet" [149]. However, these results need to be interpreted cautiously because it is possible that study participants in the treatment arm underreported fat consumption and over-reported fiber and fruit and vegetable consumption. A re-analysis of the Polyp Prevention Trial found that study participants who had high adherence to the study intervention over the 4-year trial period had a significant 32% decrease risk of adenoma recurrence, a significant 49% decrease risk of multiple adenoma recurrence, and a borderline significant 56% decreased risk of advanced adenoma recurrence [150].

Sources of naturally occurring folate include leafy green vegetables, dried beans and peas, and citrus fruits. In 1996, the U.S. Food and Drug Administration issued a regulation requiring the fortification of all enriched cereal-grain products with folic acid by January 1998 [151]. Folate has essential functions in DNA synthesis, repair, and methylation. Basic science studies have reported folate deficiency to be associated with increased chromosomal instability [138]. A methyl-donor deficient diet in rats promoted DNA strands breaks within the p53 gene [152]. Alcohol antagonizes intestinal absorption and utilization of folate [153]. However, animal studies suggest that folate supplementation following initiation of colorectal neoplasms promotes neoplastic progression [154, 155]. At least one human clinical trials [156] echoed these concerns that folic acid supplementation may promote adenoma recurrence. In the Aspirin/Folate-Polyp Prevention Study (AFPPS), participants randomized to receive 1,000 μg folic acid/d developed a higher occurrence of multiple and advanced lesions [156]. However, contrary to the AFPPS, randomized, double-blind, placebo-controlled clinical trials that evaluated the effects of supplemental folic acid in the NHS and Health Professionals Follow-up Study (HPFS), and the United Kingdom Colorectal Adenoma Prevention (UKCAP) study did not

find folic acid to increase adenoma recurrence [157, 158]. In fact, in the NHS and HPFS, folic acid supplementation was associated with a reduced risk of adenoma occurrence in participants with low baseline plasma folate (RR 0.61; 95% CI: 0.42 – 0.90) [158]. A recent study from the Cancer Prevention Study II investigated natural and synthetic folate and their associations with colorectal cancer incidence from 1999 through 2007. High intake of natural folate (≥ 316 μg/d)and folic acid (≥ 560 μg/d) was modestly associated with lower risk of colorectal cancer, although these results were not statistically significant [159]. High intake of total folate (≥1224 dietary folate equivalent/d)was significantly associated with a 19% lower risk of colorectal cancer [159]. The inverse association was limited to cancer risk after the first two years of measurement (2002-2007), suggesting that the antineoplastic effects of folate may be limited to inhibiting the initiation and not the progression of carcinogenesis [159].

Vitamin D and calcium

Ultraviolet B (UVB) radiation wavelengths (290 – 315 nm) that hit the skin are absorbed by 7-dehydrocholesterol to synthesize pre-vitamin D_3. Pre-vitamin D_3 undergoes rapid rearrangement to vitamin D_3 in a heat dependent reaction and diffuses from the epidermis into the circulation where it is bound to the vitamin D binding protein. Circulating vitamin D is transported to the liver where it is metabolized by the CYP27A1 enzyme to form 25-hydroxyvitamin D (25(OH)D), the primary circulating form of vitamin D. In the kidney the CYP27B1 enzyme synthesizes 25(OH)D to the active vitamin D metabolite $1,25(OH)_2D$. Most circulating $1,25(OH)_2D$ is synthesized in the kidney. Circulating $1,25(OH)_2D$ is responsible for the "classical" endocrine functions of vitamin D (i.e., related to calcium homeostasis). However, CYP27B1 is expressed throughout the body (i.e., the intestines, pancreas, breast, and immune cells) which synthesize $1,25(OH)_2D$, which acts in "non-classical" autocrine/paracrine functions.

The active 1,25$(OH)_2$D metabolite is short lived and is hydroxylated and inactivated by the CYP24A1 enzyme, which is also widely expressed throughout the body [160-162].

There is no consensus on the definition of vitamin D deficiency, but many experts suggest that deficiency be defined as serum 25(OH)D concentrations <20 ng/ml [163, 164]. According to such a definition of vitamin D deficiency it is estimated that approximately 50 million teenagers in the U.S. are vitamin D deficient or insufficient (serum 25(OH)D concentrations 20 – 32 ng/ml) [163, 165]. Anthropological evidence suggests that ancestral human evolution occurred in the context of abundant exposure to solar radiation [166-168]. Two traditionally living cultures in Tanzania with lifelong exposure to abundant tropical sunlight were estimated to have mean 25(OH)D concentration of 119 nmol/l (47.8 ng/ml) (range: 58 – 167 nmol/l (23.2 – 164.5 ng/ml)) and 109 nmol/l (43.7 ng/ml) (range: 71 – 171 nmol/l (28.4 – 68.5)) [168]. These vitamin D concentrations are comparable to the average 25(OH)D concentrations reported for Caucasian lifeguards working for at least 4 weeks at an open-air swimming pool in St. Louis (average 160.7 nmol/l; range: 132 – 197 nmol/l (average 64.4 ng/ml; range: 52.9 – 78.9 ng/ml)) [169].

In 1980 Garland and Garland proposed that vitamin D may be protective against colorectal cancer based on the negative association between solar radiance and colorectal cancer incidence [170]. Several prospective cohorts have investigated the association between vitamin D and colorectal neoplasms [171-186], with most suggesting an inverse association [171, 173-181, 183, 185, 186]. The highest quintile of circulating 25(OH)D (>83.4 nmol/l (33.4 ng/ml)) in the EPIC cohort was associated with a 40% lower risk of colorectal cancer [176]. The highest risk of colorectal cancer was found for individuals who were in both the lowest tertile of 25(OH)D concentrations and the lowest tertile of calcium intake (Rate Ratio 1.33; 95% CI: 1.16 – 1.55), suggesting a possible interaction between the two [176]. A meta-analysis of 5 nested

case-control studies [172, 173, 175, 183, 187] found 25(OH)D concentrations ≥33 ng/ml relative to. <12 ng/ml to be associated with a statistically significant 50% lower colorectal cancer risk; and recommended a vitamin D intake of 1000 IU/d [188]. A pooled case-control study reported an inverse association between seasonally adjusted serum 25(OH)D_3 and colorectal adenomas (highest vs lowest quartile OR = 0.33; 95% CI: 0.19 – 0.56) [189].

The association of calcium with colorectal neoplasms has been extensively investigated and has provided strong, consistent epidemiologic evidence supporting a protective role of calcium against colorectal neoplasms. Of at least 48 observational studies [171, 174, 176, 177, 179, 190-232] (24 case-control studies [190-211, 231, 232] and 24 prospective cohorts [171, 174, 176, 177, 179, 212-230]), 37 reported an inverse association between calcium intake and colorectal neoplasms [171, 174, 176, 177, 179, 192, 193, 195, 197-200, 202, 204, 206, 207, 209-213, 215-219, 221-224, 226-232]. In the EPIC cohort a high (≥1299.4 mg/d) relative to low (<667.2 mg/d) intake of dietary calcium was associated with a statistically significant 31% lower risk for colorectal cancer [176]. A pooled analysis of the NHS and HPFS reported higher total (dietary plus supplemental) calcium (>1250 mg/d vs. ≤500 mg/d) to be inversely associated with incident distal (descending and sigmoid) colon cancers (RR=0.65 95% CI: 0.43 – 0.98) [229]. The NHS and HPFS findings suggests a potential threshold effect, with most risk reduction being associated with calcium intakes between 700-800 mg/d [229].

The protective effects of calcium against colorectal neoplasms have been observed in randomized, placebo-controlled, clinical trials. The Calcium Polyp Prevention Study found 1,200 mg/d of supplemental elemental calcium to reduce adenoma recurrence among persons with at least one adenoma at baseline [233]. Following 4-years of follow-up the RR of adenoma recurrence was 0.85 (95% CI: 0.74 – 0.98) [233], and for advanced adenomas 0.65 (95% CI: 0.46 – 0.93) [234]. The treatment effects of supplemental calcium on adenoma recurrence was

modified by 25(OH)D concentrations. Among people with 25(OH)D concentrations at or below the overall baseline median (29.1 ng/ml), calcium had no effect on adenoma recurrence (RR = 1.05; 95% CI: 0.85 – 1.29), but among people with baseline 25(OH)D concentrations above the median calcium supplementation was associated with a significant reduction in adenoma recurrence (RR = 0.71; 95% CI: 0.57 – 0.89) [235]. There was also a suggestion that the treatment effects of calcium may be modified by dietary fat. In the lowest tertile of fat intake calcium supplementation markedly reduced adenoma recurrence (RR = 0.51; 95% CI = 0.25 – 1.06); however, the protective effect of calcium was attenuated in the highest tertile of fat intake (RR = 0.90; 95% CI 0.42 – 1.90) [234]. A meta-analysis of 3 clinical trials that tested the efficacy of supplemental calcium (1,200 mg, 1,600 mg, and 2,000 mg) against adenoma recurrence reported a summary RR of 0.80 (95% CI: 0.68 – 0.93) [236].

The Women's Health initiative (WHI), a randomized, double-blind, placebo-controlled clinical trial that evaluated the effects of 1,000 mg of calcium plus 400 IU vitamin D versus placebo over an average of 7 years among postmenopausal women reported no protective effect of calcium and vitamin D against colorectal cancer [187]. However, these results need to be interpreted with caution because of the low adherence in the active treat group (60% to 63% took 80% or more of their pills during the first 3 years of follow-up), women in the placebo group were allowed to use nonprotocol prescribed supplemental calcium and vitamin D, and the low dose of study vitamin D. In a re-analysis of the WHI, among women who did not report personal use of supplemental calcium or vitamin D at randomization, there was a modest 17% reduction in colorectal cancer incidence in the active treatment group; however, this was not a statistically significant finding [237].

The proposed anti-carcinogenic effects of calcium may be attributed to the binding of toxic secondary bile acids to form insoluble soaps [238-240] and to directly reducing

proliferation and promoting differentiation and apoptosis in the colorectal mucosa [241-245]. In a low calcium environment colonic cell lines undergo increased cell proliferation [241, 245]. A randomized, double-blinded, placebo-controlled clinical trial reported that calcium supplementation reduced the proportion of proliferative cells in the upper 40% of colorectal crypts [246]. Calcium's effects on the cell cycle may, in part, operate through modulating the Wnt/β-catenin signaling pathway [247-249]. Colonic cell lines exposed to high calcium concentrations increased E-cadherin expression and translocated β-catenin from the nucleus to the membrane, and these effects could be prevented by inhibiting the expression of the calcium receptor (CaR) [247-249]. A mouse model with the *CaR* gene specifically knocked-out of the intestine had increased colonic proliferation and expanded the zone of proliferation in colonic crypts, and enhanced nuclear localization of β-catenin [242]. The CaR is strongly expressed in normal appearing mucosa and in differentiated neoplasms; however, sporadic to no expression is reported in undifferentiated carcinomas [241, 247, 249-251].

Vitamin D may reduce risk of colorectal cancer through various mechanisms, including reducing cell proliferation, and promoting cell differentiation and apoptosis. *In vitro*, $1,25(OH)_2D$ promotes E-cadherin expression [248, 252] and β-catenin translocation to the membrane [252]. Binding of $1,25(OH)_2D$ to the VDR promotes the VDR to competitively bind β-catenin away from the co-transcription factor TCF-4 [252, 253]. Wild type APC may promote the interaction of the $1,25(OH)_2D$/VDR/β-catenin complex, but not truncated APC [253]. Both calcium and $1,25(OH)_2D$ promote P21 (a cyclin-dependent kinase inhibitor) expression [248, 254, 255]; however, knocking-out CaR expression prevents these effects [254]. Treatment of colon neoplasm cell lines with $1,25(OH)_2D$ inhibited the expression of the anti-apoptotic marker Bcl-2 and promoted expression of the pro-apoptotic marker Bak [256]. In a mouse model the neoplastic effects of a "Western-style" (WD) diet were attenuated by supplementing the WD

with calcium and vitamin D [257]. Compared to *APC*$^{min/+}$*VDR*$^{+/+}$ mice, *APC*$^{min/+}$*VDR*$^{-/-}$ mice had increased nuclear β-catenin expression, larger tumors, and lower E-cadherin expression [258]. A randomized, placebo-controlled, clinical trial reported supplemental calcium and vitamin D_3, alone and in combination, increased the expression of the pro-apoptotic marker Bax, and MSH2 in normal colorectal mucosa [259, 260].

The VDR, independent of 1,25$(OH)_2$D, may also protect against the cytotoxic effects of bile acids. Lithocholic acid can bind to the VDR, at a lower affinity than 1,25$(OH)_2$D, promoting transcription of CYP3A, a detoxifying enzyme that catabolizes bile acids. However, the binding of LCA to the VDR may also promote CYP24A1 expression, which may attenuate the anti-neoplastic effects of 1,25$(OH)_2$D [253, 261]. Unlike the 1,25$(OH)_2$D/VDR complex, the affinity of the LCA/VDR complex for β-catenin is low [253]. Thus, a high fat diet combined with low 25(OH)D concentrations that results in a high LCA/25(OH)D ratio, may favor colorectal proliferation and increase the risk of colorectal neoplasms.

The association between *VDR* gene polymorphisms and colorectal neoplasms is unclear. Some of the more common single nucleotide polymorphisms (SNPs) to be investigated in relation to colorectal neoplasms include *BsmI* (rs1544410, G>A), *ApaI* (rs7975232, C>A), and *FokI* (rs1073581, T/C) [162, 262]. The *ApaI* and *BsmI* polymorphisms are silent SNPs, as they do not change the coding sequence of VDR. However, they are in strong linkage disequilibrium with the *Poly(A)* microsatellite repeat polymorphism which may affect gene expression through regulation of mRNA stability [162, 263]. Located at the first potential start site of *VDR* is the *FokI* polymorphism. This polymorphism can modify the *VDR* start codon resulting in a larger, less transcriptionally active VDR protein [264, 265]. In cell lines the shorter *FokI* variant is associated with greater ability to suppress β-catenin mediated transcription [253]. Of seven reviewed studies [266-272], five [266, 267, 270-272] reported inverse association between the more

transcriptionally active *FokI* polymorphisms and colorectal cancer. A meta-analysis of 7 studies investigating the *BsmI* polymorphism and colorectal cancer reported a summary OR of 0.91 (95% CI: 0.84 – 1.00) and 0.92 (0.81 – 1.04) for the *Bb* and *BB* genotype, respectively [273]. However, a meta-analysis of 5 studies that investigated the association between *FokI* and *BsmI* polymorphisms with colorectal adenoma reported no associations for either polymorphisms [274]. In the UKCAP study the VDR polymorphisms *FokI*, *BsmI*, *ApaI* and *TaqI* were not directly associated with adenoma recurrence, but a reduction in adenoma recurrence that was associated with high intake of dairy products was limited to individuals with *ApaI aA/AA* genotype ($p_{interation}$=0.02) [275].

The expression of the VDR and CYP27B1 in colorectal mucosa increases in the early stages of the pathogenesis of colorectal cancer, but markedly decreases in undifferentiated cancers; this suggests that the VDR and CYP27B1 act as tumor suppressors, presumably by maximizing 1,25$(OH)_2$D signaling to normalize cell-cycle regulation [276-278]. CYP24A1 expression was reported to be increased in undifferentiated cancers, presumably mitigating the effects of 1,25$(OH)_2$D signaling [278, 279].

Gaps in the Literature Addressed by This Dissertation

In this dissertation I address two related needs in the literature to advance efforts to understand the etiology of colorectal cancer and how to prevent it: 1) there are no validated pre-neoplastic biomarkers of risk for colorectal neoplasms, and 2) clarification of the antineoplastic roles of calcium and vitamin D against colorectal neoplasms. Currently, the colorectal adenoma is the only validated biomarker of risk for colorectal cancer. The development of treatable, pre-neoplastic biomarkers of risk for colorectal neoplasms could be used to enhance clinical risk identification, stratification, and monitoring and to facilitate the

development of chemopreventive agents against the disease. There is considerable evidence suggesting that the Wnt/β-catenin and calcium/vitamin D metabolism pathways malfunction early in the stages of colorectal carcinogenesis, and that these pathways may be modified by diet and lifestyle behaviors, including calcium and vitamin D, making these pathways ideal candidates for investigating and developing treatable, pre-neoplastic biomarkers of risk. In this dissertation I report the results of investigations to evaluate potential of markers of the Wnt/β-catenin signaling pathway (APC, β-catenin, and E-cadherin) and markers of calcium and vitamin D metabolism (the CaR, the VDR, CYP27B1, and CYP24A1) in the normal colorectal mucosa as potential treatable, pre-neoplastic biomarkers of risk for colorectal neoplasms.

Specific Aims

To address the lack of knowledge on treatable, pre-neoplastic biomarkers of risk for colorectal neoplasms, as reported herein, in this dissertation I report findings on the investigation and development of APC, β-catenin, E-cadherin, the CaR, the VDR, CYP27B1, and CYP24A1 as potential treatable pre-neoplastic biomarkers of risk for colorectal neoplasms.

This dissertation contributes toward the development of treatable, pre-neoplastic biomarkers of risk for colorectal neoplasms with the following specific aims:

1. From the Markers of Adenomatous Polyps II (MAP II) colonoscopy-based case-control study investigate:
    a) differences between APC, β-catenin, and E-cadherin expression in patients with incident, sporadic colorectal adenomas and adenoma free controls;
    b) differences between APC, β-catenin, and E-cadherin expression in functionally distinct crypt zones (the upper 40% of the crypts (differentiation zone), the lower 60% of the crypts (proliferation zone), and the distribution index (Φh, defined as the ratio of the upper 40% of the crypts to the whole crypt)) in patients with incident, sporadic colorectal adenomas and adenoma free controls; and
    c) associations of APC, β-catenin, and E-cadherin expression with biologically plausible potential modulators.
2. From a randomized, placebo controlled, 2x2 factorial clinical trial (CaDvMAP) investigate the effects of supplemental calcium and vitamin D_3 alone and in combination over 6 months on APC, β-catenin, and E-cadherin expression and the APC/β-catenin score (developed in the MAPII study in a secondary analysis) in the normal colorectal mucosa.
3. From the randomized, placebo-controlled, 2x2 factorial clinical trial (CaDvMAP) investigate the effects of supplemental calcium and vitamin D_3 alone and in combination over 6 months on CaR, VDR, CYP27B1, and CYP24A1 expression in the normal colorectal mucosa.

Hypotheses

1. In the MAP II study greater APC and E-cadherin expression in the normal colorectal mucosa is associated with lower risk for sporadic colorectal adenoma, and greater β-catenin expression is associated with higher risk for sporadic colorectal adenoma. Protective risk factors for colorectal neoplasms are positively associated with APC and E-cadherin expression and negatively associated with β-catenin expression.
2. In the CaDvMAP study, supplemental calcium and vitamin D_3, alone and in combination, will increase APC and E-cadherin expression and the APC/β-catenin score, and decrease β-catenin expression in the normal colorectal mucosa.
3. In the CaDvMAP study, supplemental calcium and vitamin D_3, alone and in combination, will modify the expression of CaR, VDR, CYP27B1, and CYP24A1 expression in the normal colorectal mucosa.

Chapter 2. Markers of the APC/β-catenin signaling pathway as potential treatable, pre-neoplastic biomarkers of risk for colorectal neoplasms

Thomas U. Ahearn[1, 4], Aasma Shaukat[5], W. Dana Flanders[2,3,4], March E. Seabrook[6] and Roberd M. Bostick[2,4]

Authors Affiliations: [1]Nutrition and Health Sciences Program, Graduate Division of Biological and Biomedical Sciences, Departments of [2]Epidemiology and [3]Biostatistics and Bioinformatics, Rollins School of Public Health, and [4]Winship Cancer Institute, Emory University, Atlanta, GA; [5]Department of Medicine, GI Division, University of Minnesota, Minneapolis, MN; and [6]Consultants in Gastroenterology, PA, Columbia, SC.

Grant support: The Fullerton Foundation; Georgia Cancer Coalition Distinguished Scholar award (to R.M.B.); the Franklin Foundation. The Fullerton Foundation, the Georgia Cancer Coalition, and the Franklin Foundation had no influence on the design of the study; the collection, analysis, and interpretation of the data; the decision to submit the manuscript for publication; or the writing of the manuscript.

Corresponding Author:
Roberd M. Bostick, MD, MPH
Department of Epidemiology
Rollins School of Public Health
Emory University
1518 Clifton Road NE
Atlanta, GA 30322
Phone: 404-727-2671
Fax: 404-727-8737
E-mail: rmbosti@sph.emory.edu

Running title: APC and β-catenin as biomarkers of risk for colorectal neoplasms
Key words: colonic neoplasms, biomarkers, case-control study, diet, lifestyle
Word Count: 3,951
Number of figures: 2
Number of tables: 3

Abstract

Malfunctioning of the APC/β-catenin signaling pathway is both an early and common event in sporadic colorectal cancer. To assess the potential of APC/β-catenin signaling pathway markers as treatable, pre-neoplastic biomarkers of risk for colorectal neoplasms, we conducted a pilot colonoscopy-based case-control study (51 cases, 154 controls) of incident, sporadic colorectal adenoma. We evaluated APC, β-catenin, and E-cadherin expression in normal mucosa from the rectum and ascending and sigmoid colon using automated immunohistochemistry and quantitative image analysis. Diet, lifestyle, and medical history were assessed with validated questionnaires. In the normal rectal mucosa, the ratio of the proportion of APC expression in the upper 40% of crypts with total β-catenin expression (APC/β-catenin score) was 14.3% greater in controls than in cases (p=0.02) (odds ratio [OR] 0.40, 95% confidence interval [CI] 0.14 – 1.14). Compared to controls, in cases, APC expression was 3.2% lower, β-catenin expression was 3.0% higher, and E-cadherin expression was 0.7% lower; however, none of these differences was statistically significant. The APC/β-catenin score statistically significantly differed according to categories of plausible risk factors for colorectal cancer (e.g., it was 17.7% higher among those with 25[OH]-vitamin D_3 concentrations ≥ 27 ng/ml and 14.8% lower among those with high total fat consumption). These preliminary data suggest that the combined expression of APC and β-catenin in the normal rectal mucosa may be associated with risk for incident, sporadic colorectal neoplasms, as well as with modifiable risk factors for colorectal neoplasms.

Introduction

Despite advances in screening and treatment, colorectal cancer (CRC) remains the second leading cause of cancer deaths in the United States[280]. The etiology of sporadic CRC is predominately rooted in dietary and lifestyle behaviors [99, 100], suggesting that it may be preventable. The molecular basis of colorectal carcinogenesis is becoming clearer[99], but has yet to be exploited to yield validated, treatable, pre-neoplastic biomarkers of risk for colorectal neoplasms.

APC, β-catenin, and E-cadherin are appealing, potentially treatable, pre-neoplastic biomarkers of risk for colorectal adenomas. Impaired APC function/expression, which is involved in approximately 80-90% of sporadic CRCs [281], results in increased cytoplasmic β-catenin which can translocate to the nucleus and bind with TCF transcription factors and activate target genes responsible for cell proliferation and differentiation [281]. Also, β-catenin is a multi-functioning signaling protein, which, along with α-catenin, binds to the cytoplasmic tail of the calcium-dependent cell adhesion protein E-cadherin, linking E-cadherin to actin filaments and promoting cell adhesion and differentiation. E-cadherin may also antagonize APC/β-catenin signaling by sequestering β-catenin at cell adhesion junctions [282].

In normal colorectal mucosa APC, β-catenin, and E-cadherin are all strongly expressed—APC primarily in the cytoplasm, and E-cadherin and β-catenin primarily at the cell membrane. During the adenoma-carcinoma sequence APC and E-cadherin expression markedly decrease (although the increase in E-cadherin tends to occur in later stages) [47, 66, 67], and β-catenin expression steadily increases and translocates from the membrane to the cytoplasm and eventually into the nucleus [47].

We know of no other human studies that have investigated APC, β-catenin, and E-cadherin expression in normal colorectal mucosa as potentially treatable, pre-neoplastic

biomarkers of risk for sporadic colorectal neoplasms. We hypothesized that increased APC and E-cadherin expression in the normal colorectal mucosa would be associated with lower, and increased expression of β-catenin would be associated with higher, risk for adenoma. To address this, as reported herein, we conducted a colonoscopy-based case-control study of sporadic colorectal adenoma in which we evaluated normal colorectal mucosa expression of APC, β-catenin, and E-cadherin as potentially modifiable biomarkers of risk for colorectal adenoma.

Materials and Methods

Participant population and recruitment. As previously reported [283-285], the Markers of Adenomatous Polyps II (MAP II) study is a colonoscopy-based case-control study to investigate potentially treatable biomarkers of risk for incident, sporadic colorectal adenomas. Study participants were recruited through the usual scheduling of outpatient elective colonoscopies at Consultants in Gastroenterology, PA, a large private practice gastroenterology group in Columbia, SC. Briefly, eligibility requirements consisted of being 30-74 years of age, English speaking, and a resident of the Columbia, SC area. Exclusion from participation included contraindications to colonoscopic biopsies, previous adenomatous polyps, familial adenomatous polyposis, inflammatory bowel disease, incident colorectal cancer, or a history of cancer other than non-melanoma skin cancer. Cases were defined as being diagnosed with a first ever adenomatous polyp at their elective outpatient colonoscopy. Controls were persons found to have no colonic neoplasms at the elective outpatient colonoscopy. Hyperplastic polyps were not considered in the criteria for study inclusion or exclusion or in the assignment of case-control status. Over five months 351 patients were identified; of these, 305 (86.6%) were eligible to participate following initial screening, of whom 232 (76%) were successfully contacted

and provided informed consent; of these, 203 (87.5%) met final eligibility criteria; of these, 49 (56.3%) were diagnosed with incident adenomas, yielding a final sample size of 49 cases and 154 controls. Five participants were later excluded from dietary analyses because of implausibly low (females <500 kcal/d, males <800 kcal/d) or high (females >3500 kcal/d, males >4000 kcal/d) self-reported total energy intake. Due to limited funding, APC expression was evaluated only in 87 participants (42 cases, 45 controls), β-catenin in 77 participants (36 cases, 41 controls), and E-cadherin in 86 participants (40 cases, 46 controls).

***Data collection*.** Prior to colonoscopy, patients completed mailed questionnaires regarding dietary, lifestyle, and other potential risk factors for CRC. Physical activity was assessed using a modified Paffenbarger questionnaire [286]. Diet and nutritional supplement intakes were assessed using a Willett Food Frequency Questionnaire [287, 288].

Participants were asked to abstain from aspirin use seven days prior to colonoscopy. Colonoscopy of all participants was done in the usual manner following a 12-h fast and polyethylene glycol bowel cleansing preparation. Six approximately one millimeter thick "pinch" biopsy specimens were taken from the normal-appearing rectal mucosa 10 cm above the anus. On 20% of participants, biopsies from the mid-sigmoid and proximal ascending colon were also collected. No biopsies were taken within 4.0 cm of a polypoid lesion. Biopsies were placed onto a strip of bibulous paper and immediately placed in phosphate buffered saline (PBS), oriented, transferred to 10% normal buffered formalin for 24 hours, and then transferred to 70% ethanol. Then, within a week, the biopsies were processed and embedded in paraffin blocks (2 blocks of 3 biopsies per colon site per participant). For any polyp removed at colonoscopy, colon site, *in vivo* size, and shape were recorded and histologic information was further reviewed by the study index pathologist using standard criteria [289].

Immunohistochemistry protocol. For each person, for each colon site, five slides with 4 levels of 3 micron-thick biopsy sections taken 40 microns apart were prepared for each antigen, yielding a total of 20 levels for each antigen. Antigen retrieval was performed by placing the slides in a preheated Pretreatment Module (Lab Vision Corp.) with 100x citrate buffer (pH 6.0; DAKO S1699, DAKO Corp.) and steaming them for 40 min. Following antigen retrieval, slides were immunohistochemically processed in a DAKO Automated Immunostainer (DAKO Corp.) using a labeled streptavidin-biotin method for APC (Calbiochem, OP80; 1:70 dilution), β-catenin (Transduction Laboratories 610154; 1:300 dilution), and E-cadherin (Zymed 33-4000; 1:50 dilution). No slides were counterstained. After processing, slides were coverslipped with a Leica CV5000 Coverslipper (Leica Microsystems, Inc.). Each staining batch contained positive and negative slides, which were treated identically to the patients' slides except that antibody diluent was used rather than primary antibody on the negative slides.

Protocol for quantifying labeling densities of immunohistochemically detected biomarkers in normal colon crypts ("scoring"). A detailed description of the protocol used to quantify biomarker labeling optical densities ("biomarker expression") in normal colon crypts was previously described [259]. Briefly, a "scorable" crypt was defined as an intact crypt extending from the muscularis mucosa to the colon lumen [290]. Prior to "scoring", the negative and positive control slides were checked for staining adequacy. The major equipment and software for the image analysis procedures included: personal computer, light microscope (Olympus BX40, Olympus Corporation, Japan) with appropriate filters and attached digital light microscope camera (Polaroid DMC Digital Light Microscope Camera, Polaroid Corporation, USA), 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 relational database software (Microsoft Corporation, WA).

Evaluation of biomarker expression consisted of the same technician cleaning all slides, selecting the two of the three biopsies with the most scorable crypts per biopsy, creating background correction images for each slide scored, capturing 16-bit grayscale images of crypts at 200x magnification, and tracing the border of the "hemicrypt" (one half of the crypt) (**Figure 2.1**). The program then divided the outlined hemicrypt into equally spaced segments that corresponded to the average width of colonocytes, and measured the optical density of the labeling across the entire hemicrypt and within each segment, adjusting for the background. The technician then repeated this process for the adjacent hemicrypt, and proceeded to the next crypt, level, biopsy, and/or slide. The goal was to score 16-20 hemicrypts on two of three biopsies per biomarker per colon site.

Reliability control was performed by selecting samples of previously analyzed slides to be re-analyzed by the technician. The technician was blinded to the selection. Intra-reader reliability for APC, E-cadherin, and β-catenin was >0.90 throughout.

Protocol for measuring serum 25(OH)D_3. Serum 25(OH)-vitamin D_3 (25(OH)D_3) was measured using liquid chromatography/tandem mass spectrometry, as previously described [291].

***Statistical analysis*.** The characteristics of the cases and controls were compared using the *t* test for continuous variables and Fisher exact test for categorical variables.

The mean labeling optical density ("expression") of each biomarker on each study participant was calculated by summing the biomarker's expression for all analyzed crypts and dividing by the total number of analyzed crypts. Biomarker expression was standardized to adjust for possible staining batch effects by dividing an individual's mean biomarker expression by the mean biomarker expression of the staining batch in which the individual's sample was included. As a sensitivity analysis of our batch standardization, we used a robust method that

conditioned on batch and dichotomized on the batch specific median in controls [292]. Measures of biomarker expression in functional distinct crypt zones selected *a priori* were the upper 40% of the crypts (differentiation zone), the lower 60% of the crypts (proliferation zone), and the distribution index (Φh, defined as the ratio of the upper 40% of the crypts to the whole crypt).

The distributions of batch-standardized APC, E-cadherin, and β-catenin labeling optical density along the full length of the crypts were graphically plotted and evaluated using the LOESS procedure. First, each hemicrypt was standardized to 50 sections. Then, the average of each section across all crypts was predicted by the LOESS model separately for cases and controls by colon site. The results were graphically plotted along with the smoothing line.

Potential confounders were evaluated based on the biological plausibility of their being associated with the biomarker of interest and with colorectal adenomas. All nutrients were energy-adjusted using the residual regression method [293]. Continuous variables were dichotomized based on their sex-specific median in the controls.

The ratio of Φh APC to β-catenin expression (Φh APC expression/β-catenin expression) was calculated to investigate the association of combined Φh APC and β-catenin expression (APC/β-catenin score) with adenoma status, adenoma characteristics, and risk factors for colorectal neoplasms. We hypothesized that the APC/β-catenin score would be inversely associated with having an adenoma. E-cadherin was not included in the APC/β-catenin score because during carcinogenesis malfunctioning regulation of β-catenin by APC occurs earlier than E-cadherin down-regulation[294]. As a sensitivity analysis of the APC/β-catenin score we also calculated normalized z-scores for Φh APC and β-catenin expression ($z = [x – \mu]/\sigma$, where x is the biomarker value, μ is the mean biomarker expression in controls, and σ is the standard deviation

of the biomarker expression in controls) and then the β-catenin z-score was subtracted from the Φh APC z-scores (APC-β-cat z-score).

Logistic regression was used to calculate odds ratios (OR) with 95% confidence intervals (95% CI) to evaluate associations of batch-standardized expressions of APC, β-catenin, and E-cadherin, and of the APC/β-catenin score (hereafter referred to as "the biomarkers") with adenoma; for these analyses the biomarkers were dichotomized based on the mean values in the controls. For analyses of differences in mean expression of the biomarkers according to risk factors for colon cancer, given the small sample size and similarity of results across cases and controls, cases and controls were combined in analysis of covariance (ANCOVA) models with inverse probability weighting according to the relative proportions of cases and controls measured for a given biomarker. However, since there were differences in the biomarkers by nonsteroidal anti-inflammatory drug (NSAID) use according to case/control status, for this analysis cases and controls were analyzed separately. The results of the associations between E-cadherin and risk factors for CRC are not presented since no apparent difference in E-cadherin expression between adenoma cases and controls was found. All models were adjusted for age and sex. Due to limited sample sizes for the ascending and sigmoid colon, only the results for the rectal mucosa are presented in the tables.

Results

***Study population*.** Selected characteristics of the study population are presented in Table 2.1. Relative to controls, cases, on average, tended to be older and to have lower serum $25(OH)D_3$ concentrations, greater total energy, fat, and processed meat intakes, and lower total folate and calcium intakes. Cases also tended to be less likely to have a history of colorectal cancer in a first-degree relative and to regularly take a NSAID; however, only total energy

consumption statistically significantly differed between cases and controls. When restricted to cases and controls available for specific biomarkers, the case-control differences did not appreciably change (data not shown).

***Case-control differences in biomarker expression*.** The APC/β-catenin score in rectal crypts of controls was 14.3% greater (p=0.02) than in cases, and having a higher APC/β-catenin score in rectal crypts was inversely associated with adenomas (OR 0.40, 95% CI 0.14 – 1.14) (**Table 2.2**). This association did not appreciably change with multivariate adjustment.

APC expression in cases and controls along the full lengths of colorectal crypts was lowest at the crypt bases, and, beginning at approximately the upper 40th percentile of crypts, expression sharply increased towards the crypt apex (**Figure 2.2A**). Mean total APC expression in rectal crypts did not appreciably differ between cases and controls (data not shown); however, Φh APC was 3.2% lower in cases than in controls and modestly inversely associated with adenomas (OR 0.75), but these findings were not statistically significant and did not appreciably change with multivariate adjustment (**Table 2.2**). The findings for APC expression in the ascending and sigmoid colon were similar to those for the rectum (data not shown).

β-catenin expression in cases and controls was relatively uniform along the full lengths of rectal crypts (**Figure 2.2B**). Mean total β-catenin expression in rectal crypts was 3.0% higher in cases than in controls and modestly positively associated with being a case (OR 1.36), but these findings were not statistically significant and did not appreciably change with multivariate adjustment (**Table2.2**). Similar case-control differences were found in specific functional zones of crypts (data not shown).

E-cadherin expression in cases and controls, similar to β-catenin expression, was relatively uniform along the full lengths of rectal crypts (**Figure 2.2C**). Mean total E-cadherin expression in rectal crypts was 0.7% greater in controls than in cases and modestly positively

associated with adenomas (OR 1.18), but these findings were not statistically significant and did not appreciably change with multivariate adjustment (**Table 2.2**). The findings for E-cadherin expression in specific functional zones of crypts and for other colon sites were similar to the findings for total E-cadherin expression in the rectum (data not shown).

Associations of biomarkers with potential risk factors. The APC/β-catenin score tended to be lower among men (11.1%), participants with a positive family history of CRC in a first degree relative (10.7%), higher levels of physical activity (14.2%), higher WHR (11.0%), and higher intakes of total fat (14.8%) and processed meats (14.3%); and higher among participants with serum $25(OH)D_3$ concentrations >27 ng/ml (17.7%) and greater dietary fiber (13.3%) and total folate intakes (10.5%). However, only the findings for physical activity, $25(OH)D_3$ concentrations, total fat intake, and processed meat intake were statistically significant. In cases regular NSAID use was associated with a higher APC/β-catenin score (11.8%), but in controls regular NSAID use was statistically significantly associated with a lower APC/β-catenin score (20.2%; **Table 2.3**).

Φh APC expression tended to be lower among participants with a positive family history of CRC in a first degree relative (12.8%), higher levels of physical activity (9.8%), and higher intakes of total fat (9.4%) and processed meats (7.3%); and higher among participants with greater dietary fiber (15.6%) and total calcium (11.3%) and folate intakes (12.4%). However, only the findings for physical activity and intakes of total fat, calcium, fiber, and folate were statistically significant. In controls regular NSAID use was statistically significantly associated with lower Φh APC expression (11.0%) (**Table 2.3**).

β-catenin expression tended to be higher among men (5.2%), participants ≥55 years of age (6.9%), former or current smokers (5.7%) or alcohol consumers (15.7%), and those with higher levels of physical activity (5.9%), higher WHR (8.1%), and greater processed meat intakes

(8.3%); and lower among participants with higher total energy intakes (9.0%). However, only the findings for alcohol exposure, WHR, and total energy and processed meat intakes were statistically significant. In controls regular NSAID use was statistically significantly associated with higher β-catenin expression (10.6%) (**Table 2.3**).

***Sensitivity analyses*.** Alternative methods [292] noted above for accounting for staining batch yielded nearly identical associations between the biomarkers and adenoma. Also, the APC-β-catenin z-score produced results (OR 0.35, 95% CI [0.12 – 0.99]) similar to those using the APC/β-catenin score. There were no apparent differences in findings using weighted and un-weighted ANACOVA analyses.

***Adenoma characteristics*.** We found no apparent differences of expression of APC, β-catenin, or E-cadherin or the APC/β-catenin score among cases according to adenoma subtype, shape, location, or multiplicity (data not shown).

Discussion

To the best our knowledge, this is the first report to quantify and characterize the distribution of APC, β-catenin, and E-cadherin expression in the normal colorectal mucosa in incident, sporadic, colorectal adenoma cases and healthy controls. Our results suggest that persons with higher APC combined with lower β-catenin expression in the normal colorectal mucosa may be at lower risk for incident sporadic colorectal neoplasms. Also, our results suggest that these biomarkers may be associated with dietary and lifestyle risk factors for colorectal neoplasms, suggesting that these biomarkers may be modifiable. These findings, which support previous findings suggesting that molecular phenotypic differences in the normal-appearing colorectal mucosa may be associated with increased risk of colorectal neoplasms

[283-285] and are modifiable [259, 260, 295-297], are relevant because there are currently no validated treatable, pre-neoplastic biomarkers of risk for colorectal neoplasms.

In the present study having a higher proportion of APC expression in the upper 40% (differentiation zone) of colorectal crypts (Φh APC) was consistently, but not statistically significantly, greater in controls than in cases at all colon sites. These results are consistent with reports that in CRC cases [298] and those at increased risk for the disease [246, 299] the colorectal crypt proliferation zone (the lower 60%) expands upwardly into the upper 40% of crypts. That E-cadherin expression did not appreciably differ between cases and controls, and cases tended to have lower Φh APC expression, may suggest that the excess β-catenin expression found in cases could induce a greater proliferative potential. This idea is further supported by the significant inverse association of the APC/β-catenin score with adenoma. We suggest that the APC/β-catenin score may reflect the potential of β-catenin to activate proliferative genes in colorectal crypts.

A family history of CRC and increased age are well established risk factor for colorectal neoplasms[99]. Consistent with this, we found a lower APC/β-catenin score and Φh APC in people with a history of CRC in a first degree relative, and increased β-catenin expression in older participants. Consistent with the slightly higher risk of CRC in men than women [83], we found men to have a lower APC/ β-catenin ratio.

The etiology of CRC is heavily influenced by modifiable dietary and lifestyle behaviors[99, 100]; however, we are unaware of any *in vivo* investigations of associations of dietary and lifestyle behaviors with APC and β-catenin expression in the normal colorectal mucosa. Increased physical activity and NSAID use are consistently reported to reduce risk of colorectal neoplasms [83]. Unexpectedly, we found a statistically significant lower APC/β-catenin scores among people with higher physical activity, and the association between regular

NSAID use and biomarker expression differed according to adenoma status. Among sporadic adenoma cases regular NSAID use was associated with a higher APC/β-catenin score, but among controls regular NSAID use was associated with a lower APC/β-catenin score ($p_{interaction}$=0.03). We are unaware of a biologically plausible explanation for these findings, which may have been due to chance. Considering the concomitant higher E-cadherin expression (data not shown), it is plausible that the higher β-catenin expression observed with higher physical activity and NSAID use could represent β-catenin localized to the cell membrane and the cytoplasmic tail of E-cadherin and not higher levels of cytoplasmic or nuclear β-catenin. Elevated body fatness is a well-established risk factor for colorectal neoplasms, and consistent with these observations we found a lower APC/β-catenin score among people with a larger WHR and BMI. In line with reports that smoking and alcohol consumption are associated with increased proliferation [300, 301], we found that people who formerly or currently consumed alcohol or smoked had higher β-catenin expression. Higher intakes of total fat and processed meats, both of which are hypothesized to increase risk for colorectal neoplasms [100, 302], were associated with lower APC/β-catenin scores. We found a positive association between serum 25(OH)D_3 and the APC/β-catenin score, which is consistent with growing evidence suggesting that higher circulating 25(OH)D_3 reduces risk for colorectal neoplasm [189, 303]. Higher folate and fiber intakes were associated with a higher APC/β-catenin score. In mice a diet deficient in folate and other B vitamins reportedly decreased APC expression and increased β-catenin expression [304, 305]; and at least one animal study reported that pectin reduced β-catenin expression [244].

The escalating increase of APC expression in colorectal crypts beginning at approximately the junction between the proliferation and differentiation zone (**Figure 2.2A**) supports the results from previous studies that documented an important role for APC in inhibiting proliferation and promoting differentiation [281]. E-cadherin adhesion depends on

the availability of extracellular calcium. A calcium gradient from low to high is hypothesized starting at the crypt base and extending to its luminal surface [306]. The fairly uniform distribution of E-cadherin in crypts suggests that cell adhesion within colorectal crypts may depend more on the increasing extracellular calcium concentration as cells migrate towards the crypt apex, as opposed to increasing E-cadherin expression. The similar distribution patterns of E-cadherin and β-catenin may imply that most β-catenin in normal colorectal crypts is bound to the cytoplasmic tail of E-cadherin.

The primary limitation of this pilot study was the small sample size. Because of limited resources we could evaluate rectal biopsies on only a subset of study participants, and ascending and sigmoid colon biopsies on an even smaller subset. However, despite our limited sample size we found a statistically significant difference in the APC/β-catenin score between cases and controls, and statistically significant associations of the expression of APC and β-catenin and the APC/β-catenin score with plausible risk factors for colorectal neoplasms. Despite much of our immunhistochemistry procedures being automated, batch variability inevitably is a source of measurement error; however, our method of standardizing for batch variability is an efficient method of addressing it.[292] We did not specifically evaluate nuclear or cytoplasmic β-catenin because in the normal colorectal mucosa little or no nuclear and cytoplasmic β-catenin expression would be expected; however, it is plausible that total β-catenin expression is positively correlated with nuclear β-catenin expression.

The strengths of this study include: 1) that it is, to our knowledge, the first evaluation of components of the APC/β-catenin signaling pathway in the normal colorectal mucosa as potentially treatable, pre-neoplastic biomarkers of risk for incident, sporadic colorectal adenoma; 2) the automated immunostaining and novel image analysis software to quantify the crypt distribution of the expression of APC, β-catenin, and E-cadherin; 3) the assignment of

case/control status based on colonoscopy, minimizing the chances of misclassification; and 4) the assessment of dietary and lifestyle behaviors prior to case/control assignment to reduce recall bias.

In summary, the results of this pilot study suggest that lower APC expression, especially the proportion of APC in the upper 40% of crypts, combined with higher β-catenin expression in the normal colorectal mucosa may be associated with increased risk for incident, sporadic colorectal adenoma. Our results do not support E-cadherin as a pre-neoplastic biomarker of risk for colorectal adenoma. Also, we found that the APC/β-catenin score and APC and β-catenin expression may be associated with modifiable plausible dietary and lifestyle risk factors for colorectal neoplasms, suggesting that the biomarkers may potentially be modifiable. These results, taken together with our previous reports [259, 260, 283-285, 295-297], provide support for conducting further, larger investigations to evaluate APC and β-catenin as potentially treatable, pre-neoplastic biomarkers of risk for colorectal neoplasms.

Table 2.1. Selected characteristics of incident, sporadic colorectal adenoma cases and healthy controls; Markers of Adenomatous Polyps study II

Characteristic[a]	Controls (n=51)	Cases (n=47)	p-value[b]
Demographics, medical history, habits, anthropometrics			
Age, years	55.5 (8.3)	56.1 (7.2)	0.68
Men (%)	47	53	0.69
White (%)	98	96	0.61
Physical activity, METs/d	26.6 (20.5)	29.6 (23.2)	0.50
History of colorectal cancer in 1° relative (%)	16	13	0.78
Take NSAID[c] regularly (%)	40	34	0.67
Current smoker (%)	8	17	0.38
Consume alcohol currently (%)	60	64	0.91
Body mass index (BMI), kg/m^2	30.8 (7.0)	30.9 (7.3)	0.93
Waist-to-hip ratio (WHR)	0.9 (0.14)	0.9 (0.09)	0.69
Serum $25(OH)D_3$, ng/mL	28.3 (12)	26.9 (13)	0.62
Dietary intakes[d]			
Total energy, kcal/d	1545 (443)	1931 (759)	0.003
Total fat, g/d	65.9 (14)	66.8 (16)	0.77
Dietary fiber, g/d	15.1 (6)	15.0 (5)	0.94
Total[e] folate, g/d	528 (272)	479 (229)	0.34
Total[e] calcium, g/d	951 (487)	907 (483)	0.66
Processed meat intake, servings/wk	2.3 (2)	2.7 (3)	0.33

[a] Continuous variables presented as mean ± SD and categorical variables as percent
[b] Based on *t* test for continuous variables and Fisher's exact test for dichotomous variables
[c] Take non-sterodial anti-inflammatory drugs (not including aspirin) ≥ once a week
[d] Energy adjusted using residual method
[e] Total = diet + supplements

Table 2.2. Differences in APC and APC/β-catenin score[a] in normal-appearing rectal mucosa between incident sporadic colorectal adenoma cases and controls, the MAPII study

Batch-adjusted biomarker labeling optical density				Prop diff (%)[b]	*P*[c]	OR[d]	95% CI	Model covariates[e]
APC/β-catenin score								
Controls (N=35)	**(SE)**	**Cases (N=32)**	**(SE)**					
0.55	0.02	0.47	0.02	-14.3	0.02	0.40	0.14 - 1.14	Age and sex only
0.53	0.03	0.45	0.03	-15.5	0.02	0.38	0.13 - 1.09	Family hx CRC
0.56	0.02	0.47	0.03	-16.2	0.01	0.31	0.09 - 1.05	$25(OH)D_3$[f]
0.55	0.02	0.47	0.02	-13.8	0.03	0.47	0.16 - 1.36	Physical activity
0.55	0.02	0.47	0.02	-13.9	0.03	0.45	0.15 - 1.32	Total calcium intake
0.53	0.03	0.45	0.03	-15.0	0.03	0.42	0.14 - 1.28	Family hx CRC, physical activity, calcium
APC[g]								
(N=45)		**(N=42)**						
0.50	0.02	0.49	0.02	-3.2	0.54	0.75	0.31 - 1.68	Age and sex only
0.48	0.02	0.46	0.02	-3.9	0.47	0.68	0.29 - 1.61	Family hx CRC
0.50	0.02	0.49	0.02	-2.8	0.63	0.88	0.34 - 2.27	$25(OH)D_3$[f]
0.50	0.02	0.49	0.02	-2.5	0.63	0.90	0.37 - 2.14	Physical activity
0.50	0.02	0.49	0.02	-1.6	0.75	0.96	0.39 - 2.33	Total calcium intake
0.48	0.02	0.47	0.02	-2.1	0.70	0.82	0.37 - 2.30	Family hx CRC, physical activity, calcium

[a] APC/β-catenin score = ΦAPC/β-catenin

[b] Proportional difference = [(mean of cases - mean of controls)/mean of controls]*100%

[c] Based on F-test for significance in a linear model

[d] The labeling optical density was dichotomized on the mean of the colon site-specific distributions in the controls

[e] All estimates adjusted for age and sex; Abbreviations: family hx CRC = family history of colorectal cancer in a first degree relative, NSAID = take nonsteroidal anti-inflammatory drug ≥ once/week, 25(OH)D3 = serum 25-OH vitamin D3 level

[f] Sample size for 25(OH)D3 adjustment: APC/β-catenin ratio - 28 controls, 25 cases; APC - 35 controls, 34 cases

[g] Ratio of upper 40% of crypt to whole crypt

Table 2.3. Differences in β-catenin[a] and E-cadherin[a] in normal-appearing rectal mucosa between incident sporadic colorectal adenoma cases and controls, the MAPII study

Batch-adjusted biomarker labeling optical density				Prop diff (%)[b]	*P*[c]	OR[d]	95% CI	Model covariates[e]
β-catenin								
Controls (N=41)	**(SE)**	**Cases (N=36)**	**(SE)**					
0.98	0.03	1.01	0.03	3.0	0.40	1.36	0.54 - 3.40	Age and sex only
0.98	0.03	1.01	0.03	3.0	0.40	1.35	0.54 - 3.38	Family hx CRC
0.97	0.03	1.01	0.03	4.2	0.30	1.53	0.54 - 4.32	$25(OH)D_3$[f]
0.98	0.02	1.00	0.03	2.6	0.46	1.41	0.54 - 3.69	Physical activity
0.98	0.02	1.01	0.03	3.3	0.36	1.54	0.60 - 3.95	Total calcium intake
0.99	0.03	1.02	0.03	3.2	0.37	1.45	0.55 - 3.84	Family hx CRC, physical activity, calcium
E-cadherin								
(N=46)		**(N=40)**						
1.01	0.03	1.00	0.03	-0.7	0.88	1.18	0.49 - 2.82	Age and sex only
1.03	0.04	1.02	0.04	-0.6	0.89	1.19	0.50 - 2.88	Family hx CRC
0.97	0.03	1.00	0.04	2.4	0.64	1.37	0.50 - 3.74	$25(OH)D_3$[f]
1.01	0.03	1.00	0.03	-0.9	0.84	1.25	0.50 - 3.10	Physical activity
1.01	0.03	1.00	0.03	-0.9	0.86	1.27	0.52 - 3.15	Total calcium intake
1.03	0.04	1.02	0.04	-1.2	0.80	1.24	0.49 - 3.15	Family hx CRC, physical activity, calcium

[a] Whole crypt

[b] Proportional difference = [(mean of cases - mean of controls)/mean of controls]*100%

[c] Based on F-test for significance in a linear model

[d] The labeling optical density was dichotomized on the mean of the colon site-specific distributions in the controls

[e] All estimates adjusted for age and sex; Abbreviations: family hx CRC = family history of colorectal cancer in a first degree relative, NSAID = take nonsteroidal anti-inflammatory drug ≥ once/week, 25(OH)D3 = serum 25-OH vitamin D3 level

[f] Sample size for 25(OH)D3 adjustment: β-catenin - 34 controls, 29 cases; E-cadherin - 37 controls, 32 cases

Table 2.4. Differences in expression of APC, β-catenin in normal-appearing colorectal mucosa according to potential risk factors for colorectal neoplasms, the MAPII study

Characteristic[a,b]	*N*	APC[c]	SE	Pro. Diff. (%)[d]	*P*[e]	N	β-cat[c]	SE	Pro. Diff. (%)[d]	*P*[e]
Sex										
Female	45	0.49	0.02			37	0.96	0.02		
Male	42	0.51	0.02	4.2	0.43	40	1.01	0.02	5.2	0.15
Age (yrs.)										
< 55	43	0.50	0.02			42	0.95	0.02		
≥ 55	44	0.50	0.02	-1.7	0.73	35	1.02	0.03	6.9	0.07
Family hx of CRC										
No	75	0.51	0.01			67	0.98	0.02		
Yes	12	0.44	0.03	-12.8	0.07	10	0.98	0.05	-0.4	0.94
Smoking status										
Never	41	0.51	0.02			34	0.95	0.03		
Former/Current	45	0.49	0.02	-4.4	0.39	43	1.01	0.02	5.7	0.12
Alcohol intake										
Never	10	0.49	0.04			8	0.86	0.06		
Former/Current	76	0.50	0.01	2.3	0.80	69	1.00	0.02	15.7	0.03
Physical activity (METS/d)										
Low	36	0.52	0.02			33	0.95	0.03		
High	47	0.47	0.02	-9.8	0.05	42	1.00	0.02	5.9	0.12
BMI (Kg/m2)										
< 30	44	0.51	0.02			37	0.95	0.02		
≥ 30	42	0.49	0.02	-4.7	0.35	40	1.00	0.02	6.0	0.27
WHR										
Low	42	0.51	0.02			35	0.94	0.02		
High	44	0.49	0.02	-5.2	0.30	41	1.02	0.02	8.1	0.03
NSAID - cases										
No	26	0.49	0.03			23	1.02	0.03		
Yes	16	0.48	0.03	-2.0	0.82	13	0.98	0.04	-4.1	0.44
NSAID - controls										
No	24	0.53	0.02			24	0.93	0.03		
Yes	20	0.47	0.03	-11.0	0.01	17	1.03	0.04	10.6	0.05
$p_{interaction}$[f]					0.54					0.10
Serum 25(OH)D3 (ng/ml)										
< 27	33	0.48	0.02			33	0.99	0.03		
≥ 27	37	0.51	0.02	5.3	0.37	30	0.95	0.03	-4.1	0.31
Total energy intake										
Low	29	0.49	0.02			28	1.04	0.03		
High	54	0.50	0.02	2.9	0.59	47	0.94	0.02	-9.0	0.01
Total fat										
Low	40	0.52	0.02			36	0.96	0.02		
High	43	0.47	0.02	-9.4	0.05	39	1.00	0.03	4.2	0.27

Table 2.4. con't

Characteristic[a,b]	*N*	APC[c]	SE	Pro. Diff. (%)[d]	*P*[e]	N	β-cat[c]	SE	Pro. Diff. (%)[d]	*P*[e]
Total[g] calcium										
Low	42	0.47	0.02			37	0.97	0.03		
High	41	0.52	0.02	11.3	0.04	38	0.98	0.03	0.9	0.81
Dietary fiber										
Low	38	0.46	0.02			37	1.00	0.03		
High	45	0.53	0.02	15.6	0.01	38	0.95	0.03	-4.8	0.19
Total[g] folate										
Low	41	0.46	0.02			38	0.96	0.03		
High	42	0.52	0.02	12.4	0.02	37	0.99	0.03	3.7	0.35
Processed meat intake										
Low	31	0.52	0.02			29	0.93	0.03		
High	52	0.48	0.02	-7.3	0.15	46	1.01	0.02	8.3	0.04

[a] Referent category = "Female", the youngest or lowest category, or "No" groups

[b] Abbreviations: Family hx of CRC = family history of colorectal cancer in a first degree relative, BMI = body mass index, WHR = waist to hip ratio, NSAID = take nonsteroidal anti-inflammatory drug ≥ once/week

[c] All estimates adjusted for age and sex; dietary and physical activity covariates also adjusted for energy consumption; and total energy consumption adjusted for physical activity

[d] Proportional difference = [(mean of comparative category - mean of referent category)/mean of referent category]*100%

[e] p-value for comparison of means (analysis of covariance)

[f] p-value of multiplicative interaction term between regular NSAID use and case/control status in ANACOVA models

[g] Total = dietary + supplemental

Table 2.5. Differences in expression of the APC/β-catenin score[a] in normal-appearing colorectal mucosa according to potential risk factors for colorectal neoplasms, the MAPII study

Characteristic[b,c]	*N*	APC/ β-catenin score[d]	SE	Pro. Diff. (%)[e]	*P*[f]
Sex					
Female	33	0.57	0.03		
Male	34	0.50	0.02	-11.1	0.08
Age (yrs.)					
< 55	36	0.52	0.03		
≥ 55	31	0.50	0.03	-5.3	0.45
Family hx of CRC					
No	59	0.54	0.02		
Yes	8	0.48	0.05	-10.7	0.22
Smoking status					
Never	32	0.54	0.02		
Former/Current	35	0.53	0.03	-2.9	0.66
Alcohol intake					
Never	6	0.56	0.07		
Former/Current	61	0.53	0.02	-4.2	0.75
Physical activity (METS/d)					
Low	27	0.58	0.03		
High	38	0.50	0.02	-14.2	0.02
BMI (Kg/m2)					
< 30	32	0.56	0.02		
≥ 30	35	0.51	0.02	-9.9	0.11
WHR					
Low	31	0.57	0.02		
High	36	0.50	0.02	-11.0	0.08
NSAID - cases					
No	19	0.45	0.03		
Yes	13	0.50	0.04	11.8	0.29
NSAID - controls					
No	18	0.61	0.03		
Yes	17	0.49	0.03	-20.2	0.01
$p_{interaction}$[g]					0.03
Serum 25(OH)D3 (ng/ml)					
< 27	27	0.50	0.03		
≥ 27	26	0.59	0.02	17.7	0.02
Total energy intake					
Low	21	0.52	0.03		
High	44	0.54	0.02	4.8	0.51

Table 2.5. Con't

Characteristic[a,b]		*N*	APC/ β-catenin score[d]	SE	Pro. Diff. (%)[e]	*P*[f]
Total fat						
	Low	31	0.57	0.02		
	High	34	0.49	0.02	-14.8	0.02
Total[h] calcium						
	Low	31	0.51	0.03		
	High	34	0.55	0.02	7.0	0.33
Dietary fiber						
	Low	31	0.50	0.03		
	High	34	0.56	0.02	13.3	0.07
Total[h] folate						
	Low	34	0.51	0.03		
	High	31	0.56	0.03	10.5	0.15
Processed meat intake						
	Low	24	0.59	0.03		
	High	41	0.50	0.02	-14.3	0.02

[a] APC/β-catenin score = ΦAPC/β-catenin

[b] Referent category = "Female", the youngest or lowest category, or "No" groups

[c] Abbreviations: Family hx of CRC = family history of colorectal cancer in a first degree relative, BMI = body mass index, WHR = waist to hip ratio, NSAID = take nonsteroidal anti-inflammatory drug ≥ once/week

[d] All estimates adjusted for age and sex; dietary and physical activity covariates also adjusted for energy consumption; and total energy consumption adjusted for physical activity

[e] Proportional difference = [(mean of comparative category - mean of referent category)/mean of referent category]*100%

[f] p-value for comparison of means (analysis of covariance)

[g] p-value of multiplicative interaction term between regular NSAID use and case/control status in ANACOVA models

[h] Total = dietary + supplemental

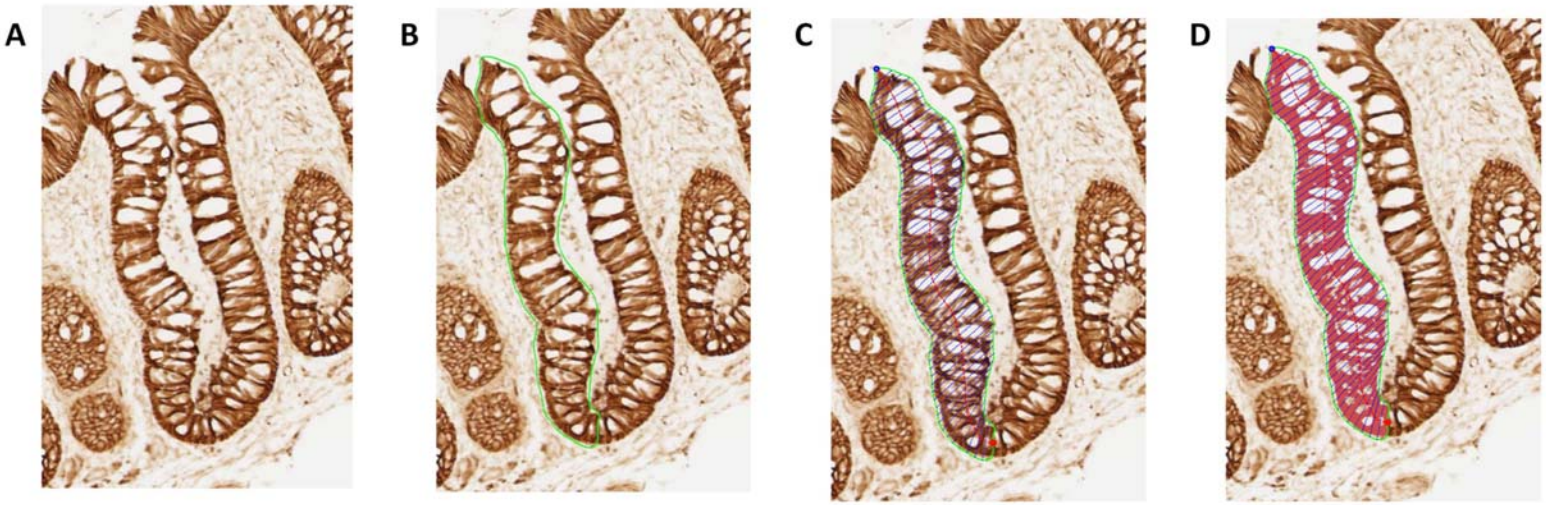
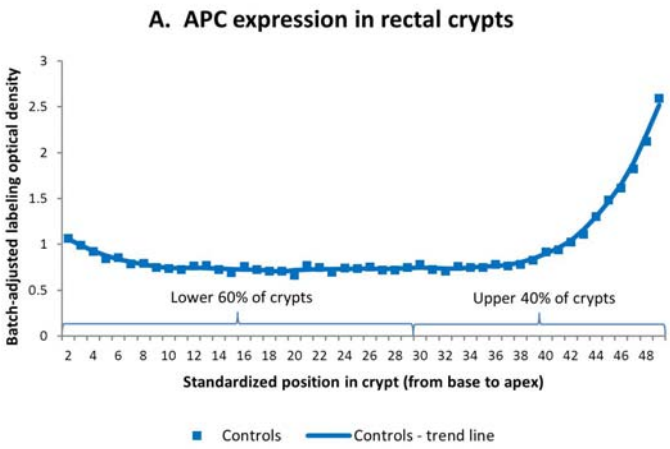
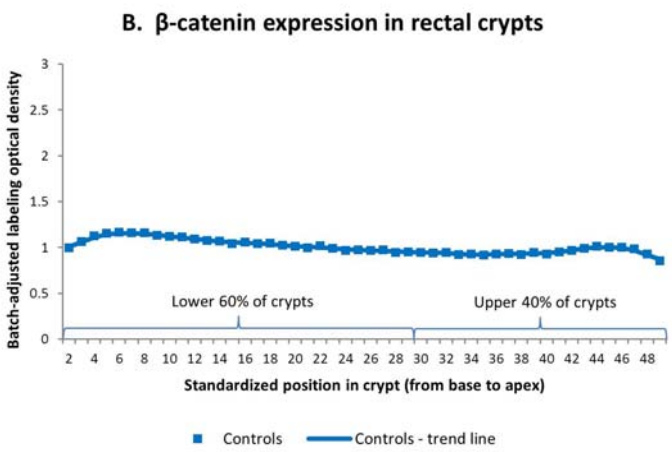
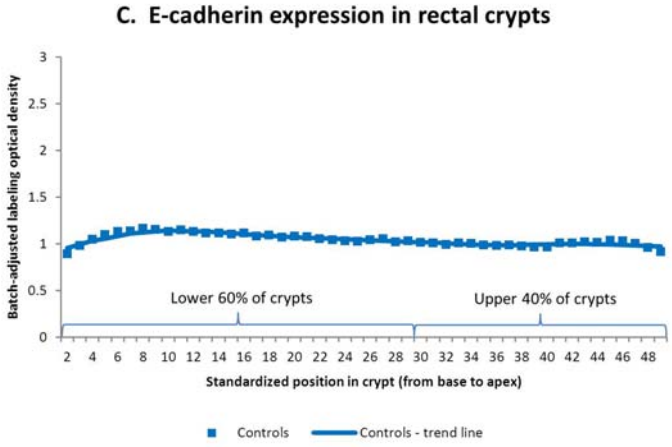


Figure 2.1. Quantitative image analysis. A, finding scorable crypts; B, tracing the hemicrypt; C, automated sectioning of the trace; and D, automated quantification of β-catenin labeling optical density.

Figure 2.2. Representative examples of labeling expression distribution (distribution plots represent labeling optical density in rectal crypts) of the (A) APC (B) β-catenin and (C) E-cadherin.







Chapter 3. A randomized clinical trial of the effects of supplemental calcium and vitamin D_3 on APC, β-catenin, and E-cadherin expression in the normal mucosa of colorectal adenoma patients

Thomas U. Ahearn[1,4], Aasma Shaukat[5], W. Dana Flanders[2,3,4], Robin E. Rutherford[6], and Roberd M. Bostick[2,4]

Author Affiliations: [1]Nutrition and Health Sciences Program, Graduate Division of Biological and Biomedical Sciences, Departments of [2]Epidemiology and [3]Biostatistics and Bioinformatics, Rollins School of Public Health, and [4]Winship Cancer Institute, Emory University; [5]Department of Medicine, GI Division, University of Minnesota, Minneapolis, Minnesota; [6]Division of Digestive Diseases, Emory University School of Medicine, Atlanta, Georgia

Grant support: National Cancer Institute, National Institutes of Health (R01 CA104637 and R03 CA115230 to R.M.B.); Georgia Cancer Coalition Distinguished Scholar award (to R.M.B.); the Franklin Foundation. The National Cancer Institute, the Georgia Cancer Coalition, and the Franklin Foundation had no influence on the design of the study; the collection, analysis, and interpretation of the data; the decision to submit the manuscript for publication; or the writing of the manuscript.

Corresponding Author:
Roberd M. Bostick
Department of Epidemiology
Rollins School of Public Health
Emory University
1518 Clifton Road, NE
Atlanta, GA 30322
Phone: 404-727-2671; Fax: 404-727-8737
E-mail: rmbosti@sph.emory.edu

Running title: Calcium and vitamin D effects on Wnt markers in human colon
Key words: calcium, vitamin D, colonic neoplasms, biomarkers, clinical trial

Abstract

APC/β-catenin pathway perturbation is a common early event in colorectal carcinogenesis and is affected by calcium and vitamin D in basic science studies. To assess the effects of calcium and vitamin D on APC, β-catenin, and E-cadherin expression in the normal appearing colorectal mucosa of sporadic colorectal adenoma patients, we conducted a randomized, double-blinded, placebo-controlled 2x2 factorial clinical trial. Pathology-confirmed colorectal adenoma cases were treated with 2 g/day elemental calcium and/or 800 IU/day vitamin D_3 versus placebo over 6 months (N=92; 23/group). Overall APC, β-catenin, and E-cadherin expression and distributions in colon crypts in normal-appearing rectal mucosa biopsies were detected by standardized automated immunohistochemistry and quantified by image analysis. In the vitamin D_3-supplemented group relative to placebo, the proportion of APC in the upper 40% of crypts (Φh APC) increased 21% (p=0.01), β-catenin decreased 10% (p=0.28), E-cadherin increased 77% (p=0.02), and the Φh APC/β-catenin ratio (APC/β-catenin score) increased 31% (p=0.02). In the calcium-supplemented group Φh APC increased 10% (p=0.12), β-catenin decreased 15% (p=0.06), and the APC/β-catenin score increased 41% (p=0.01). In the calcium/vitamin D_3 supplemented group β-catenin decreased 11% (p=0.19), E-cadherin increased 39% (p=0.17), and the APC/β-catenin score increased 16% (p=0.26). These results support 1) that calcium and vitamin D modify APC, β-catenin, and E-cadherin expression in humans in directions hypothesized to reduce risk for colorectal neoplasms, 2) calcium and vitamin D as potential chemopreventive agents against colorectal neoplasms, and 3) the potential of APC, β-catenin, and E-cadherin expression as modifiable, pre-neoplastic risk biomarkers for colorectal neoplasms.

Introduction

Colorectal cancer (CRC), the second leading cause of cancer deaths in the United States [280], is responsible for approximately 8% of all cancer deaths worldwide [100, 307]. The etiology of sporadic CRC is predominately rooted in dietary and lifestyle behaviors [99, 100], suggesting that it may be preventable. The molecular basis of colorectal carcinogenesis is becoming clearer [99]; however, there are no validated, treatable, pre-neoplastic biomarkers of risk for colorectal neoplasms.

Malfunction of the APC/β-catenin signaling pathway is an early and common event in the pathogenesis of colorectal neoplasms. Impaired APC function occurs in approximately 80-90% of sporadic CRCs[281], resulting in the increased potential of β-catenin to translocate to the nucleus and activate target genes responsible for promoting cell proliferation and inhibiting differentiation [28, 281, 282]. E-cadherin may also antagonize β-catenin nuclear expression by sequestering β-catenin to its cytoplasmic tail, linking E-cadherin to actin filaments and promoting cell adhesion and differentiation [281, 282]. We reported that APC expression (especially the proportion of APC in the upper 40% of colorectal crypts (Φh APC)), β-catenin expression, and the Φh APC/β-catenin ratio (APC/β-catenin score) in normal colorectal mucosa may be modifiable, pre-neoplastic biomarkers of risk for colorectal neoplasms.

Convincing evidence from experimental and observational studies and randomized, placebo-controlled clinical trials suggests that calcium and vitamin D have cheompreventive effects against colorectal neoplasms [162]. The beneficial effects of calcium may partially be attributed to its binding of toxic secondary bile acids and ionized fatty acids, and/or by directly inhibiting proliferation and promoting differentiation and apoptosis [162]. Vitamin D signaling may induce cell-cycle arrest and promote differentiation and apoptosis directly through vitamin D-mediated gene transcription and indirectly through modifying growth factors, and play roles

in promoting oxidative DNA damage repair, inhibiting angiogenesis, and regulating immune cell function [162]. Also, prospective cohort studies have consistently found higher total calcium intake to be associated with lower risk for colorectal neoplasms [162], calcium supplementation reduces colorectal adenoma recurrence (modified by vitamin D status) [308], and higher circulating 25(OH)-vitamin D (25(OH)D) is inversely associated with colorectal neoplasms [162, 188, 309].

Evidence from animal models [244, 310] and *in vitro* [247-249] studies suggest that the chemoprentive effects of calcium and vitamin D may, in part, include modification of the APC/β-catenin signaling pathway. However, to our knowledge there are no reported human *in vivo* investigations on the effects of supplemental calcium and vitamin D_3 on the expression of APC, β-catenin, and E-cadherin in the normal colorectal mucosa. To address this, as reported herein, 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, versus placebo over 6 months, to estimate the efficacy of these agents on APC, β-catenin, and E-cadherin expression in the normal colorectal mucosa.

Study Participants and Methods

Participant population. A detailed description of the study protocol for recruitment procedures and detailed specific exclusions was published previously [259]. Briefly, eligible participants were 30 to 75 years of age, in general good health, and had a history of at least one pathology-confirmed adenomatous colorectal polyp within the past 36 months. Exclusions from participation included contraindications to calcium or vitamin D supplementation or rectal biopsy procedures, and medical conditions, habits, or medication usage that potentially could

interfere with the study. Participants were recruited from patients attending the Digestive Diseases Clinic of the Emory Clinic, Emory University.

Clinical trial protocol. Between April 2005 and January 2006, 522 potentially eligible patients were identified through initial screening of electronic medical records; of these, 244 (43%) were contacted, and of these 105 (47%) attended the eligibility visit to be interviewed, sign a consent form, complete questionnaires, and provide blood samples [259]. Diet was assessed using a semi-quantitative Willett Food Frequency Questionnaire [254]. Medical and pathology records were reviewed. Following a 30-day placebo run-in trial, 92 (88%) participants with no significant perceived side effects and who took at least 80% of their assigned tablets underwent a baseline rectal biopsy, and, with additional consent, were randomly assigned to the following four treatment groups: 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 vitamin D_3 supplementation ($n = 23$). Additional details and rationale for the doses and forms of calcium and vitamin D_3 supplements were previously published [259]. Participants were instructed to maintain their usual diet and not take any new nutritional supplements they were not taking at the time of entry into the study. All aspects of the trial were approved by the Institutional Review Board of Emory University.

During the 6-month treatment period, participants attended follow-up visits 2 and 6 months after randomization. At follow-up visits participants completed questionnaires and were interviewed about adherence and adverse events. At the 6-month follow-up, participants again underwent a venipuncture and rectal biopsy. All visits for a given participant were scheduled for the same time of day to control for potential circadian variation. Dietary, lifestyle, and other factors hypothesized to modify biomarker expression in normal colon mucosa were

assessed at baseline and at 6-months follow-up. Participants were asked to abstain from aspirin use seven days prior to each biopsy. Participants were not required to be fasting for their visits and did not take a bowel cleansing preparation or enema.

Six approximately one millimeter thick biopsy specimens were taken from the normal-appearing rectal mucosa 10 cm above the level of the external anal aperture through a short rigid sigmoidoscope using a jumbo cup flexible biopsy forceps mounted on a semi-rigid rod. No biopsies were taken within 4.0 cm of a polypoid lesion. Biopsies were placed onto a strip of bibulous paper and immediately placed in phosphate buffered saline (PBS), oriented, transferred to 10% normal buffered formalin for 24 hours, and then transferred to 70% ethanol. Then, within a week, the biopsies were processed and embedded in paraffin blocks (2 blocks of 3 biopsies per participant, per biopsy visit).

Immunohistochemistry protocol. Five slides with 4 levels of 3 micron-thick biopsy sections taken 40 microns apart were prepared for each antigen, yielding a total of 20 levels for each antigen. Antigen retrieval was performed by placing the slides in a preheated Pretreatment Module (Lab Vision Corp.) with 100x Citrate Buffer (pH 6.0; DAKO S1699, DAKO Corp.) and steaming them for 40 min. Following antigen retrieval, slides were immunohistochemically processed in a DAKO Automated Immunostainer (DAKO Corp.) using a labeled streptavidin-biotin method for APC (Calbiochem, OP80; 1:70 dilution), β-catenin (Transduction Laboratories 610154; 1:300 dilution), and E-cadherin (Zymed 33-4000; 1:50 dilution). No slides were counterstained. After processing, slides were coverslipped with a Leica CV5000 Coverslipper (Leica Microsystems, Inc.). The negative and positive control slides were treated identically to the patients' slides except that antibody diluent was used rather than primary antibody on the negative slides.

Protocol for quantifying labeling densities of immunohistochemically detected biomarkers in normal colon crypts ("scoring"). A detailed description of the protocol used to quantify biomarker labeling optical densities ("biomarker expression") in normal colon crypts was previously described [259]. Briefly, a scorable crypt was defined as an intact crypt extending from the muscularis mucosa to the colon lumen [290]. Prior to "scoring", the negative and positive control slides were checked for staining adequacy. The major equipment and software for the image analysis procedures included: personal computer, light microscope (Olympus BX40, Olympus Corporation, Japan) with appropriate filters and attached digital light microscope camera (Polaroid DMC Digital Light Microscope Camera, Polaroid Corporation, USA), 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 2003 relational database software (Microsoft Corporation, WA).

Evaluation of biomarker expression consisted of the same technician cleaning all slides, selecting the two of the three biopsies with the most scorable crypts per biopsy, creating background correction images for each slide scored, capturing 16-bit grayscale images of crypts at 200x magnification, and tracing the border of the "hemicrypt" (one half of the crypt). The program then divided the outlined hemicrypt into equally spaced segments that corresponded to the average width of colonocytes, and measured the optical density of the labeling across the entire hemicrypt and within each segment, adjusting for the background. The technician then repeated this process for the adjacent hemicrypt, and proceeded to the next crypt, level, biopsy, and/or slide. The goal was to score 16 to 20 hemicrypts per biopsy visit for each biomarker (**Figure 3.1**).

Reliability control was performed by selecting samples of previously analyzed slides to be re-analyzed by the technician, who was blinded to the selection. Intra-reader reliability was greater than 0.90 for APC, β-catenin, and E-cadherin.

Protocol for measuring serum vitamin D levels. Serum 25-OH-vitamin D and 1,25-(OH)2-vitamin D were measured by Dr. Bruce W. Hollis at the Medical University of South Carolina using a RIA method as previously described [311, 312]. Serum 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 was 2.3% and 6.2% for serum 25-OH-vitamin D and for 1,25-(OH)2-vitamin D, respectively.

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 intra-class correlation coefficients.

The mean labeling optical density expression of each biomarker on each study participant, at baseline and 6-month follow-up, was calculated by summing the biomarker's expression for all analyzed crypts and dividing by the total number of analyzed crypts. Biomarker expression was transformed to adjust for possible staining batch effects by dividing an individual's mean biomarker expression by their batch mean biomarker expression [259]. To evaluate distinct functional zones of crypts, measures of crypt biomarker distribution selected *a priori* were the upper 40% of the crypts (differentiation zone), the lower 60% of the crypts (proliferation zone), and the ratio of the upper 40% of crypts to the whole crypt to (Φh).

Primary analyses were based on assigned treatment at the time of randomization, regardless of adherence status (intent-to-treat analysis). Treatment effects were evaluated by

assessing the differences in the transformed biomarker expression from baseline to the 6-month follow-up between participants in the active treatment groups and those in the placebo group by a repeated-measures linear MIXED effects model. The model included the intercept, follow-up visit effects (baseline and follow-up), treatment groups, and interactions between treatment groups and the follow-up visit effect. Because optical density is measured in arbitrary units, to provide perspective on the magnitude of the treatment effects, we also calculated the relative effect. The relative effect was calculated as the (treatment group at follow-up / treatment group at baseline) / (placebo group at follow-up / placebo group at baseline). The relative effect provides a conservative estimate of the proportional change in the treatment group relative to that in the placebo group, and its interpretation is somewhat analogous to that of an odds ratio (e.g., a relative effect of 2.0 would mean that the proportional change in the treatment group was two times that in the placebo group).

The distributions of batch standardized APC, β-catenin, and E-cadherin labeling optical densities along the full length of the crypts were graphically plotted and evaluated using the LOESS procedure. First, each hemicrypt was standardized to 50 sections. Then, the average of each section across all crypts was predicted by the LOESS model separately for each patient and then for each treatment group by visit. The results were graphically plotted along with the smoothing lines. Although the plots illustrate the distribution of expression, they do not provide a complete analysis of treatment effects because they do not account for changes in the placebo group.

The APC/β-catenin score was calculated by dividing an individual's Φh APC by their β-catenin expression in the whole crypt (Φh APC expression/β-catenin expression). E-cadherin was not included in the APC/β-catenin score because during carcinogenesis malfunctioning regulation of β-catenin by APC occurs most often earlier than E-cadherin down-regulation [313].

We hypothesize that a higher score is associated with reduced potential of β-catenin to promote proliferation.

Statistical analyses were performed using SAS 9.2 statistical software (SAS Institute Inc.). A *P* value ≤ 0.05 (two-sided) was considered statistically significant.

Results

Characteristics of study participants. Baseline characteristics of study participants did not significantly differ by treatment group (**Table 3.1**). The mean age of study participants was 61 years, 70% 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 significantly differ among the four treatment groups. On average, at least 80% of pills were taken by 93% of participants at the first follow-up visit and by 84% of participants at the final follow-up visit. No adverse events were attributed to study procedures or treatments. Seven participants (8%) were lost to follow-up. Dropouts included one person from the vitamin D_3 supplementation group and two from each of the other three groups [259].

Baseline serum 25-OH-vitamin D and 1,25-$(OH)_2$-vitamin D levels did not differ between the four treatment groups. At the conclusion of the study, serum 25-OH-vitamin D levels had increased 60% ($p<0.0001$) and 56% ($p<0.0001$) in the vitamin D_3 and calcium / vitamin D_3 groups, respectively, relative to placebo; however, mean serum 25-OH-vitamin D concentrations remained below 32 ng/ml in all treatment groups [259]. There was no evidence of treatment effect modification by obesity status (body mass index ≥30).

APC. A graphical comparison of APC crypt expression distribution at baseline and 6-month follow-up indicated that APC expression decreased in approximately the lower 40% of

the crypt and increased in the upper 60% of the crypt (**Figure 3.2A**). As shown in **Table 3.2**, following 6 months of treatment, APC expression increased in the vitamin D_3 treatment group 25% (p=0.14) in the full length of crypts, 48% (p=0.03) in the upper 40% of crypts, 11% in the lower 60% of crypts (p=0.47), and 21% (p=0.01) in the Φh of crypts, relative to the placebo group. In the calcium group APC expression decreased 2% (p=0.91) in the full length of crypts, increased 7% (p=0.66) in the upper 40% of crypts, decreased 10% (p=0.51) in the lower 60% of crypts, and increased 10% (p=0.12) in the Φh of crypts, relative to the placebo group. APC expression tended to increase in the calcium/vitamin D_3 less than in the vitamin D_3 group, and these findings were not statistically significant (**Table 3.2A**).

β-catenin. A graphical evaluation of β-catenin crypt expression distribution at baseline and 6-month follow-up indicated that β-catenin expression did not change in approximately the lower 20% of the crypt, but steadily decreased towards the crypt apex (**Figure 3.2B**). As shown in **Table 3.2**, following 6 months of treatment, β-catenin expression decreased along the full length of crypts by 15% (p=0.06), 11% (p=0.18), and 11% (p=0.20) in the calcium, vitamin D_3 and calcium/vitamin D_3 groups, respectively, relative to the placebo group. The findings in the upper 40% and lower 60% of crypts did not appreciably differ from those observed in the full length of crypts. There were no apparent treatment effects on β-catenin expression in the Φh of crypts.

E-cadherin. A graphical evaluation of E-cadherin crypt expression distribution at baseline and 6-month follow-up indicated that E-cadherin expression uniformly increased along the full length of the crypt (**Figure 3.2C**). As shown in **Table 3.2**, following 6 months of treatment, E-cadherin expression increased in the vitamin D_3 group 78% (p=0.03) in the full length of crypts, 78% (p=0.02) in the upper 40% of crypts, 68% (p=0.05) in the lower 60% of crypts, and 14% (p=0.10) in the Φh of crypts. E-cadherin expression also increased in the calcium/vitamin D_3 group, but less so than in the vitamin D_3 group, except in the Φh of crypts

where E-cadherin expression increased 18% (p=0.03). In the calcium group E-cadherin did not appreciably change relative to the placebo group (**Table 3.2C**).

APC/β-catenin score. The APC/β-catenin score increased 41% (p=0.01), 31% (p=0.02), and 16% (p=0.26) in the calcium, vitamin D_3, and calcium/vitamin D_3 groups, respectively, relative to the placebo group (**Table 3.2D**).

Discussion

The results of this pilot, randomized, placebo-controlled clinical trial provides the first human *in* vivo evidence that supplemental calcium and vitamin D_3, alone or in combination, may increase APC and E-cadherin expression and the APC/β-catenin score and decrease β-catenin expression in the normal colorectal mucosa of sporadic adenoma patients. These finding support the hypothesis that the anti-carcinogenic effects calcium and vitamin D, alone or in combination, may in part operate by modifying the APC/β-catenin signaling pathway. These findings are relevant because, in light of our previous report of differences in APC and β-catenin expression between persons with incident sporadic adenomas and persons with no past or current adenomas, they 1) provide further support that APC and β-catenin expression and the APC/β-catenin score in the normal colorectal mucosa may be modifiable, pre-neoplastic biomarkers of risk for colorectal adenomas, and 2) provide human *in vivo* mechanistic evidence of the possible protective effects of calcium and vitamin D_3 against colorectal neoplasms.

APC and β-catenin are appealing candidates for being pre-neoplastic biomarkers of risk because malfunctioning of the APC/β-catenin signaling pathway is a common and early event in the colorectal neoplastic transition [281]. In normal colorectal mucosa APC, axin, glycogen synthase kinase 3, and casein kinase negatively regulate Wnt signaling by forming the "β-catenin destruction" complex, and, in the absence of Wnt signaling, phosphorylate and promote the

degradation of free β-catenin [281]. Normal functioning of the *APC* gene is inhibited in approximately 80-90% of sporadic CRC, resulting in increased potential for β-catenin to translocate to the nucleus and activate Wnt target genes [281]. In approximately 7% of sporadic CRC Wnt signaling is constitutively activated by stabilizing mutations in the *β-catenin* gene [314]. In normal colorectal mucosa APC, β-catenin, and E-cadherin are all strongly expressed—APC primarily in the cytoplasm, and E-cadherin and β-catenin primarily at the cell membrane. During the adenoma-carcinoma sequence APC and E-cadherin expression markedly decrease (although the decrease in E-cadherin tends to occur in later stages) [47, 66, 67], and β-catenin expression appears to steadily increase and translocate from the membrane to the cytoplasm and eventually into the nucleus [47, 315]. We previously proposed that the APC/β-catenin score may represent the potential of β-catenin to translocate to the nucleus and promote proliferative signaling [316]. We found the APC/β-catenin score in the normal colorectal mucosa of sporadic colorectal adenoma patients to be statistically significantly lower than in the normal colorectal mucosa of healthy controls; and that Φh APC and β-catenin expression and the APC/β-catenin score may be modifiable as suggested by their being associated with lifestyle and dietary risk factors for colorectal neoplasms [316].

The etiology of CRC is heavily influenced by modifiable dietary and lifestyle behaviors. Dietary-induced epigenetic modifications to the APC/β-catenin signaling pathway may initiate or be a "second hit" in the adenoma-carcinoma pathway. Calcium and vitamin D are two promising chemopreventive agents that may act against colorectal neoplasms; however, the mechanisms by which they operate are not entirely clear [162]. Colorectal cancer cell line studies suggest that calcium and $1,25(OH)_2D$ up-regulate E-cadherin expression and promote the translocation of β-catenin from the nucleus and cytoplasm to the plasma membrane [247-249, 252]. Mice fed a diet comparable to a typical "Western" diet had increased *β-catenin* and

Tcf gene expression and decreased *APC* gene expression; however, supplementation of the "Western" diet with increased dietary calcium and vitamin D decreased *β-catenin* and *Tcf* gene expression, but had no apparent effect on *APC* gene expression [310]. In a transmissible murine colonic hyperplasia model high dietary calcium modestly reduced total β-catenin expression [244]. A diet supplemented with the vitamin D analog $1\alpha(OH)D_5$ inhibited β-catenin nuclear expression in azoxymethane-treated mice [317], and endometrial E-cadherin expression increased in mice fed a diet high in vitamin D_3 [318]. Investigations of the *in vivo* effects of calcium and/or vitamin D on APC expression are limited [310], but dietary modification of APC expression is supported by reports that diets moderately deficient in B-vitamin methyl donors reduced APC expression and increased β-catenin/TCF signaling in rodents [304, 305].

Our results are consistent with the hypothesis that calcium and vitamin D reduce cell proliferation and promote differentiation in the colorectal mucosa. To our knowledge this is the first human *in vivo* study to suggest that calcium and/or vitamin D_3 may increase APC expression in the normal colorectal mucosa; however, the mechanism by which calcium and/or vitamin D_3 may modify APC expression remains unclear. We did not evaluate β-catenin localization, but consistent with the observed increase in APC expression, particularly Φh APC, we observed decreased β-catenin expression and an increased APC/β-catenin score in all three active treatment groups, suggesting that calcium and vitamin D_3 treatment may decrease the potential of β-catenin to promote proliferative signaling. These results are in line with our reports that suggest calcium and/or vitamin D_3 treatment reduced hTERT expression (a potential cofactor in the β-catenin transcriptional complex [319]) and increased p21 expression (which is negatively regulated by β-catenin/TCF signaling [320]) [296]. These results also corroborate our report that the APC/β-catenin score may be a modifiable, pre-neoplastic biomarker of risk for colorectal

neoplasms, providing *in vivo* evidence that suggests supplemental calcium and/or vitamin D_3 promotes a greater APC/β-catenin score in the normal rectal mucosa.

Conflicting with previous *in vitro* studies [247, 249] we did not observe increased E-cadherin expression in the calcium group. The explanation for this lack of consistency in findings is not clear and may be a chance finding; however, the difference may be attributed to our investigation evaluating the effects of calcium on E-cadherin expression in the normal mucosa of free living humans rather than *in vitro*, or that the ability of calcium to increase E-cadherin expression may be limited to neoplastic mucosa.

Contrary to our hypothesis and what has been reported in some studies [308, 321], the estimated treatment effect of calcium plus vitamin D_3 was not greater than that of either the calcium or vitamin D_3 alone in increasing Φh APC, E-cadherin, or the APC/β-catenin score, or in decreasing β-catenin. We previously reported that calcium combined with vitamin D_3 may mitigate treatment effects of calcium and vitamin D_3 alone on colorectal mucosa markers of apoptosis and differentiation [259, 296]. There are several plausible explanations for why this was observed, the first being that these could have been chance findings given the small sample size of our study. At least one study reported that calcium and vitamin D individually suppressed tumorigenesis in rodents, but the combination of the two was ineffective [322]. We previously observed that calcium combined with vitamin D_3 increased CYP24A1 expression [295], which may reduce the effects of $1,25(OH)_2D$ in the colorectal mucosa. A large clinical trial of colorectal adenoma recurrence suggested that calcium supplementation was primarily effective among people with 25(OH)D concentrations greater than the median in the study population (29.1 ng/ml) [308]. In our study population only participants in the vitamin D_3 group reached 25(OH)D concentrations greater than 29.1 ng/ml [259], suggesting the possibility of a threshold effect.

We previously reported that calcium and vitamin D_3 supplementation in this same trial favorably modified the expression of markers of calcium and vitamin D metabolism [295], proliferation [296], differentiation [296], apoptosis [259], mismatch repair [260], and oxidative DNA damage [297] in the normal human colorectal mucosa. Our current results, taken together with our previous findings, support the hypothesized effects of calcium and vitamin D on favorably modulating the phenotype of the colorectal mucosa and reducing risk for colorectal neoplasms.

Our study had several limitations. First, it was a pilot study with a relatively small sample size, increasing the role of chance observations and limiting our ability to perform stratified analyses. We were unable to evaluate β-catenin sub-cellular localization; however, our previous findings suggest that sporadic colorectal adenoma cases relative to normal controls may have greater total β-catenin expression in the normal colorectal mucosa. We propose that the APC/β-catenin score may represent the potential of β-catenin to promote proliferative signaling, and needs to be investigated in basic science studies. Also, we only examined the rectal mucosa and therefore treatment effects in other parts of the colon remain unknown. Another limitation is that we measured protein expression but not protein activity, and, therefore, could not correlate changes in expression with changes in protein activity. Finally, these markers are not proven biomarkers of risk; however, evidence from our pilot case-control study suggests that APC, β-catenin expression, and the APC/β-catenin score may be pre-neoplastic biomarkers of risk.

The strengths of this study include 1) that it is, to our knowledge, the first randomized, double-blind, placebo-controlled clinical trial to test the effects of supplemental calcium and vitamin D_3, alone and in combination, on components of the APC/β-catenin signaling pathway in the normal colorectal epithelium in sporadic adenoma patients, 2) the high protocol adherence

by study participants, and 3) the automated immunostaining and newly-designed image analysis software to quantify the crypt distribution of the expression of APC, β-catenin, and E-cadherin, resulting in high biomarker measurement reliability.

In summary, the results of this pilot, randomized, placebo-controlled clinical-trial provide human *in vivo* evidence that supplemental calcium and vitamin D_3, alone and in combination, may increase APC and E-cadherin expression and the APC/β-catenin score and decrease β-catenin expression in the normal colorectal mucosa of sporadic colorectal adenoma cases. These results suggest that the anti-carcinogenetic effects of supplemental calcium and vitamin D_3 may, in part, depend on the ability of these agents to favorably modulate the expression of the APC, β-catenin, and E-cadherin and thus, possibly, inhibit proliferative β-catenin signaling. Taken together with our previous findings, APC (especially ϕh APC) and β-catenin expression, the APC/β-catenin score, and E-cadherin may be modifiable, pre-neoplastic biomarkers of risk for colorectal neoplasms and warrant further investigation. Finally, our results support further investigation of calcium and vitamin D_3 as chemopreventive agents against colorectal neoplasms.

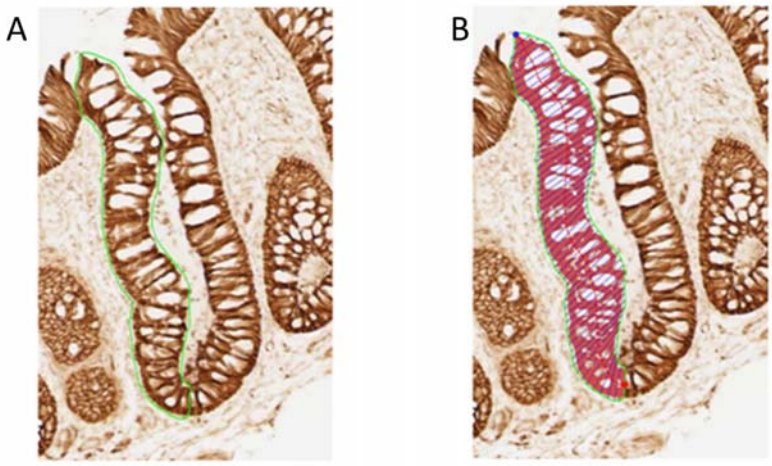


Figure 3.1. Quantitative image analysis. A, finding and tracing the hemicrypt; B, automated sectioning and quantification of β-catenin labeling optical density

Figure 3.2. Representative examples of labeling expression distribution* of (A) APC†, (B) β-catenin‡, and (C) E-cadherin† along normal colorectal crypts by treatment group at baseline and 6-month follow-up

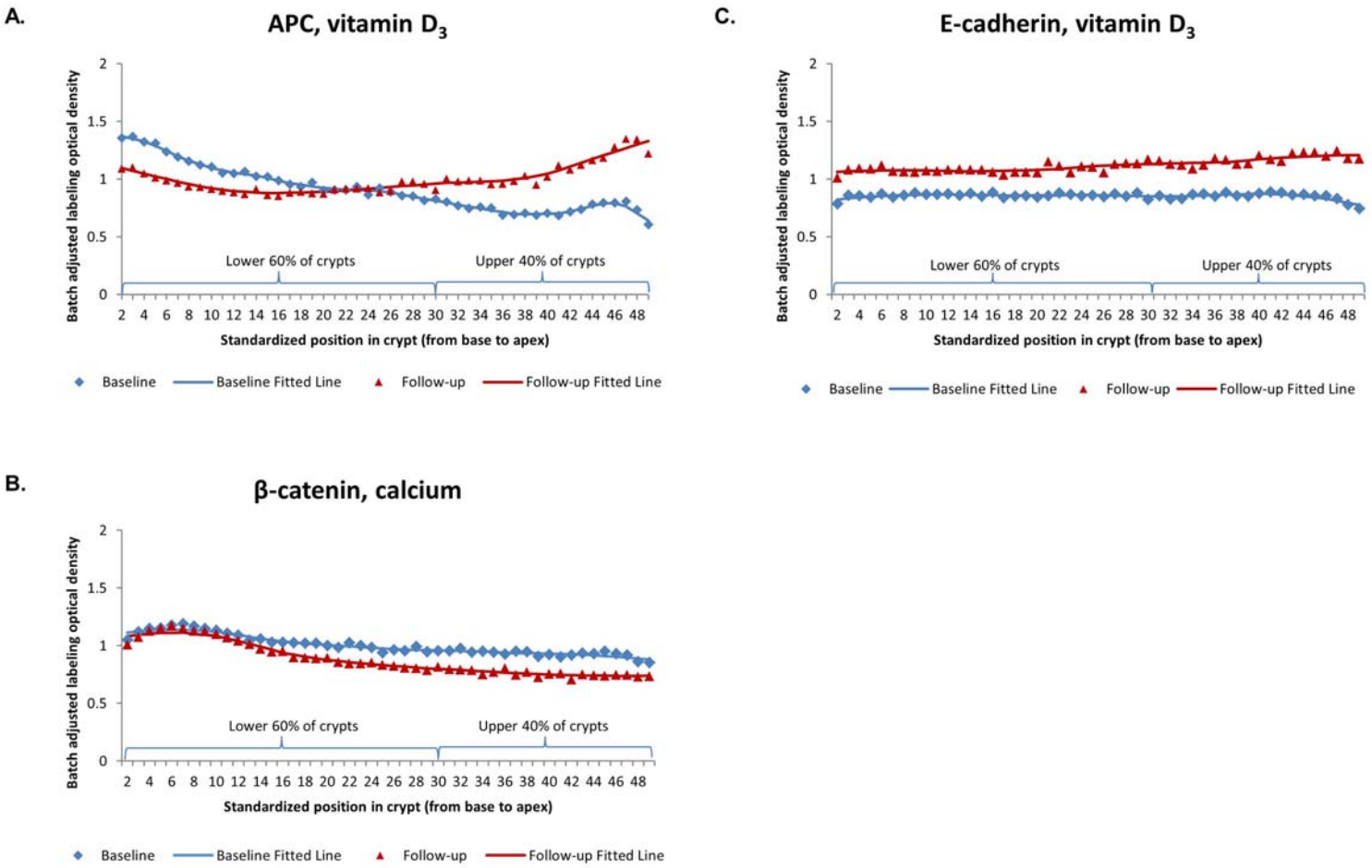


Table 3.1. Selected baseline characteristics of the study participants[*] (n=92)

Characteristics	Treatment Group: Placebo (n=23)	Calcium (n=23)	Vitamin D (n=23)	Calcium + Vit. D (n=23)	*P*-value[**]
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 CRC in 1° relative (%)	17	30	17	13	0.60
Take NSAID[¥] regularly[§] (%)	22	13	9	22	0.60
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	625	678	753	733	0.75
Total[§§] vitamin D, IU/d	279	326	348	401	0.50
Total fat, gm/d	66	66	61	65	0.59
Dietary fiber, gm/d	15	16	16	15	0.97
Alcohol intake, gm/d	8	10	13	9	0.84
Total serum 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

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

[**] By Fisher's exact $\chi 2$ test for categorical variables, and ANOVA for continuous variables.

[¥] Nonsteroidal anti-inflammatory drug.

[§] At least once a week.

[***] All nutrients energy adjusted using residual method [293].

[§§] Diet plus supplements.

Table 3.2. Expression of APC in the normal-appearing colorectal mucosa during the clinical trial

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	***n***	**Mean**	**Std Err**	***P***	***n***	**Mean**	**Std Err**	***P***	**Rx effect***	**Std Err**	***P*** **	**Relative Effect¥**
Whole crypts												
Placebo	22	1.09	0.08		21	0.97	0.08		0.00	.	.	1.00
Calcium	23	1.07	0.08	0.90	21	0.94	0.08	0.80	-0.02	0.15	0.91	0.98
Vitamin D	23	0.90	0.08	0.10	22	1.00	0.08	0.80	0.22	0.14	0.14	1.25
Ca + Vit D	23	0.97	0.08	0.29	21	0.91	0.08	0.61	0.06	0.15	0.68	1.06
Upper 40% of crypts												
Placebo	22	0.39	0.04		21	0.41	0.04		0.00	.	.	1.00
Calcium	23	0.40	0.04	0.91	21	0.44	0.04	0.51	0.03	0.06	0.66	1.07
Vitamin D	23	0.30	0.04	0.07	22	0.46	0.04	0.31	0.15	0.06	0.03	1.48
Ca + Vit D	23	0.36	0.04	0.47	21	0.41	0.04	0.39	0.04	0.06	0.58	1.10
Lower 60% of crypts												
Placebo	22	0.68	0.05		21	0.57	0.05		0.00	.	.	1.00
Calcium	23	0.70	0.05	0.81	21	0.53	0.05	0.53	-0.06	0.09	0.51	0.90
Vitamin D	23	0.60	0.05	0.24	22	0.56	0.05	0.84	0.07	0.09	0.47	1.11
Ca + Vit. D	23	0.60	0.05	0.24	21	0.52	0.05	0.49	0.03	0.09	0.73	1.04
Φh°°												
Placebo	22	0.35	0.01		21	0.42	0.01		0.00	.	.	1.00
Calcium	23	0.34	0.01	0.06	21	0.46	0.01	0.06	0.04	0.03	0.12	1.10
Vitamin D	23	0.31	0.01	0.10	22	0.46	0.01	0.05	0.07	0.03	0.01	1.21
Ca + Vit. D	23	0.35	0.01	0.86	21	0.44	0.01	0.30	0.01	0.03	0.64	1.03

* Rx 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)]

°° Φh = proportion of APC expression in the upper 40% of crypt

Table 3.3. Expression of β-catenin in the normal-appearing colorectal mucosa during the clinical trial

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	***n***	**Mean**	**Std Err**	***P***	***n***	**Mean**	**Std Err**	***P***	**Rx effect***	**Std Err**	***P*** **	**Relative Effect¥**
Whole crypts												
Placebo	21	0.99	0.04		18	0.98	0.04		0.00	.	.	1.00
Calcium	22	1.01	0.04	0.69	19	0.85	0.05	0.04	-0.15	0.08	0.08	0.85
Vitamin D	21	0.94	0.04	0.45	21	0.82	0.04	0.03	-0.11	0.08	0.18	0.88
Ca + Vit D	22	1.04	0.04	0.38	17	0.92	0.05	0.37	-0.11	0.08	0.20	0.89
Upper 40% of crypts												
Placebo	21	0.38	0.02		18	0.37	0.02		0.00	.	.	1.00
Calcium	22	0.40	0.02	0.52	19	0.33	0.02	0.07	-0.06	0.03	0.06	0.85
Vitamin D	21	0.36	0.02	0.36	21	0.33	0.02	0.12	-0.02	0.03	0.61	0.95
Ca + Vit D	22	0.39	0.02	0.69	17	0.36	0.02	0.61	-0.02	0.03	0.50	0.94
Lower 60% of crypts												
Placebo	21	0.61	0.02		18	0.62	0.03		0.00	.	.	1.00
Calcium	22	0.63	0.02	0.44	19	0.56	0.02	0.11	-0.08	0.05	0.09	0.87
Vitamin D	21	0.58	0.02	0.38	21	0.54	0.02	0.02	-0.05	0.05	0.27	0.91
Ca + Vit. D	22	0.63	0.02	0.58	17	0.59	0.03	0.46	-0.05	0.05	0.36	0.93
Φh°°												
Placebo	21	0.39	0.01		18	0.38	0.01		0.00	.	.	1.00
Calcium	22	0.38	0.01	0.87	19	0.36	0.01	0.16	-0.01	0.01	0.31	0.97
Vitamin D	21	0.39	0.01	0.94	21	0.38	0.01	0.81	0.00	0.01	0.80	1.01
Ca + Vit D	22	0.38	0.01	0.88	17	0.37	0.01	0.67	0.00	0.01	0.81	0.99

* Rx 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)]

°° Φh = proportion of β-catenin expression in the upper 40% of crypt

Table3.4. Expression of E-cadherin in the normal-appearing colorectal mucosa during the clinical trial

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	***n***	**Mean**	**Std Err**	***P***	***n***	**Mean**	**Std Err**	***P***	**Rx effect***	**Std Err**	***P*** **	**Relative Effect¥**
Whole crypts												
Placebo	21	1.13	0.12		19	1.04	0.13		0.00	.	.	1.00
Calcium	21	1.23	0.12	0.69	21	1.09	0.12	0.78	-0.05	0.25	0.85	1.01
Vitamin D	21	0.82	0.12	0.05	18	1.29	0.13	0.17	0.56	0.25	0.03	1.78
Ca + Vit. D	22	0.91	0.12	0.14	19	1.26	0.13	0.23	0.46	0.25	0.08	1.56
Upper 40% of crypts												
Placebo	21	0.44	0.05		19	0.42	0.05		0.00	.	.	1.00
Calcium	21	0.47	0.05	0.70	21	0.45	0.05	0.70	0.00	0.10	0.99	1.01
Vitamin D	21	0.33	0.05	0.09	18	0.56	0.05	0.06	0.25	0.10	0.02	1.78
Ca + Vit. D	22	0.37	0.05	0.26	19	0.55	0.05	0.09	0.20	0.10	0.05	1.56
Lower 60% of crypts												
Placebo	21	0.64	0.07		19	0.60	0.08		0.00	.	.	1.00
Calcium	21	0.69	0.07	0.68	21	0.63	0.07	0.78	-0.01	0.15	0.93	0.98
Vitamin D	21	0.48	0.07	0.12	18	0.75	0.08	0.19	0.31	0.16	0.05	1.68
Ca + Vit. D	22	0.55	0.07	0.38	19	0.70	0.08	0.39	0.19	0.15	0.23	1.35
Φh°°												
Placebo	21	0.41	0.02		19	0.40	0.02		0.00	.	.	1.00
Calcium	21	0.41	0.02	0.92	21	0.41	0.02	0.66	0.01	0.03	0.71	1.03
Vitamin D	21	0.40	0.02	0.61	18	0.45	0.02	0.06	0.05	0.03	0.10	1.14
Ca + Vit. D	22	0.40	0.02	0.60	19	0.46	0.02	0.01	0.07	0.03	0.03	1.18

* Rx 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)]

°° Φh = proportion of E-cadherin expression in the upper 40% of crypt

Table 3.5. Expression of the APC/β-catenin score° in the normal-appearing colorectal mucosa during the clinical trial

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	*n*	Mean	Std Err	*P*	*n*	Mean	Std Err	*P*	Rx effect*	Std Err	*P***	Relative Effect¥
Placebo	21	0.37	0.03		18	0.44	0.03		0.00	.	.	1.00
Calcium	22	0.35	0.03	0.49	18	0.58	0.03	0.003	0.16	0.06	0.01	1.41
Vitamin D	21	0.36	0.03	0.83	20	0.57	0.03	0.004	0.13	0.06	0.02	1.31
Ca + Vit. D	22	0.37	0.03	0.84	17	0.50	0.03	0.18	0.07	0.06	0.26	1.16

° APC/β-catenin score = Φh APC/β-catenin expression in the whole crypt, where Φh APC = ratio of APC expression in the upper 40% of the crypts to the whole crypt.

* Rx 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)]

Chapter 4. A randomized clinical trial of the effects of supplemental calcium and vitamin D_3 on markers of their metabolism in normal mucosa of colorectal adenoma patients

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

Authors Affiliations: [1]Nutrition and Health Sciences Program, Graduate Division of Biological and Biomedical Sciences, Departments of [2]Epidemiology and [3]Biostatistics and Bioinformatics, Rollins School of Public Health, and [4]Winship Cancer Institute, Emory University; [5]Epidemiology Research Program, American Cancer Society; [6]Division of Digestive Diseases, Emory University School of Medicine, Atlanta, Georgia; and [7]Department of Medicine, GI Division, University of Minnesota, Minneapolis, Minnesota

Grant support: National Cancer Institute, National Institutes of Health (R01 CA104637 and R03 CA121873 to R.M.B.); Georgia Cancer Coalition Distinguished Scholar award (to R.M.B.); the Franklin Foundation. The National Cancer Institute, the Georgia Cancer Coalition, and the Franklin Foundation had no influence on the design of the study; the collection, analysis, and interpretation of the data; the decision to submit the manuscript for publication; or the writing of the manuscript.

Corresponding Author:
Roberd M. Bostick
Department of Epidemiology,
Rollins School of Public Health, Emory University
1518 Clifton Road
Northeast, Atlanta, GA 30322.
Phone: 404-727-2671; Fax: 404-727-8737.
E-mail: rmbosti@sph.emory.edu

Running title: Effects of supplemental calcium and vitamin D in human colon
Key words: calcium, vitamin D, colonic neoplasms, biomarkers, clinical trial

Published in: Ahearn, T.U., et al., *A randomized clinical trial of the effects of supplemental calcium and vitamin D3 on markers of their metabolism in normal mucosa of colorectal adenoma patients.* Cancer Res, 2011. 71(2): p. 413-23.

Abstract

In cancer cell lines and rodent models calcium and vitamin D favorably modulate cell proliferation, differentiation, and apoptosis in colonic epithelia. These effects may be modulated by local expression of the calcium receptor (CaR), the vitamin D receptor (VDR), and the P450 cytochromes CYP27B1 and CYP24A1, however, they have yet to be investigated in humans. To address this gap, we conducted a randomized, double-blinded, placebo-controlled 2x2 factorial clinical trial. Patients with at least one pathology-confirmed colorectal adenoma were treated with 2 g/day elemental calcium and/or 800 IU/day vitamin D_3 versus placebo over 6 months (N=92; 23/group). CaR, VDR, CYP27B1, and CYP24A1 expression and distribution in biopsies of normal-appearing rectal mucosa were detected by standardized automated immunohistochemistry and quantified by image analysis. In the calcium-supplemented group CaR expression increased 27% (p=0.03) and CYP24A1 expression decreased 21% (p=0.79). In the vitamin D_3-supplemented group CaR expression increased 39% (p=0.01) and CYP27B1 expression increased 159% (p=0.06). In patients supplemented with both calcium and vitamin D3 VDR expression increased 19% (p=0.13) and CaR expression increased 24% (p=0.05). These results provide mechanistic support for further investigation of calcium and vitamin D_3 as chemopreventive agents against colorectal neoplasms, and CaR, VDR, CYP27B1, and CYP24A1 as modifiable, pre-neoplastic risk biomarkers for colorectal neoplasms.

Introduction

Colorectal cancer remains the second leading cause of cancer deaths in the United States, despite advances in cancer screening and treatment [323]. The molecular basis for colon carcinogenesis has become clearer [99], aiding in the development of much needed treatable pre-neoplastic biomarkers of risk for colorectal neoplasms.

There is strong biological plausibility and animal and human observational evidence for protective effects of calcium and vitamin D, acting separately and synergistically through, at least in part, their respective receptors against colorectal neoplasms. Colonic luminal calcium binding to the calcium receptor (CaR, also referred to as the calcium sensing receptor) may directly modulate the cell cycle of colonocytes, partly by 1) inhibiting the β-catenin/TCF transcription complex [324, 325], 2) promoting activation of E-cadherin [324], and 3) reducing the concentration of 25-hydroxyvitamin D 24-hydroxylase (CYP24A1) [326]. Luminal calcium is also hypothesized to bind pro-inflammatory secondary bile acids and ionized fatty acids [324, 327]. Binding of vitamin D to the vitamin D receptor (VDR) may modulate the cell cycle, partly by 1) competitively binding β-catenin [328], 2) up-regulating p21 [296] and E-cadherin expression [329], and 3) regulating growth factors [330]. The VDR also promotes detoxification of the secondary bile acid lithocholic acid [330]. Also, prospective cohort studies have consistently found higher total calcium intake to be associated with reduced risk for colorectal neoplasms [330], calcium supplementation reduces colorectal adenoma recurrence (modified by vitamin D status) [308], and higher circulating concentrations of 25(OH)D are inversely associated with colorectal neoplasms [188, 309, 330].

The CaR is expressed in all parts of the gastrointestinal tract and sporadically expressed in well- to moderately-differentiated colon carcinomas; however, little to no expression of the CaR is found in undifferentiated carcinomas [331]. Colorectal mucosa expresses the VDR; the

$1,25(OH)_2D$ synthesizing enzyme, 25(OH)D-1α-hydroxylase (CYP27B1); and the catabolizing enzyme CYP24A1, suggesting an autocrine/paracrine function for vitamin D in colorectal mucosa that is separate from its role in systemic calcium homeostasis [332]. Expression of the VDR and CYP27B1 was reported to be increased in well- to moderately-differentiated colon carcinomas; however, little to no expression was observed in undifferentiated colon carcinomas [276-278, 333]. Expression of CYP24A1 was reported to be increased in undifferentiated colon carcinomas relative to moderately differentiated colon carcinomas [278, 333].

CaR, VDR, CYP27B1, and CYP24A1 are appealing potential modifiable biomarkers of risk for colorectal neoplasms given the reported differences in their expression between cancerous and normal mucosa and their functional importance in modulating the disease-reducing actions of calcium and vitamin D. We know of no other reported human studies in which the effects of calcium and vitamin D supplementation on the expression of these markers in normal human colorectal mucosa were evaluated. To address this, as reported herein, 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 versus placebo over 6 months, to estimate the efficacy of these agents on markers of calcium and vitamin D metabolism in the normal colorectal mucosa.

Study Participants and Methods

Participant population. A detailed description of the study protocol for recruitment procedures and detailed specific exclusions was published previously [259]. Briefly, eligible participants were 30 to 75 years of age, in general good health, and had a history of at least one pathology-confirmed adenomatous colorectal polyp within the past 36 months. Exclusions from participation included contraindications to calcium or vitamin D supplementation or rectal

biopsy procedures, and medical conditions, habits, or medication usage that potentially could interfere with the study. Participants were recruited from patients attending the Digestive Diseases Clinic at the Emory Clinic, Emory University.

Clinical trial protocol. Between April 2005 and January 2006, 522 potentially eligible patients were identified through initial screening of electronic medical records; of these, 244 (43%) were contacted, and of these 105 (47%) attended the eligibility visit to be interviewed, sign a consent form, complete questionnaires, and provide blood samples [259]. Diet was assessed using a semi-quantitative Willett Food Frequency Questionnaire [334]. Medical and pathology records were reviewed. Following a 30-day placebo run-in trial, 92 (88%) participants with no significant perceived side effects and who took at least 80% of their assigned tablets underwent a baseline rectal biopsy, and, with additional consent, were randomly assigned to the following four treatment groups: 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 vitamin D_3 supplementation ($n = 23$). Additional details and rationale for the doses and forms of calcium and vitamin D_3 supplements were previously published [259]. Participants were instructed to maintain their usual diet and not take any new nutritional supplements they were not taking at the time of entry into the study. All aspects of the trial were approved by the Institutional Review Board of Emory University.

During the 6-month treatment period, participants attended follow-up visits 2 and 6 months after randomization. At follow-up visits participants completed questionnaires and were interviewed about adherence and adverse events. At the 6-month follow-up, participants again underwent a venipuncture and rectal biopsy. All visits for a given participant were scheduled for the same time of day to control for potential circadian variation. Dietary, lifestyle,

and other factors hypothesized to modify biomarker expression in normal colon mucosa were assessed at baseline and at 6-months follow-up. Participants were asked to abstain from aspirin use seven days prior to each biopsy. Participants were not required to be fasting for their visits and did not take a bowel cleansing preparation or enema.

Six approximately one millimeter thick biopsy specimens were taken from the normal appearing rectal mucosa 10 cm above the level of the external anal aperture through a short rigid sigmoidoscope using a jumbo cup flexible biopsy forceps mounted on a semi-rigid rod. No biopsies were taken within 4.0 cm of a polypoid lesion. Biopsies were placed onto a strip of bibulous paper and immediately placed in phosphate buffered saline (PBS), oriented, transferred to 10% normal buffered formalin for 24 hours, and then transferred to 70% ethanol. Then, within a week, the biopsies were processed and embedded in paraffin blocks (2 blocks of 3 biopsies per participant, per biopsy visit).

Immunohistochemistry protocol. Five slides with 4 levels of 3 micron thick biopsy sections taken 40 microns apart were prepared for each antigen, yielding a total of 20 levels for each antigen. Antigen retrieval was performed by placing the slides in a preheated Pretreatment Module (Lab Vision Corp.) with 100x Citrate Buffer (pH 6.0; DAKO S1699, DAKO Corp.) and steaming them for 40 min. Following antigen retrieval, slides were immunohistochemically processed in a DAKO Automated Immunostainer (DAKO Corp.) using a labeled streptavidin-biotin method for CaR, VDR, CYP27B1, and CYP24A1 (**Table 4.1, Figure 4.1**). No slides were counterstained. After processing, slides were coverslipped with a Leica CV5000 Coverslipper (Leica Microsystems, Inc.). Each staining batch contained positive and negative slides; normal kidney was the positive control tissue for CYP27B1 and CYP24A1, and normal colon tissue was the positive control for CaR and VDR. The negative and positive control slides

were treated identically to the patients' slides except that antibody diluent was used rather than primary antibody on the negative slides.

Protocol for quantifying labeling densities of immunohistochemically detected biomarkers in normal colon crypts ("scoring"). A detailed description of the protocol used to quantify biomarker labeling optical densities ("biomarker expression") in normal colon crypts was previously described [259]. Briefly, a scorable crypt was defined as an intact crypt extending from the muscularis mucosa to colon lumen [290]. Prior to "scoring", the negative and positive control slides were checked for staining adequacy. The major equipment and software for the image analysis procedures included: personal computer, light microscope (Olympus BX40, Olympus Corporation, Japan) with appropriate filters and attached digital light microscope camera (Polaroid DMC Digital Light Microscope Camera, Polaroid Corporation, USA), 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 2003 relational database software (Microsoft Corporation, WA).

Evaluation of biomarker expression consisted of the same technician cleaning all slides, selecting the two of the three biopsies with the most scorable crypts per biopsy, creating background correction images for each slide scored, capturing 16-bit grayscale images of crypts at 200x magnification, and tracing the border of the "hemicrypt" (one half of the crypt). The program then divided the outlined hemicrypt into equally spaced segments that corresponded to the average width of colonocytes, and measured the optical density of the labeling across the entire hemicrypt and within each segment, adjusting for the background. The technician then repeated this process for the adjacent hemicrypt, and proceeded to the next crypt, level, biopsy, and/or slide. The goal was to score 16 to 20 hemicrypts per biopsy visit for each biomarker (**Figure 4.2**).

Reliability control was performed by selecting samples of previously analyzed slides to be re-analyzed by the technician. The technician was blinded to the selection. Intra-reader reliability for CaR, VDR, CYP27B1, and CYP24A1 was between 0.95 – 0.99 throughout.

Protocol for measuring serum 25-OH-vitamin D and 1,25-(OH)2-vitamin D levels. Serum 25-OH-vitamin D and 1,25-(OH)2-vitamin D were measured by Dr. Bruce W. Hollis at the Medical University of South Carolina using a RIA method as previously described [311, 312]. Serum 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 was 2.3% and 6.2% for serum 25-OH-vitamin D and for 1,25-(OH)2-vitamin D, respectively.

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 intra-class correlation coefficients.

The mean labeling optical density expression of each biomarker on each study participant, at baseline and 6-month follow-up, was calculated by summing the biomarker's expression for all analyzed crypts and dividing by the total number of analyzed crypts. Biomarker expression was transformed to adjust for possible staining batch effects by dividing an individual's mean biomarker expression by their batch mean biomarker expression [259]. Measures of crypt biomarker distribution selected *a priori* were the upper 40% of the crypts (differentiation zone) and the lower 60% of the crypts (proliferation zone), to represent distinct functional zones of colon crypts. In a sensitivity analysis, we also transformed biomarker expression by subtracting from an individual's mean biomarker expression their batch mean expression; the results did not materially differ from those reported.

Primary analyses were based on assigned treatment at the time of randomization, regardless of adherence status (intent-to-treat analysis). Treatment effects were evaluated by assessing the differences in the transformed biomarker expression from baseline to the 6-month follow-up between participants in the active treatment groups and those in the placebo group by a repeated-measures linear MIXED effects model. The model included the intercept, follow-up visit effects (baseline and follow-up), and interactions between treatment groups and the follow-up visit effect. Because optical density is measured in arbitrary units, to provide perspective on the magnitude of the treatment effects, we also calculated the relative effect. The relative effect was calculated as the (treatment group at follow-up / treatment group at baseline) / (placebo group at follow-up / placebo group at baseline). The relative effect provides a conservative estimate of the proportional change in the treatment group relative to that in the placebo group, and its interpretation is somewhat analogous to that of an odds ratio (e.g., a relative effect of 2.0 would mean that the proportional change in the treatment group was two times that in the placebo group).

The distributions of batch standardized CaR, VDR, CYP27B1, and CYP24A1 labeling optical densities along the full length of the crypts were graphically plotted and evaluated using the LOESS procedure. First, each hemicrypt was standardized to 50 sections. Then, the average of each section across all crypts was predicted by the LOESS model separately for each patient and then for each treatment group by visit. The results were graphically plotted along with the smoothing lines. Although the plots illustrate the distribution of expression, they do not provide a complete analysis of treatment effects because they do not account for changes in the placebo group.

Statistical analyses were performed using SAS 9.2 statistical software (SAS Institute Inc.). A P value ≤ 0.05 (two-sided) was considered statistically significant.

Results

***Characteristics of study participants*.** Baseline characteristics of study participants did not significantly differ by treatment group (**Table 4.2**). The mean age of study participants was 61 years, 70% 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 significantly differ among the four treatment groups. On average, at least 80% of pills were taken by 93% of participants at the first follow-up visit and by 84% of participants at the final follow-up visit. No adverse events were attributed to study procedures or treatments. Seven participants (8%) were lost to follow-up. Dropouts included one person from the vitamin D_3 supplementation group and two from each of the other three groups.

Baseline serum 25-OH-vitamin D and 1,25-$(OH)_2$-vitamin D levels did not differ between the four treatment groups. At the conclusion of the study, serum 25-OH-vitamin D levels had increased 60% ($p<0.0001$) and 56% ($p<0.0001$) in the vitamin D_3 and calcium / vitamin D_3 groups, respectively, relative to placebo; however, mean serum 25-OH-vitamin D concentrations remained below 32 ng/ml in all treatment groups [259]. There was no evidence of treatment effect modification by obesity status (body mass index ≥30).

Calcium receptor. CaR expression along the full lengths of colorectal crypts appeared highest at the base of the crypt and gradually decreased towards the crypt apex (**Figure 4.3A**). After 6 months of treatment, CaR expression statistically significantly increased along the full length of crypts by 27% ($p=0.03$), 39% ($p=0.01$), and 24% ($p=0.05$) in the calcium, vitamin D_3, and calcium/vitamin D_3 groups, respectively, relative to the placebo group. In the upper 40% of crypts the CaR expression increased 21% ($p=0.13$), 38% ($p=0.02$), and 26% ($p=0.07$) in the

calcium, vitamin D_3, and calcium/vitamin D_3 groups, respectively, relative to the placebo group. In the lower 60% of crypts CaR expression increased, 17% (p=0.14), 26% (p=0.06), and 17% (p=0.15) in the calcium, vitamin D_3, and calcium/vitamin D_3 groups, respectively, relative to the placebo group (**Table 4.3A**).

Vitamin D receptor. VDR expression along the full lengths of colorectal crypts appeared lowest at the base of crypts and gradually increased towards the crypt apex (**Figure 4.3B**). After 6 months of treatment, VDR expression increased in the calcium/vitamin D_3 group 19% (p=0.13) along the full length of crypts, 19% (p=0.09) in the upper 40% of crypts, and 31% (p=0.10) in the lower 60% of crypts, relative to the placebo group. In the calcium and vitamin D_3 groups there were also estimated increases in VDR expression, but they were of lower magnitude and not statistically significant (**Table 4.3B**).

CYP27B1. CYP27B1 expression along the full lengths of colorectal crypts at baseline tended to decrease from the base to the mid-third of the crypt where it remained level and then tended to increase slightly from there to the crypt apex (**Figure 4.3C**). After 6 months of treatment, CYP27B1 expression increased in the vitamin D_3 group 159% (p=0.06) along the full length of crypts, 111% (p=0.04) in the upper 40% of crypts, and 110% (p=0.04) in the lower 60% of crypts, relative to the placebo group. In the calcium and the calcium/vitamin D_3 groups, there were also estimated increases in CYP27B1 expression, similar in both treatment groups, but of much lower magnitude than in the vitamin D_3 alone group and not statistically significant (**Table 4.4A**).

CYP24A1. CYP24A1 expression along the full lengths of colorectal crypts appeared highest at the base of crypts and sharply declined towards the upper 40% of the crypts, followed by a leveling off of expression throughout the upper parts of crypts (**Figure 4.3D**). After 6 months of treatment CYP24A1 expression in the calcium group tended to decrease, particularly

in the upper 40% of the crypts, whereas in the vitamin D_3 groups it tended to increase throughout the crypts (**Table 4.4B**); however, despite the large estimated treatment effects none of these findings was statistically significant.

Discussion

The results of this pilot randomized, controlled clinical trial provide the first evidence that supplemental calcium and vitamin D_3, alone or in combination, modify CaR, VDR, CYP27B1, and CYP24A1 expression in the normal colorectal mucosa of sporadic adenoma patients. Following six months of treatment, 2.0 g of calcium daily significantly increased CaR expression, but did not substantially change VDR expression. Although not statistically significant, the results also suggested that calcium substantially increased CYP27B1 and decreased CYP24A1 expression. Treatment with 800 IU of vitamin D_3 daily significantly increased expression of the CaR (slightly more than did calcium) and CYP27B1. Although not statistically significant, the results also suggested that vitamin D_3 increased VDR and CYP24A1 expression. The combined calcium and vitamin D_3 treatment statistically significantly increased CaR expression to about the same degree as calcium treatment alone, and thus less than vitamin D_3 alone. Although not statistically significant, the results also suggested that the combined treatment increased expression of the VDR (more than did calcium or vitamin D_3 alone), CYP27B1 (about the same as did calcium alone, but less than vitamin D_3 alone), and CYP24A1 (about the same as did vitamin D_3 alone). The findings from this study are relevant because there is substantial epidemiological [308, 335, 336] and basic science [333, 337] evidence that calcium and vitamin D_3 may reduce risk of incident or recurrent colorectal neoplasms, and that any protective effects of calcium and vitamin D_3 may, at least in part, operate through the CaR, the VDR, and the vitamin D metabolizing enzymes CYP27B1 and CYP24A1.

Expression of the CaR was reported in normal appearing mucosa in humans [247, 248, 251, 338, 339] and rats [340], and in differentiated carcinomas [247, 248, 251, 338, 339]; however, sporadic to no expression was reported in undifferentiated carcinomas [247, 248, 251, 338, 339]. It was proposed that in normal colon crypts there is a calcium gradient with low calcium concentrations at the base of the crypts and high concentrations at the crypt apex [306]. Decreased calcium concentrations were reported to increase cellular proliferation [245, 325, 339, 341], while increased calcium concentrations were reported to decrease proliferation and promote differentiation [247, 248, 325, 338, 339, 341]. Loss of function of the CaR disrupts the signaling actions from increased calcium concentrations, such as activating E-cadherin and downregulating β-catenin/TCF signaling [338].

There are no reported human randomized clinical trials on the effects of calcium and vitamin D on CaR expression in normal colon mucosa; however, these effects have been evaluated in colon cancer cell lines. In cell culture, incubation with calcium and/or $1,25(OH)_2D_3$ increased CaR expression [248, 341]; calcium and $1,25(OH)_2D_3$ combined increased CaR expression more than did calcium or $1,25(OH)_2D_3$ alone [248]. We therefore hypothesized that supplementation with calcium and vitamin D_3 alone and combined would increase CaR expression in the normal colon mucosa. Our hypothesis was further supported by the presence of vitamin D response elements in both the P1 and P2 promoters of the CaR gene [342]. As hypothesized, we observed increased CaR expression in the calcium, vitamin D, and the combination calcium/vitamin D_3 supplementation groups. Vitamin D_3 supplementation alone appeared to have had the largest treatment effect, while calcium alone and in combination with vitamin D_3 appeared to yield lesser, approximately equal treatment effects. We expected that calcium plus vitamin D_3 supplementation would have the greatest treatment effect. There are several plausible explanations for why this was not observed, the first being that our findings

may have been due to chance. At least one experiment in rodents reported that calcium and vitamin D individually suppressed tumorigenesis, but the combination of the two was ineffective [322]. However, some studies reported stronger combined effects of calcium and vitamin D_3 in reducing adenoma and colorectal cancer risk [343, 344]. Some of the strongest evidence comes from a large clinical trial of colorectal adenoma recurrence that suggested that calcium supplementation was primarily effective among those with 25(OH)D concentrations greater than the median in the study population (29.1 ng/ml) [308]. In our trial only the vitamin D_3 supplementation group reached 25(OH)D concentrations greater than 29.1 ng/ml [259], suggesting the possibility of a threshold effect.

There is little consensus on the distribution of the CaR in the normal colon crypt [248, 251, 338, 340]. Our study, the largest to investigate this thus far, used disease free colon tissue rather than normal appearing sections of colon mucosa adjacent to neoplastic tissue, and, to the best of our knowledge, is the first to use image analysis software to analyze CaR distribution in the normal colon mucosa. We found CaR expression to be highest at the base of the crypts, amongst the most proliferative cells, and to gradually decline in the differentiation zone towards the crypt apex. These results suggest that CaR expression may undergo negative feed-back regulation by luminal calcium.

An autocrine/paracrine function for vitamin D in the normal colon mucosa is suggested by the expression of the VDR and the vitamin D metabolizing enzymes CYP27B1 and CYP24A1 in the colon mucosa [332]. The ability of colon cells to produce the active vitamin D metabolite $1,25(OH)_2D$ was recently demonstrated [279, 345, 346]. Evidence from animal models suggests that the colonic vitamin D system is regulated separately from the renal vitamin D system [326]. It was reported that renal synthesis of $1,25(OH)_2D$ does not produce $1,25(OH)_2D$ in the nanomolar concentrations needed to halt proliferation and induce apoptosis, suggesting that

colonic metabolism of $1,25(OH)_2D$ is needed to explain vitamin D's possible anti-colon carcinogenesis effects [333]. The expression of the VDR and CYP27B1 was reported to increase in the early stages of cancer development, but to be greatly reduced in undifferentiated cancers; this suggests that the VDR and CYP27B1 act as tumor suppressors, presumably by maximizing $1,25(OH)_2D$ signaling to normalize cell cycle regulation [276-278]. CYP24A1 expression was reported to be increased in undifferentiated cancers, presumably mitigating the effects of $1,25(OH)_2D$ signaling [278, 279]. Our observation of increased VDR and CYP27B1 expression in the active treatment groups is in agreement with previous reports, which suggests that the anti-proliferative and pro-apoptotic effects of supplemental vitamin D_3 may in part be due to increased VDR and CPY27B1 expression. We also observed an insignificant increase in CYP24A1 expression in the vitamin D and calcium plus vitamin D treatment groups, which may minimize the effects of increased VDR and CYP27B1 expression. However, the apparent decrease of CYP24A1 and increase in CYP27B1 in the calcium treatment group may lead to increased vitamin D signaling despite no increase in VDR expression; however, these estimated treatment effects were not statistically significant. The distribution of the VDR and CYP27B1 in the upper 40% of crypts provides additional evidence of the importance of vitamin D signaling for promoting differentiation and apoptosis. CYP24A1 also appeared to be expressed most strongly in the bottom half of the colon crypts, presumably lessening the effects of 1,25(OH)D and thus favoring proliferation.

There are only a few cell culture and animal studies, and no large randomized human trials on the effects of calcium and/or vitamin D on the expression of the VDR, CYP27B1, or CYP24A1 in the normal human colon mucosa. The effects of $25(OH)D_3$ or $1,25,(OH)_2D_3$ on VDR and CYP27B1 expression in colon cancer cells appear to vary depending upon the differentiation status of the cells; however, CYP24A1 expression consistently increases in response to $25(OH)D_3$

or 1,25,$(OH)_2D_3$ treatment [345, 347, 348]. Two separate studies in mice found that low calcium diets increased CYP24A1 mRNA expression, especially in male mice and in the right colon. The low calcium diet also increased VDR and CYP27B1 mRNA expression in the proximal colon and reduced VDR expression in the distal colon; however, this was only observed in female mice. No significant change in VDR or CYP27B1 expression was observed in either the left or right colon in male mice fed a low calcium diet [349, 350].

We previously reported that calcium and vitamin D_3 supplementation in this same trial favorably modified expression of markers of proliferation [296], differentiation [296], apoptosis [259], and oxidative DNA damage [297] in the normal human colorectal mucosa. Previous reports in cancer cell lines [254] and human studies [297] suggest that some of the chemopreventative effects of supplemental calcium and vitamin D_3 may depend on the expression of the CaR and the VDR. Our results provide *in vivo* human evidence that supplemental calcium and vitamin D_3 modify the expression of the CaR, the VDR, CYP27B1, and CYP24A1 in the normal colorectal epithelium, which may then promote calcium and vitamin D anti-neoplastic signaling pathways.

Our study had several limitations. First, it was a pilot study with a relatively small sample size, increasing the role of chance observations and limiting our ability to perform stratified analyses. Also, we only examined the rectal mucosa and therefore treatment effects in other parts of the colon remain unknown. Another limitation is that we measured protein expression but not protein activity, and, therefore, could not correlate changes in expression with changes in protein activity. Finally, these markers are not proven biomarkers of risk and we do not yet know whether changes in CaR, VDR, CYP27B1, or CYP24A1 affect risk for colorectal neoplasms in humans.

The strengths of this study include 1) that it is, to our knowledge, the first randomized, double-blind, placebo-controlled clinical trial to test the effects of supplemental calcium and vitamin D_3, alone and in combination, on components of the calcium and vitamin D metabolizing system in the normal colorectal epithelium in sporadic adenoma patients, 2) the high protocol adherence by study participants, and 3) the automated immunostaining and newly-designed image analysis software to quantify the crypt distribution of the expression of the CaR, the VDR, CYP27B1, and CYP24A1, resulting in high biomarker measurement reliability.

In summary, the results of this pilot clinical trial indicate that calcium and vitamin D_3 supplementation, alone and in combination, may increase expression of the CaR, the VDR, and CYP27B1, and that vitamin D_3 supplementation, alone and in combination with calcium supplementation, may increase CYP24A1 expression in the normal colorectal mucosa of sporadic colorectal adenoma patients. The anti-carcinogenesis effects of supplemental calcium and vitamin D_3 may in part depend on the ability of these agents to favorably modulate the expression of the CaR, the VDR, CYP27B1, and CYP24A1 in the colorectal mucosa. These results, taken together with previous reports [259, 296, 297], suggest that calcium and vitamin D_3 supplementation modulate the colorectal mucosa molecular phenotype to inhibit proliferation and promote cellular differentiation and apoptosis. Our results also suggest that the CaR, the VDR, CYP27B1, and CYP24A1 may be treatable biomarkers of risk for colorectal neoplasms and warrant further investigation. Finally, our results support further investigation of calcium and vitamin D_3 as chemopreventive agents against colorectal neoplasms.

Table 4.1. Summary of biomarker immunohistochemical protocols for: calcium receptor, vitamin D receptor, CYP27B1, and CYP24A1

Antibody	Manufacturer	Catalog No.	Dilution	Detection kit*
CaR	Sigma-Aldrich Co., St. Louis, MO	C0493	1:200	LSAB2
VDR	Santa Cruz Inc., Santa Cruz, CA	SC-13133	1:7500	LSAB2
CYP27B1	Santa Cruz Inc., Santa Cruz, CA	SC-49642	1:100	LSAB+
CYP24A1	Santa Cruz Inc., Santa Cruz, CA	SC-32166	1:50	LSAB+

* DAKO Corp., Carpinteria, CA

Table 4.2. 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*-value[**]**
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 CRC in 1° relative (%)	17	30	17	13	0.60
Take NSAID[¥] regularly[§] (%)	22	13	9	22	0.60
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	625	678	753	733	0.75
Total[§§] vitamin D, IU/d	279	326	348	401	0.50
Total fat, gm/d	66	66	61	65	0.59
Dietary fiber, gm/d	15	16	16	15	0.97
Alcohol intake, gm/d	8	10	13	9	0.84
Total serum 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

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

[**] By Fisher's exact $\chi 2$ test for categorical variables, and ANOVA for continuous variables.

[¥] Nonsteroidal anti-inflammatory drug.

[§] At least once a week.

[***] All nutrients energy adjusted using residual method [293].

[§§] Diet plus supplements.

Table 4.3. Expression of calcium receptor in the normal-appearing colorectal mucosa during the clinical trial

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	***n***	**Mean**	**Std Err**	***P***	***n***	**Mean**	**Std Err**	***P***	**Rx effect***	**Std Err**	***P*** **	**Relative Effect**¥
Whole crypts												
Placebo	20	1.11	0.07		19	1.00	0.07		0.00	.	.	1.00
Calcium	22	1.01	0.06	0.28	20	1.16	0.07	0.09	0.26	0.12	0.03	1.27
Vitamin D	23	0.87	0.06	0.01	19	1.09	0.07	0.37	0.33	0.12	0.01	1.39
Ca + Vit D	22	1.04	0.06	0.45	20	1.16	0.07	0.10	0.23	0.12	0.05	1.24
Upper 40% of crypts												
Placebo	20	0.34	0.02		19	0.32	0.02		0.00	.	.	1.00
Calcium	22	0.31	0.02	0.37	20	0.35	0.02	0.33	0.06	0.04	0.13	1.21
Vitamin D	23	0.25	0.02	0.01	19	0.33	0.02	0.89	0.09	0.04	0.02	1.38
Ca + Vit D	22	0.29	0.02	0.13	20	0.35	0.02	0.45	0.07	0.04	0.07	1.26
Lower 60% of crypts												
Placebo	20	0.76	0.05		19	0.74	0.05		0.00	.	.	1.00
Calcium	22	0.73	0.04	0.63	20	0.84	0.05	0.16	0.12	0.08	0.14	1.17
Vitamin D	23	0.63	0.04	0.04	19	0.77	0.05	0.66	0.16	0.08	0.06	1.26
Ca + Vit D	22	0.73	0.04	0.57	20	0.83	0.05	0.18	0.12	0.08	0.15	1.17

* Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

Table 4.4 Expression of vitamin D receptor in the normal-appearing colorectal mucosa during the clinical trial

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	***n***	**Mean**	**Std Err**	***P***	***n***	**Mean**	**Std Err**	***P***	**Rx effect***	**Std Err**	***P*** **	**Relative Effect¥**
Whole crypts												
Placebo	21	1.06	0.06		18	1.00	0.07		0.00	.	.	1.00
Calcium	21	0.95	0.06	0.19	21	0.93	0.06	0.46	0.05	0.12	0.67	1.05
Vitamin D	22	0.93	0.06	0.14	17	0.97	0.07	0.70	0.09	0.12	0.43	1.10
Ca + Vit D	21	1.06	0.06	0.92	18	1.18	0.07	0.07	0.18	0.12	0.13	1.19
Upper 40% of crypts												
Placebo	21	0.58	0.03		18	0.59	0.04		0.00	.	.	1.00
Calcium	21	0.52	0.03	0.19	21	0.56	0.03	0.47	0.03	0.06	0.66	1.05
Vitamin D	22	0.49	0.03	0.04	17	0.55	0.04	0.38	0.05	0.06	0.41	1.11
Ca + Vit D	21	0.54	0.03	0.42	18	0.66	0.04	0.19	0.10	0.06	0.09	1.19
Lower 60% of crypts												
Placebo	21	0.47	0.04		18	0.41	0.04		0.00	.	.	1.00
Calcium	21	0.46	0.04	0.81	21	0.41	0.04	0.92	0.01	0.07	0.92	1.01
Vitamin D	22	0.45	0.04	0.58	17	0.44	0.04	0.58	0.06	0.08	0.44	1.14
Ca + Vit D	21	0.45	0.04	0.69	18	0.52	0.04	0.06	0.13	0.08	0.10	1.31

* Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

Table 4.5. Expression of CYP27B1[§] in the normal-appearing colorectal mucosa during the clinical trial.

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	***n***	**Mean**	**Std Err**	***P***	***n***	**Mean**	**Std Err**	***P***	**Rx effect[*]**	**Std Err**	***P*[**]**	**Relative Effect[¥]**
Whole crypts												
Placebo	20	1.07	0.18		20	1.00	0.09		0.00	.	.	1.00
Calcium	21	0.89	0.10	0.38	16	1.11	0.36	0.76	0.29	0.42	0.49	1.34
Vitamin D	22	0.85	0.12	0.31	18	2.06	0.61	0.10	1.28	0.66	0.06	2.59
Ca + Vit D	22	1.20	0.15	0.56	19	1.46	0.41	0.29	0.32	0.48	0.50	1.30
Upper 40% of crypts												
Placebo	20	0.40			20	0.45	0.10	0.10	0.00	.	.	1.00
Calcium	21	0.38	0.10	0.86	16	0.51	0.11	0.70	0.08	0.21	0.69	1.21
Vitamin D	22	0.35	0.10	0.73	18	0.84	0.11	0.01	0.44	0.20	0.04	2.11
Ca + Vit D	22	0.46	0.10	0.67	19	0.57	0.10	0.43	0.05	0.20	0.79	1.09
Lower 60% of crypts												
Placebo	20	0.65	0.13		20	0.58	0.13		0.00	.	.	1.00
Calcium	21	0.52	0.13	0.47	16	0.60	0.14	0.92	0.15	0.25	0.55	1.30
Vitamin D	22	0.51	0.13	0.44	18	0.96	0.14	0.05	0.52	0.25	0.04	2.10
Ca + Vit D	22	0.67	0.13	0.95	19	0.72	0.13	0.43	0.14	0.25	0.59	1.23

[§] CYP27B1 = Cytochrome P450 family 27B1

[*] Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

Table 4.6. Expression of CYP24A1[§] in the normal-appearing colorectal mucosa during the clinical trial.

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	***n***	**Mean**	**Std Err**	***P***	***n***	**Mean**	**Std Err**	***P***	**Rx effect[*]**	**Std Err**	***P*[**]**	**Relative Effect[¥]**
Whole crypts												
Placebo	21	1.04	0.21		18	1.00	0.23		0.00	.	.	1.00
Calcium	20	1.05	0.21	0.96	20	0.80	0.21	0.52	-0.22	0.43	0.62	0.79
Vitamin D	21	0.85	0.21	0.52	19	1.34	0.22	0.28	0.53	0.43	0.22	1.65
Ca + Vit D	22	1.06	0.20	0.94	17	1.54	0.23	0.10	0.52	0.43	0.24	1.51
Upper 40% of crypts												
Placebo	21	0.37	0.09		18	0.40	0.10		0.00	.	.	1.00
Calcium	20	0.42	0.09	0.70	20	0.27	0.09	0.35	-0.18	0.19	0.35	0.59
Vitamin D	21	0.31	0.09	0.68	19	0.51	0.10	0.40	0.17	0.19	0.37	1.51
Ca + Vit D	22	0.40	0.09	0.81	17	0.59	0.10	0.17	0.16	0.19	0.39	1.38
Lower 60% of crypts												
Placebo	21	0.69	0.13		18	0.63	0.14		0.00	.	.	1.00
Calcium	20	0.66	0.13	0.89	20	0.58	0.13	0.79	-0.03	0.25	0.92	0.96
Vitamin D	21	0.61	0.13	0.67	19	0.78	0.14	0.46	0.22	0.25	0.39	1.38
Ca + Vit D	22	0.74	0.13	0.78	17	0.89	0.14	0.21	0.21	0.26	0.43	1.31

[§] CYP24A1 = Cytochrome P450 family 24A1

[*] Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

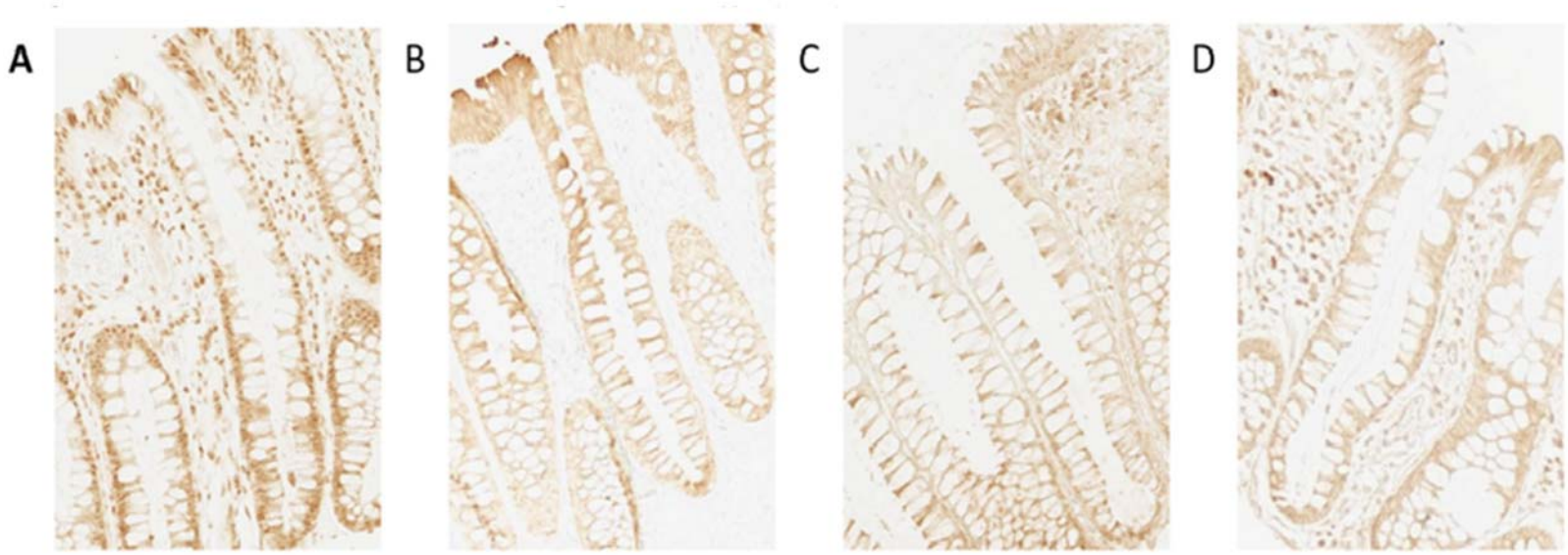


Figure 4.1 Biomarker immunohistochemical images of colon crypts (200x)

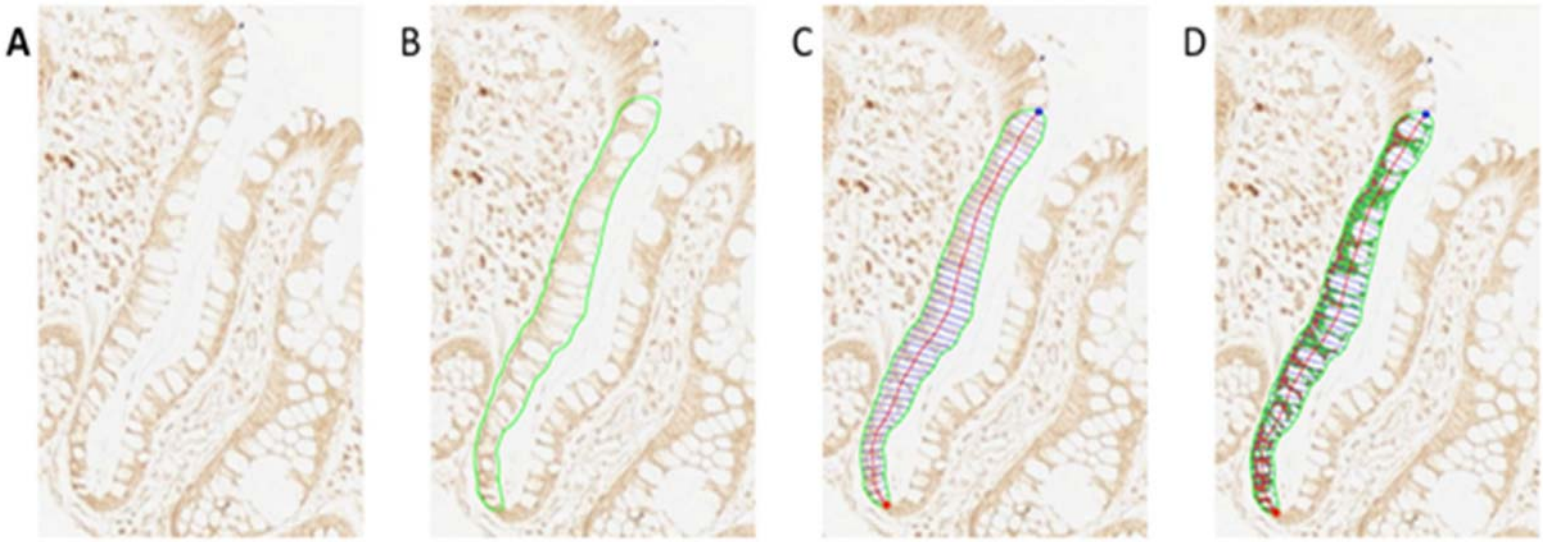
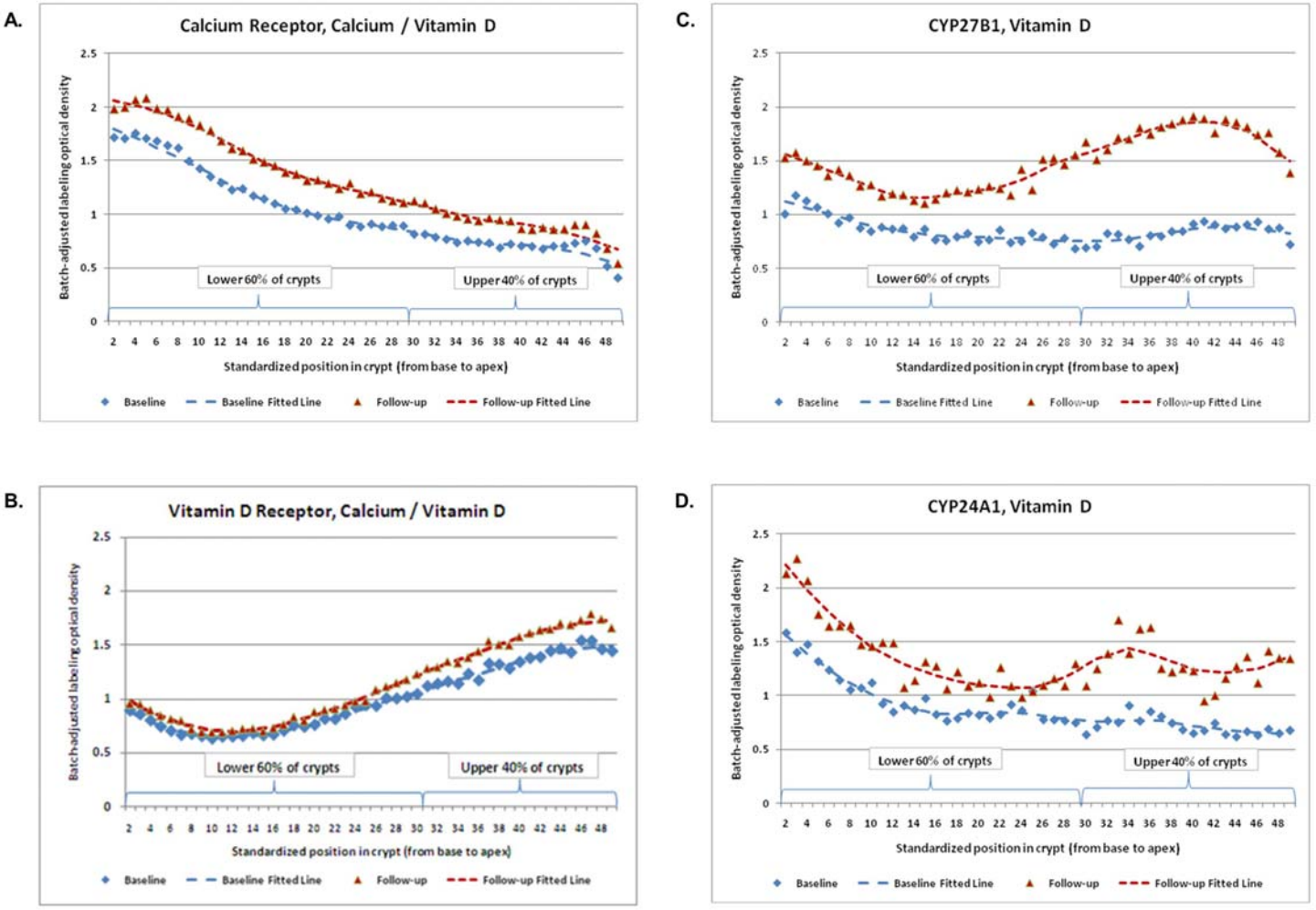


Figure 4.2. Quantitative image analysis

Figure 4.3. Representative examples of labeling expression distribution* of the (A) calcium receptor†, the (B) vitamin D receptor†, (C) CYP27B1‡, and (D) CYP24A1‡ along normal colorectal crypts by treatment group at baseline and 6-month follow-up



Chapter 5. Summary and Public Health Implications

Multiple lines of evidence including migration, basic science, animal, and observational studies and randomized, placebo-controlled clinical trials suggest an important role for diet and life-style factors in the etiology of colorectal cancer [8, 123, 124, 257, 308, 351]. There is an urgent need to develop treatable, pre-neoplastic biomarkers of risk for colorectal neoplasms that can be used to enhance clinical risk identification, stratification, monitoring, and facilitate the development of chemopreventive agents against the disease. Currently the colorectal adenoma is the only validated biomarker of risk for colorectal cancer. The removal of adenomas during colonoscopy is an effective procedure for reducing colorectal cancer incidence and mortality [22]; however, the benefit of colonoscopies are highly operator dependent, with adenoma miss rates between 17 – 24% [16, 18, 19]. Treatable, pre-neoplastic biomarkers of risk have the potential of being valuable resources to accompany colonoscopy screening. In a scenario of a missed neoplasm during a colonoscopy, a person's biomarker profile may recommend that they undergo a follow-up colonoscopy sooner rather than wait the 10 years recommended for an average risk individual. Their biomarker profile may also help guide an appropriate life style and dietary intervention to further reduce the risk of colorectal cancer developing or progressing. This dissertation sought to address this lack in knowledge by investigating the potential of markers of the Wnt/β-catenin signaling pathway (APC, β-catenin, and E-cadherin) and markers of calcium and vitamin D metabolism (CaR, VDR, CYP27B1, and CYP24A1) as potential treatable, pre-neoplastic biomarkers of risk for colorectal neoplasms.

In the MAP II study we found controls to have a statistically significantly greater APC/ β-catenin score than cases. We suggest that the APC/β-catenin score may reflect the potential of β-catenin to promote proliferation. The APC/β-catenin score was positively associated plausible protective risk factors for colorectal neoplasms. Controls had greater Φh APC and lower β-

catenin expression than cases; however, neither of these findings was statistically significant, and E-cadherin expression (**Appendix Table A1**) did not appreciably differ between cases and controls. E-cadherin expression was associated with risk factors for colorectal neoplasms, but often in the opposite of the hypothesized direction (**Appendix Table A2**)**.**

Results from the CaDvMAP study suggest supplemental calcium and vitamin D_3, alone and in combination, may modify the Wnt/β-catenin signaling pathway and markers of calcium and vitamin D metabolism in the normal colorectal mucosa of cases of incident, sporadic colorectal adenoma. In the vitamin D_3 treatment group APC expression increased in all functional zones of the crypt, and in the calcium treatment group APC expression increased in the Φh of the crypt. In all active treatment groups β-catenin expression uniformly decreased and the APC/β-catenin score increased. E-cadherin expression uniformly increased in both vitamin D_3 treatment groups. In all active treatment groups CaR expression uniformly increased, VDR increased (most in the bottom 60% of the crypt), and CYP27B1 expression uniformly increased. CYP24A1 expression uniformly decreased in the calcium treatment group and uniformly increased in the vitamin D_3 treatment groups. Unexpectedly, with the exception of the VDR, the treatment effects in the calcium/vitamin D_3 treatment group tended to be attenuated compared to supplemental calcium and vitamin D_3 alone.

Stratified analyses of the CaDvMAP study were conducted by biologically plausible modifiers of vitamin D metabolism (sex, age, obesity, baseline 25(OH)D_3 concentrations, and *BsmI* genotype) to evaluate for potential modification of treatment effects (**Appendix Table B1-B20, C5- C24**). We found a lack of consistent evidence to suggest the treatment effects were modified by potential modifiers of vitamin D metabolism. This may be due to the relatively small sample size of the CaDvMAP, limiting our statistical power and increasing the role of chance observations.

Overall, the results of this dissertation provide the first human *in vivo* evidence that 1) suggest APC and β-catenin expression as potentially treatable, pre-neoplastic biomarkers of risk for colorectal neoplasms; 2) suggest that the antineoplastic effects of calcium and vitamin D_3 may, in part, depend of their ability to favorably modify APC, β-catenin, E-cadherin, CaR, VDR, CYP27B1, and CYP24A1 expression in the normal colorectal mucosa; and 3) provide support for conducting further, larger investigations to evaluate the potential of APC, β-catenin, E-cadherin, CaR, VDR, CYP27B1, and CYP24A1 expression in the normal colorectal mucosa as potentially treatable, pre-neoplastic biomarkers of risk for colorectal neoplasms.

Chapter 6. Future Directions

The results of this dissertation are based on pilot studies with limited sample sizes. I propose conducting a larger follow-up case-control study to the MAPII study that will have greater power to further clarify the role of APC, β-catenin, E-cadherin, CaR, VDR, CYP27B1, and CYP24A1 expression (and other potential biomarkers) as potential treatable, pre-neoplastic biomarkers of risk for colorectal neoplasms. A larger study will also permit increased potential to evaluate potential effect-modifiers (i.e. NSAIDs, family history of colorectal cancer, race, and sex) that we were limited evaluating in the MAPII study.

In the MAP II and CaDvMAP studies total β-catenin expression was measured, limiting our ability to evaluate β-catenin localized at the membrane from β-catenin localized in the nucleus. We hypothesized that total β-catenin expression would be positively correlated with nuclear β-catenin expression; therefore people with greater total β-catenin expression may also have greater nuclear β-catenin expression. I propose that follow-up studies test this hypothesis by using methods to distinguish membrane β-catenin from nuclear β-catenin. One potential method to accomplish this may be to use quantum dot immunohistochemistry (Q-dot IHC) [352]. Using fluorescent quantum dots to stain for total β-catenin, in combination with 4',6-diamidino-2-phenylindole (DAPI) to stain the nuclear region of cells, it may be possible (using image analysis software) to evaluate membrane β-catenin from nuclear β-catenin.

We proposed that the APC/β-catenin score may represent the proliferative potential of β-catenin, but this score needs to be validated. Another motivation to measure nuclear β-catenin is that it may be used to help validate the APC/β-catenin score. If our hypothesis about the APC/β-catenin score is correct, then the APC/β-catenin score will be positively associated with nuclear β-catenin expression. Other potential down-stream markers of β-catenin

transcription that could also be used to validate the APC/β-catenin score include c-myc and cyclin D1.

There is strong evidence that hyperinsulinemia may be associated with colorectal neoplasms [353-356]; however, there is a lack of human *in vivo* studies that have investigated the association between circulating insulinogenic markers and tissue insulinogenic markers in the normal colorectal mucosa or the potential of these circulating and tissue markers as pre-neoplastic biomarkers of risk for colorectal adenomas. Animal model and *in vitro* studies have demonstrated the pro-proliferating, anti-differentiating, and anti-apoptotic effects of insulin, insulin analogs, and insulin-like growth factor (IGF) signaling [357-375]. I propose a case-control study to investigate the validity of blood markers of insulinogenic signaling (insulin, IGF-1, IGF-2, insulin-like growth factor binding protein-3 [IGFBP-3; intact not total], C-peptide, glucose, and HbA1C) and tissue markers of insulinogenic signaling (insulin receptor, insulin like growth factor-1 receptor, insulin-like growth factor binding protein-3, insulin receptor substrate-1, PI3K, Akt, IκB, mTOR, and FOXO) as potential treatable, pre-neoplastic biomarkers of risk for incident sporadic colorectal adenomas.

Based on the results of the CaDvMAP study, I propose conducting a large randomized clinical trial to investigate the effects of several doses of vitamin D_3 (1,000, 2,000, and 4,000 IU/day) alone and in combination with calcium (1.0 g/day). This will allow us to investigate dose-response trends on 25(OH)D concentrations, to clarify potential interactions between vitamin D and calcium, and to further investigate the effects of vitamin D and calcium on potential biomarkers of risk. A large clinical trial will also give us the opportunity to investigate potential modification of the effects of vitamin D and calcium by sex, race, age, and other dietary and lifestyle behaviors.

References

1. Siegel, R., et al., *Cancer statistics, 2011.* CA: A Cancer Journal for Clinicians, 2011. **61**(4): p. 212-236.
2. Jemal, A., et al., *Global cancer statistics.* CA: A Cancer Journal for Clinicians, 2011. **61**(2): p. 69-90.
3. Center, M.M., A. Jemal, and E. Ward, *International Trends in Colorectal Cancer Incidence Rates.* Cancer Epidemiology Biomarkers & Prevention, 2009. **18**(6): p. 1688-1694.
4. *Amercian Cancer Society. Colorectal Cancer Facts & Figures 2011-2013*, 2011, American Cancer Society: Atlanta.
5. O'Connell, J.B., et al., *Rates of colon and rectal cancers are increasing in young adults.* Am Surg, 2003. **69**(10): p. 866-72.
6. Kohler, B.A., et al., *Annual report to the nation on the status of cancer, 1975-2007, featuring tumors of the brain and other nervous system.* J Natl Cancer Inst, 2011. **103**(9): p. 714-36.
7. Kolonel, L.N., D. Altshuler, and B.E. Henderson, *The multiethnic cohort study: exploring genes, lifestyle and cancer risk.* Nat Rev Cancer, 2004. **4**(7): p. 519-527.
8. Stirbu, I., et al., *Cancer mortality rates among first and second generation migrants in the Netherlands: Convergence toward the rates of the native Dutch population.* International Journal of Cancer, 2006. **119**(11): p. 2665-2672.
9. Stewart, S.L., et al., *A population-based study of colorectal cancer histology in the United States, 1998–2001.* Cancer, 2006. **107**(S5): p. 1128-1141.
10. Levine, J.S. and D.J. Ahnen, *Adenomatous Polyps of the Colon.* New England Journal of Medicine, 2006. **355**(24): p. 2551-2557.
11. Winawer, S.J., et al., *Guidelines for Colonoscopy Surveillance After Polypectomy: A Consensus Update by the US Multi-Society Task Force on Colorectal Cancer and the American Cancer Society.* Gastroenterology, 2006. **130**(6): p. 1872-1885.
12. Lieberman, D.A., et al., *Five-Year Colon Surveillance After Screening Colonoscopy.* Gastroenterology, 2007. **133**(4): p. 1077-1085.
13. Kozuka, S., et al., *Premalignancy of the mucosal polyp in the large intestine: II. Estimation of the periods required for malignant transformation of mucosal polyps.* Diseases of the colon and rectum, 1975. **18**(6): p. 494-500.
14. Kelloff, G.J., et al., *Colorectal Adenomas.* Clinical Cancer Research, 2004. **10**(11): p. 3908-3918.
15. Bressler, B., et al., *Colonoscopic miss rates for right-sided colon cancer: A population-based analysis.* Gastroenterology, 2004. **127**(2): p. 452-456.
16. Rex, D.K., et al., *Colonoscopic miss rates of adenomas determined by back-to-back colonoscopies.* Gastroenterology, 1997. **112**(1): p. 24-28.
17. Pickhardt, P.J., et al., *Location of adenomas missed by optical colonoscopy.* Annals of internal medicine, 2004. **141**(5): p. 352-9.
18. Ahn, S.B., et al., *The Miss Rate for Colorectal Adenoma Determined by Quality-Adjusted, Back-to-Back Colonoscopies.* Gut and liver, 2012. **6**(1): p. 64-70.
19. Heresbach, D., et al., *Miss rate for colorectal neoplastic polyps: a prospective multicenter study of back-to-back video colonoscopies.* Endoscopy, 2008. **40**(04): p. 284,290.
20. Levin, B., et al., *Screening and Surveillance for the Early Detection of Colorectal Cancer and Adenomatous Polyps, 2008: A Joint Guideline From the American Cancer Society, the US Multi-Society Task Force on Colorectal Cancer, and the American College of Radiology.* Gastroenterology, 2008. **134**(5): p. 1570-1595.

21. Zauber, A.G., et al., *Colonoscopic polypectomy and long-term prevention of colorectal-cancer deaths.* The New England journal of medicine, 2012. **366**(8): p. 687-96.
22. Wilkins, J.T. and D.M. Lloyd-Jones, *Biomarkers for Coronary Heart Disease Clinical Risk Prediction: A Critical Appraisal.* Preventive Cardiology, 2010. **13**(4): p. 160-165.
23. Marshman, E., C. Booth, and C.S. Potten, *The intestinal epithelial stem cell.* BioEssays, 2002. **24**(1): p. 91-98.
24. Pinto, D., et al., *Canonical Wnt signals are essential for homeostasis of the intestinal epithelium.* Genes & Development, 2003. **17**(14): p. 1709-1713.
25. Pinto, D. and H. Clevers, *Wnt, stem cells and cancer in the intestine.* Biol Cell, 2005. **97**(3): p. 185-96.
26. Fevr, T., et al., *Wnt/β-Catenin Is Essential for Intestinal Homeostasis and Maintenance of Intestinal Stem Cells.* Molecular and Cellular Biology, 2007. **27**(21): p. 7551-7559.
27. Fearon, E.R. and B. Vogelstein, *A genetic model for colorectal tumorigenesis.* Cell, 1990. **61**(5): p. 759-767.
28. Markowitz, S.D. and M.M. Bertagnolli, *Molecular Basis of Colorectal Cancer.* New England Journal of Medicine, 2009. **361**(25): p. 2449-2460.
29. Vogelstein, B., et al., *Genetic Alterations during Colorectal-Tumor Development.* New England Journal of Medicine, 1988. **319**(9): p. 525-532.
30. Jasperson, K.W., et al., *Hereditary and familial colon cancer.* Gastroenterology, 2010. **138**(6): p. 2044-58.
31. Fearnhead, N.S., J.L. Wilding, and W.F. Bodmer, *Genetics of colorectal cancer: hereditary aspects and overview of colorectal tumorigenesis.* British Medical Bulletin, 2002. **64**(1): p. 27-43.
32. Burt, R., *Colon Cancer Screening.* Gastroenterology, 2000. **119**(3): p. 837-853.
33. Burt, R., *Inheritance of Colorectal Cancer.* Drug Discov Today Dis Mech, 2007. **4**(4): p. 293-300.
34. Kinzler, K.W. and B. Vogelstein, *Lessons from Hereditary Colorectal Cancer.* Cell, 1996. **87**(2): p. 159-170.
35. Sena, P., et al., *Subcellular localization of beta-catenin and APC proteins in colorectal preneoplastic and neoplastic lesions.* Cancer Letters, 2006. **241**(2): p. 203-212.
36. Jass, J.R., *Pathways and Patholgy*. Genetics of Colorectal Cancer, ed. J.D. Potter and N.M. Lindor2009, New York: Springer Science and Business Media
37. Takayama, T., et al., *Colorectal cancer: genetics of development and metastasis.* Journal of Gastroenterology, 2006. **41**(3): p. 185-192.
38. Jass, J.R., *Classification of colorectal cancer based on correlation of clinical, morphological and molecular features.* Histopathology, 2007. **50**(1): p. 113-130.
39. Azcarate-Peril, M.A., M. Sikes, and J.M. Bruno-Barcena, *The intestinal microbiota, gastrointestinal environment and colorectal cancer: a putative role for probiotics in prevention of colorectal cancer?* American journal of physiology. Gastrointestinal and liver physiology, 2011. **301**(3): p. G401-24.
40. Terzić, J., et al., *Inflammation and Colon Cancer.* Gastroenterology, 2010. **138**(6): p. 2101-2114.e5.
41. Greenhough, A., et al., *The COX-2/PGE2 pathway: key roles in the hallmarks of cancer and adaptation to the tumour microenvironment.* Carcinogenesis, 2009. **30**(3): p. 377-386.
42. Fearnhead, N.S., M.P. Britton, and W.F. Bodmer, *The ABC of APC.* Human Molecular Genetics, 2001. **10**(7): p. 721-733.

43. Valvezan, A.J., et al., *Adenomatous Polyposis Coli (APC) Regulates Multiple Signaling Pathways by Enhancing Glycogen Synthase Kinase-3 (GSK-3) Activity.* The Journal of biological chemistry, 2012. **287**(6): p. 3823-32.
44. Su, Y., et al., *APC is essential for targeting phosphorylated beta-catenin to the SCFbeta-TrCP ubiquitin ligase.* Molecular cell, 2008. **32**(5): p. 652-61.
45. Kimelman, D. and W. Xu, *beta-catenin destruction complex: insights and questions from a structural perspective.* Oncogene, 2006. **25**(57): p. 7482-91.
46. Clevers, H., *Wnt/[beta]-Catenin Signaling in Development and Disease.* Cell, 2006. **127**(3): p. 469-480.
47. Iwamoto, M., et al., *Expression of β-catenin and full-length APC protein in normal and neoplastic colonic tissues.* Carcinogenesis, 2000. **21**(11): p. 1935-1940.
48. Anderson, C.B., K.L. Neufeld, and R.L. White, *Subcellular distribution of Wnt pathway proteins in normal and neoplastic colon.* Proceedings of the National Academy of Sciences, 2002. **99**(13): p. 8683-8688.
49. Larriba, M.J., et al., *Vitamin D receptor deficiency enhances Wnt/beta-catenin signaling and tumor burden in colon cancer.* PLoS ONE, 2011. **6**(8): p. e23524.
50. Phelps, R.A., et al., *A Two-Step Model for Colon Adenoma Initiation and Progression Caused by APC Loss.* Cell, 2009. **137**(4): p. 623-634.
51. Phelps, R.A., et al., *New perspectives on APC control of cell fate and proliferation in colorectal cancer.* Cell Cycle, 2009. **8**(16): p. 2549-56.
52. Johnson, V., et al., *Exon 3 beta-catenin mutations are specifically associated with colorectal carcinomas in hereditary non-polyposis colorectal cancer syndrome.* Gut, 2005. **54**(2): p. 264-7.
53. Segditsas, S. and I. Tomlinson, *Colorectal cancer and genetic alterations in the Wnt pathway.* Oncogene, 2006. **25**(57): p. 7531-7.
54. Dikovskaya, D., et al., *Loss of APC induces polyploidy as a result of a combination of defects in mitosis and apoptosis.* The Journal of Cell Biology, 2007. **176**(2): p. 183-95.
55. Etienne-Manneville, S. and A. Hall, *Cdc42 regulates GSK-3beta and adenomatous polyposis coli to control cell polarity.* Nature, 2003. **421**(6924): p. 753-6.
56. Kroboth, K., et al., *Lack of adenomatous polyposis coli protein correlates with a decrease in cell migration and overall changes in microtubule stability.* Molecular biology of the cell, 2007. **18**(3): p. 910-8.
57. Munemitsu, S., et al., *The APC gene product associates with microtubules in vivo and promotes their assembly in vitro.* Cancer Res, 1994. **54**(14): p. 3676-81.
58. Matsumine, A., et al., *Binding of APC to the human homolog of the Drosophila discs large tumor suppressor protein.* Science, 1996. **272**(5264): p. 1020-3.
59. Su, L.K., et al., *APC binds to the novel protein EB1.* Cancer Res, 1995. **55**(14): p. 2972-7.
60. Fodde, R., et al., *Mutations in the APC tumour suppressor gene cause chromosomal instability.* Nat Cell Biol, 2001. **3**(4): p. 433-438.
61. Hiltunen, M.O., et al., *Hypermethylation of the APC (adenomatous Polyposis Coli) gene promoter region in human colorectal carcinoma.* International Journal of Cancer, 1997. **70**(6): p. 644-648.
62. Esteller, M., et al., *Analysis of Adenomatous Polyposis Coli Promoter Hypermethylation in Human Cancer.* Cancer Research, 2000. **60**(16): p. 4366-4371.
63. Bai, A.H.C., et al., *Promoter hypermethylation of tumor-related genes in the progression of colorectal neoplasia.* International Journal of Cancer, 2004. **112**(5): p. 846-853.
64. Masatoshi, T., *Morphogenetic roles of classic cadherins.* Current Opinion in Cell Biology, 1995. **7**(5): p. 619-627.

65. Gottardi, C.J. and B.M. Gumbiner, *Adhesion signaling: How β-catenin interacts with its partners.* Current Biology, 2001. **11**(19): p. R792-R794.
66. Day, R.M., et al., *Changes in the expression of syndecan-1 in the colorectal adenoma–carcinoma sequence.* Virchows Archiv, 1999. **434**(2): p. 121-125.
67. Pap, Z., et al., *An Immunohistochemical Study of Colon Adenomas and Carcinomas: E-cadherin, Syndecan-1, Ets-1.* Pathology & Oncology Research, 2009
68. Gottardi, C.J., E. Wong, and B.M. Gumbiner, *E-cadherin suppresses cellular transformation by inhibiting beta-catenin signaling in an adhesion-independent manner.* The Journal of Cell Biology, 2001. **153**(5): p. 1049-60.
69. Orsulic, S., et al., *E-cadherin binding prevents beta-catenin nuclear localization and beta-catenin/LEF-1-mediated transactivation.* Journal of Cell Science, 1999. **112 (Pt 8)**: p. 1237-45.
70. Sasaki, C.Y., et al., *Truncation of the Extracellular Region Abrogrates Cell Contact but Retains the Growth-suppressive Activity of E-cadherin.* Cancer Research, 2000. **60**(24): p. 7057-7065.
71. Qian, X., et al., *E-cadherin-mediated adhesion inhibits ligand-dependent activation of diverse receptor tyrosine kinases.* EMBO J, 2004. **23**(8): p. 1739-1784.
72. Thiery, J.P., *Epithelial-mesenchymal transitions in tumour progression.* Nat Rev Cancer, 2002. **2**(6): p. 442-454.
73. Morali, O.G., et al., *IGF-II induces rapid beta-catenin relocation to the nucleus during epithelium to mesenchyme transition.* Oncogene, 2001. **20**(36): p. 4942-50.
74. Efstathiou, J.A., et al., *Mutated epithelial cadherin is associated with increased tumorigenicity and loss of adhesion and of responsiveness to the motogenic trefoil factor 2 in colon carcinoma cells.* Proceedings of the National Academy of Sciences of the United States of America, 1999. **96**(5): p. 2316-21.
75. Wheeler, J.M., et al., *Hypermethylation of the promoter region of the E-cadherin gene (CDH1) in sporadic and ulcerative colitis associated colorectal cancer.* Gut, 2001. **48**(3): p. 367-71.
76. Schuhmacher, C., et al., *Loss of immunohistochemical E-cadherin expression in colon cancer is not due to structural gene alterations.* Virchows Archiv : an international journal of pathology, 1999. **434**(6): p. 489-95.
77. Kim, Y.H., et al., *CpG island methylation of genes accumulates during the adenoma progression step of the multistep pathogenesis of colorectal cancer.* Genes, chromosomes & cancer, 2006. **45**(8): p. 781-9.
78. Johns, L.E. and R.S. Houlston, *A systematic review and meta-analysis of familial colorectal cancer risk.* The American journal of gastroenterology, 2001. **96**(10): p. 2992-3003.
79. Lieberman, D.A., et al., *Use of colonoscopy to screen asymptomatic adults for colorectal cancer. Veterans Affairs Cooperative Study Group 380.* The New England journal of medicine, 2000. **343**(3): p. 162-8.
80. Imperiale, T.F., et al., *Risk of advanced proximal neoplasms in asymptomatic adults according to the distal colorectal findings.* The New England journal of medicine, 2000. **343**(3): p. 169-74.
81. Schoenfeld, P., et al., *Colonoscopic screening of average-risk women for colorectal neoplasia.* The New England journal of medicine, 2005. **352**(20): p. 2061-8.
82. de Jonge, V., et al., *Systematic literature review and pooled analyses of risk factors for finding adenomas at surveillance colonoscopy.* Endoscopy, 2011. **43**(7): p. 560-72.

83. Potter, J.D., et al., *Colon Cancer: A Review of the Epidemiology.* Epidemiol Rev, 1993. **15**(2): p. 499-545.
84. Sidney J, W., *Natural history of colorectal cancer.* The American Journal of Medicine, 1999. **106**(1, Supplement 1): p. 3-6.
85. Koo, J.H. and R.W. Leong, *Sex differences in epidemiological, clinical and pathological characteristics of colorectal cancer.* J Gastroenterol Hepatol, 2010. **25**(1): p. 33-42.
86. Simon, M.S., et al., *Racial differences in colorectal cancer incidence and mortality in the Women's Health Initiative.* Cancer Epidemiol Biomarkers Prev, 2011. **20**(7): p. 1368-78.
87. Robbins, A.S., R.L. Siegel, and A. Jemal, *Racial Disparities in Stage-Specific Colorectal Cancer Mortality Rates From 1985 to 2008.* Journal of Clinical Oncology, 2012. **30**(4): p. 401-405.
88. Barnes, C.J., et al., *Non-steroidol anti-inflammatory drug effect on crypt cell proliferation and apoptosis during initiation of rat colon carcinogenesis.* British journal of cancer, 1998. **77**(4): p. 573-80.
89. Chan, A.T., et al., *Aspirin in the Chemoprevention of Colorectal Neoplasia: An Overview.* Cancer Prevention Research, 2012. **5**(2): p. 164-178.
90. Tsujii, M., et al., *Cyclooxygenase regulates angiogenesis induced by colon cancer cells.* Cell, 1998. **93**(5): p. 705-16.
91. Rothwell, P.M., et al., *Long-term effect of aspirin on colorectal cancer incidence and mortality: 20-year follow-up of five randomised trials.* The Lancet, 2010. **376**(9754): p. 1741-1750.
92. Cole, B.F., et al., *Aspirin for the chemoprevention of colorectal adenomas: meta-analysis of the randomized trials.* Journal of the National Cancer Institute, 2009. **101**(4): p. 256-66.
93. Arber, N., et al., *Celecoxib for the Prevention of Colorectal Adenomatous Polyps.* New England Journal of Medicine, 2006. **355**(9): p. 885-895.
94. Baron, J.A., et al., *A Randomized Trial of Rofecoxib for the Chemoprevention of Colorectal Adenomas.* Gastroenterology, 2006. **131**(6): p. 1674-1682.
95. Bertagnolli, M.M., et al., *Celecoxib for the Prevention of Sporadic Colorectal Adenomas.* New England Journal of Medicine, 2006. **355**(9): p. 873-884.
96. Cuzick, J., et al., *Aspirin and non-steroidal anti-inflammatory drugs for cancer prevention: an international consensus statement.* Lancet Oncol, 2009. **10**(5): p. 501-7.
97. Botteri, E., et al., *Smoking and Colorectal Cancer.* JAMA: The Journal of the American Medical Association, 2008. **300**(23): p. 2765-2778.
98. Paskett, E.D., et al., *Association Between Cigarette Smoking and Colorectal Cancer in the Women's Health Initiative.* Journal of the National Cancer Institute, 2007. **99**(22): p. 1729-1735.
99. Potter, J.D., *Colorectal Cancer: Molecules and Populations.* J. Natl. Cancer Inst., 1999. **91**(11): p. 916-932.
100. *Food, Nutrition, Physical Activity, and the Prevention of Cancer: a Global Perspective*, 2007, World Cancer Research Fund / American Institute for Cancer Research: Washington DC.
101. Fedirko, V., et al., *Alcohol drinking and colorectal cancer risk: an overall and dose–response meta-analysis of published studies.* Annals of Oncology, 2011. **22**(9): p. 1958-1972.
102. Pronko, P., et al., *Effect of chronic alcohol consumption on the ethanol- and acetaldehyde-metabolizing systems in the rat gastrointestinal tract.* Alcohol and alcoholism, 2002. **37**(3): p. 229-35.

103. Homann, N., J. Tillonen, and M. Salaspuro, *Microbially produced acetaldehyde from ethanol may increase the risk of colon cancer via folate deficiency.* International journal of cancer. Journal international du cancer, 2000. **86**(2): p. 169-73.
104. Farinati, F., et al., *Effect of chronic ethanol consumption on activation of nitrosopyrrolidine to a mutagen by rat upper alimentary tract, lung, and hepatic tissue.* Drug metabolism and disposition: the biological fate of chemicals, 1985. **13**(2): p. 210-4.
105. Simanowski, U.A., et al., *Increased rectal cell proliferation following alcohol abuse.* Gut, 2001. **49**(3): p. 418-22.
106. Lund, E.K., et al., *Recent advances in understanding the role of diet and obesity in the development of colorectal cancer.* Proceedings of the Nutrition Society, 2011. **70**(02): p. 194-204.
107. Vanamala, J., C.C. Tarver, and P.S. Murano, *Obesity-Enhanced Colon Cancer: Functional Food Compounds and their Mechanisms of Action.* Current Cancer Drug Targets, 2008. **8**(7): p. 611-633.
108. Rinaldi, S., et al., *Serum levels of IGF-I, IGFBP-3 and colorectal cancer risk: results from the EPIC cohort, plus a meta-analysis of prospective studies.* International Journal of Cancer, 2010. **126**(7): p. 1702-1715.
109. Harriss, D.J., et al., *Lifestyle factors and colorectal cancer risk (2): a systematic review and meta-analysis of associations with leisure-time physical activity.* Colorectal disease : the official journal of the Association of Coloproctology of Great Britain and Ireland, 2009. **11**(7): p. 689-701.
110. Wolin, K.Y., et al., *Physical activity and colon cancer prevention: a meta-analysis.* British journal of cancer, 2009. **100**(4): p. 611-6.
111. Wolin, K.Y., et al., *Leisure-time physical activity patterns and risk of colon cancer in women.* Int J Cancer, 2007. **121**(12): p. 2776-81.
112. Gingras, D. and R. Beliveau, *Colorectal cancer prevention through dietary and lifestyle modifications.* Cancer Microenviron, 2011. **4**(2): p. 133-9.
113. Randi, G., et al., *Dietary patterns and the risk of colorectal cancer and adenomas.* Nutrition Reviews, 2010. **68**(7): p. 389-408.
114. Chan, D.S.M., et al., *Red and Processed Meat and Colorectal Cancer Incidence: Meta-Analysis of Prospective Studies.* PLoS ONE, 2011. **6**(6): p. e20456.
115. Sinha, R., et al., *Pan-Fried Meat Containing High Levels of Heterocyclic Aromatic Amines but Low Levels of Polycyclic Aromatic Hydrocarbons Induces Cytochrome P4501A2 Activity in Humans.* Cancer Research, 1994. **54**(23): p. 6154-6159.
116. Solyakov, A. and K. Skog, *Screening for heterocyclic amines in chicken cooked in various ways.* Food and Chemical Toxicology, 2002. **40**(8): p. 1205-1211.
117. Yoon, H., et al., *Systematic review of epidemiological studies on meat, dairy products and egg consumption and risk of colorectal adenomas.* European journal of cancer prevention : the official journal of the European Cancer Prevention Organisation, 2000. **9**(3): p. 151-64.
118. Santarelli, R.L., F. Pierre, and D.E. Corpet, *Processed meat and colorectal cancer: a review of epidemiologic and experimental evidence.* Nutr Cancer, 2008. **60**(2): p. 131-44.
119. Bingham, S.A., et al., *Does increased endogenous formation of N-nitroso compounds in the human colon explain the association between red meat and colon cancer?* Carcinogenesis, 1996. **17**(3): p. 515-23.
120. Lewin, M.H., et al., *Red meat enhances the colonic formation of the DNA adduct O6-carboxymethyl guanine: implications for colorectal cancer risk.* Cancer Research, 2006. **66**(3): p. 1859-65.

121. Lin, J., et al., *Dietary Fat and Fatty Acids and Risk of Colorectal Cancer in Women.* American Journal of Epidemiology, 2004. **160**(10): p. 1011-1022.
122. Pietinen, P., et al., *Diet and risk of colorectal cancer in a cohort of Finnish men.* Cancer Causes and Control, 1999. **10**(5): p. 387-396.
123. Giovannucci, E., et al., *Intake of Fat, Meat, and Fiber in Relation to Risk of Colon Cancer in Men.* Cancer Research, 1994. **54**(9): p. 2390-2397.
124. Bostick, R.M., et al., *Sugar, Meat, and Fat Intake, and Non-Dietary Risk Factors for Colon Cancer Incidence in Iowa Women (United States).* Cancer Causes & Control, 1994. **5**(1): p. 38-52.
125. Flood, A., et al., *Meat, Fat, and Their Subtypes as Risk Factors for Colorectal Cancer in a Prospective Cohort of Women.* American Journal of Epidemiology, 2003. **158**(1): p. 59-68.
126. Daniel, C.R., et al., *Dietary intake of omega-6 and omega-3 fatty acids and risk of colorectal cancer in a prospective cohort of U.S. men and women.* Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology, 2009. **18**(2): p. 516-25.
127. Kim, S., et al., *Intake of Polyunsaturated Fatty Acids and Distal Large Bowel Cancer Risk in Whites and African Americans.* American Journal of Epidemiology, 2010. **171**(9): p. 969-979.
128. Alexander, D.D., et al., *Meta-analysis of animal fat or animal protein intake and colorectal cancer.* The American Journal of Clinical Nutrition, 2009. **89**(5): p. 1402-1409.
129. Berr, F., et al., *7 alpha-dehydroxylating bacteria enhance deoxycholic acid input and cholesterol saturation of bile in patients with gallstones.* Gastroenterology, 1996. **111**(6): p. 1611-20.
130. Reddy, B.S., J.H. Weisburger, and E.L. Wynder, *Effects of high risk and low risk diets for colon carcinogenesis on fecal microflora and steroids in man.* The Journal of Nutrition, 1975. **105**(7): p. 878-84.
131. Hamada, K., et al., *In vitro formation of DNA adducts with bile acids.* Carcinogenesis, 1994. **15**(9): p. 1911-5.
132. Nagengast, F.M., M.J. Grubben, and I.P. van Munster, *Role of bile acids in colorectal carcinogenesis.* European Journal of Cancer, 1995. **31A**(7-8): p. 1067-70.
133. Zhu, Y., et al., *Ca2+- and PKC-dependent stimulation of PGE2 synthesis by deoxycholic acid in human colonic fibroblasts.* American journal of physiology. Gastrointestinal and liver physiology, 2002. **283**(3): p. G503-10.
134. Pai, R., A.S. Tarnawski, and T. Tran, *Deoxycholic acid activates beta-catenin signaling pathway and increases colon cell cancer growth and invasiveness.* Molecular biology of the cell, 2004. **15**(5): p. 2156-63.
135. Lechner, S., et al., *Bile acids mimic oxidative stress induced upregulation of thioredoxin reductase in colon cancer cell lines.* Carcinogenesis, 2002. **23**(8): p. 1281-8.
136. Cockbain, A.J., G.J. Toogood, and M.A. Hull, *Omega-3 polyunsaturated fatty acids for the treatment and prevention of colorectal cancer.* Gut, 2012. **61**(1): p. 135-149.
137. Kelsay, J.L., K.M. Behall, and E.S. Prather, *Effect of fiber from fruits and vegetables on metabolic responses of human subjects I. Bowel transit time, number of defecations, fecal weight, urinary excretions of energy and nitrogen and apparent digestibilities of energy, nitrogen, and fat.* The American Journal of Clinical Nutrition, 1978. **31**(7): p. 1149-53.

138. Steinmetz, K.A. and J.D. Potter, *Vegetables, Fruit, and Cancer. II. Mechanisms.* Cancer Causes & Control, 1991. **2**(6): p. 427-442.
139. Shaughnessy, D.T., et al., *Inhibition of Fried Meat-Induced Colorectal DNA Damage and Altered Systemic Genotoxicity in Humans by Crucifera, Chlorophyllin, and Yogurt.* PLoS ONE, 2011. **6**(4): p. e18707.
140. Phillips, J., et al., *Effect of resistant starch on fecal bulk and fermentation-dependent events in humans.* Am J Clin Nutr, 1995. **62**(1): p. 121-30.
141. Sie, K.K., et al., *Effect of maternal and postweaning folic acid supplementation on colorectal cancer risk in the offspring.* Gut, 2011. **60**(12): p. 1687-94.
142. Fini, L., et al., *Chemoprevention of intestinal polyps in ApcMin/+ mice fed with western or balanced diets by drinking annurca apple polyphenol extract.* Cancer Prev Res (Phila), 2011. **4**(6): p. 907-15.
143. Koushik, A., et al., *Fruits, Vegetables, and Colon Cancer Risk in a Pooled Analysis of 14 Cohort Studies.* Journal of the National Cancer Institute, 2007. **99**(19): p. 1471-1483.
144. Aune, D., et al., *Nonlinear Reduction in Risk for Colorectal Cancer by Fruit and Vegetable Intake Based on Meta-analysis of Prospective Studies.* Gastroenterology, 2011. **141**(1): p. 106-118.
145. Hague, A. and C. Paraskeva, *The short-chain fatty acid butyrate induces apoptosis in colorectal tumour cell lines.* European journal of cancer prevention : the official journal of the European Cancer Prevention Organisation, 1995. **4**(5): p. 359-64.
146. Aune, D., et al., *Dietary fibre, whole grains, and risk of colorectal cancer: systematic review and dose-response meta-analysis of prospective studies.* BMJ, 2011. **343**.
147. Alberts, D.S., et al., *Lack of Effect of a High-Fiber Cereal Supplement on the Recurrence of Colorectal Adenomas.* New England Journal of Medicine, 2000. **342**(16): p. 1156-1162.
148. Bonithon-Kopp, C., et al., *Calcium and fibre supplementation in prevention of colorectal adenoma recurrence: a randomised intervention trial.* The Lancet, 2000. **356**(9238): p. 1300-1306.
149. Schatzkin, A., et al., *Lack of Effect of a Low-Fat, High-Fiber Diet on the Recurrence of Colorectal Adenomas.* New England Journal of Medicine, 2000. **342**(16): p. 1149-1155.
150. Sansbury, L.B., et al., *The effect of strict adherence to a high-fiber, high-fruit and -vegetable, and low-fat eating pattern on adenoma recurrence.* Am J Epidemiol, 2009. **170**(5): p. 576-84.
151. Choumenkovitch, S.F., et al., *Folic acid intake from fortification in United States exceeds predictions.* The Journal of Nutrition, 2002. **132**(9): p. 2792-8.
152. Pogribny, I.P., et al., *Breaks in genomic DNA and within the p53 gene are associated with hypomethylation in livers of folate/methyl-deficient rats.* Cancer Research, 1995. **55**(9): p. 1894-901.
153. Giovannucci, E., *Alcohol, One-Carbon Metabolism, and Colorectal Cancer: Recent Insights from Molecular Studies.* The Journal of Nutrition, 2004. **134**(9): p. 2475S-2481S.
154. Song, J., et al., *Chemopreventive effects of dietary folate on intestinal polyps in Apc+/-Msh2-/- mice.* Cancer Research, 2000. **60**(12): p. 3191-9.
155. Kim, Y.-I., *Folate, colorectal carcinogenesis, and DNA methylation: Lessons from animal studies.* Environmental and Molecular Mutagenesis, 2004. **44**(1): p. 10-25.
156. Cole, B.F., et al., *Folic acid for the prevention of colorectal adenomas: a randomized clinical trial.* JAMA : the journal of the American Medical Association, 2007. **297**(21): p. 2351-9.
157. Logan, R.F., et al., *Aspirin and folic acid for the prevention of recurrent colorectal adenomas.* Gastroenterology, 2008. **134**(1): p. 29-38.

158. Wu, K., et al., *A randomized trial on folic acid supplementation and risk of recurrent colorectal adenoma.* The American Journal of Clinical Nutrition, 2009. **90**(6): p. 1623-1631.
159. Stevens, V.L., et al., *High Levels of Folate From Supplements and Fortification Are Not Associated With Increased Risk of Colorectal Cancer.* Gastroenterology, 2011. **141**(1): p. 98-105.e1.
160. Holick, M.F., *Vitamin D: a D-Lightful health perspective.* Nutrition Reviews, 2008. **66**: p. S182-S194.
161. Holick, M.F., *Vitamin D: evolutionary, physiological and health perspectives.* Current drug targets, 2011. **12**(1): p. 4-18.
162. Bostick, R.M., M. Goodman, and E. Sidelnikov, *Calcium and Vitamin D*. Genetics of Colorectal Cancer, ed. J.D. Potter and L. NM2009, New York: Springer Science + Business Media, LLC.
163. Holick, M.F., *Vitamin D Deficiency.* New England Journal of Medicine, 2007. **357**(3): p. 266-281.
164. Holick, M.F., *Vitamin D deficiency in 2010: Health benefits of vitamin D and sunlight: a D-bate.* Nat Rev Endocrinol, 2011. **7**(2): p. 73-75.
165. Reis, J.P., et al., *Vitamin D Status and Cardiometabolic Risk Factors in the United States Adolescent Population.* Pediatrics, 2009. **124**(3): p. e371-e379.
166. Eaton, S.B. and M. Konner, *Paleolithic nutrition. A consideration of its nature and current implications.* The New England journal of medicine, 1985. **312**(5): p. 283-9.
167. Konner, M. and S.B. Eaton, *Paleolithic Nutrition.* Nutrition in Clinical Practice, 2010. **25**(6): p. 594-602.
168. Luxwolda, M.F., et al., *Traditionally living populations in East Africa have a mean serum 25-hydroxyvitamin D concentration of 115 nmol/l.* The British journal of nutrition, 2012: p. 1-5.
169. Haddad, J.G. and K.J. Chyu, *COMPETITIVE PROTEIN-BINDING RADIOASSAY FOR 25-HYDROXYCHOLECALCIFEROL.* Journal of Clinical Endocrinology & Metabolism, 1971. **33**(6): p. 992-995.
170. Garland, C.F. and F.C. Garland, *Do sunlight and vitamin D reduce the likelihood of colon cancer?* Int J Epidemiol, 1980. **9**(3): p. 227-31.
171. Bostick, R.M., et al., *Relation of calcium, vitamin D, and dairy food intake to incidence of colon cancer among older women. The Iowa Women's Health Study.* Am J Epidemiol, 1993. **137**(12): p. 1302-17.
172. Braun, M.M., et al., *Colon cancer and serum vitamin D metabolite levels 10-17 years prior to diagnosis.* Am J Epidemiol, 1995. **142**(6): p. 608-11.
173. Feskanich, D., et al., *Plasma vitamin D metabolites and risk of colorectal cancer in women.* Cancer Epidemiol Biomarkers Prev, 2004. **13**(9): p. 1502-8.
174. Garland, C., et al., *Dietary vitamin D and calcium and risk of colorectal cancer: a 19-year prospective study in men.* Lancet, 1985. **1**(8424): p. 307-9.
175. Garland, C.F., et al., *Serum 25-hydroxyvitamin D and colon cancer: eight-year prospective study.* Lancet, 1989. **2**(8673): p. 1176-8.
176. Jenab, M., et al., *Association between pre-diagnostic circulating vitamin D concentration and risk of colorectal cancer in European populations:a nested case-control study.* BMJ, 2010. **340**: p. b5500.
177. Kearney, J., et al., *Calcium, vitamin D, and dairy foods and the occurrence of colon cancer in men.* Am J Epidemiol, 1996. **143**(9): p. 907-17.

178. Lee, J.E., et al., *Circulating levels of vitamin D and colon and rectal cancer: the Physicians' Health Study and a meta-analysis of prospective studies.* Cancer Prev Res (Phila), 2011. **4**(5): p. 735-43.
179. Martinez, M.E., et al., *Calcium, vitamin D, and the occurrence of colorectal cancer among women.* J Natl Cancer Inst, 1996. **88**(19): p. 1375-82.
180. McCullough, M.L., et al., *Calcium, vitamin D, dairy products, and risk of colorectal cancer in the Cancer Prevention Study II Nutrition Cohort (United States).* Cancer Causes and Control, 2003. **14**(1): p. 1-12.
181. Otani, T., et al., *Plasma vitamin D and risk of colorectal cancer: the Japan Public Health Center-Based Prospective Study.* Br J Cancer, 2007. **97**(3): p. 446-451.
182. Platz, E.A., et al., *Plasma 1,25-dihydroxy- and 25-hydroxyvitamin D and adenomatous polyps of the distal colorectum.* Cancer Epidemiol Biomarkers Prev, 2000. **9**(10): p. 1059-65.
183. Tangrea, J., et al., *Serum levels of vitamin D metabolites and the subsequent risk of colon and rectal cancer in Finnish men.* Cancer Causes Control, 1997. **8**(4): p. 615-25.
184. Terry, P., et al., *Dietary calcium and vitamin D intake and risk of colorectal cancer: a prospective cohort study in women.* Nutrition and cancer, 2002. **43**(1): p. 39-46.
185. Woolcott, C.G., et al., *Plasma 25-Hydroxyvitamin D Levels and the Risk of Colorectal Cancer: The Multiethnic Cohort Study.* Cancer Epidemiology Biomarkers & Prevention, 2010. **19**(1): p. 130-134.
186. Wu, K., et al., *A Nested Case–Control Study of Plasma 25-Hydroxyvitamin D Concentrations and Risk of Colorectal Cancer.* Journal of the National Cancer Institute, 2007. **99**(14): p. 1120-1129.
187. Wactawski-Wende, J., et al., *Calcium plus vitamin D supplementation and the risk of colorectal cancer.* The New England journal of medicine, 2006. **354**(7): p. 684-96.
188. Gorham, E.D., et al., *Optimal Vitamin D Status for Colorectal Cancer Prevention: A Quantitative Meta Analysis.* American Journal of Preventive Medicine, 2007. **32**(3): p. 210-216.
189. Fedirko, V., et al., *Blood 25-hydroxyvitamin D3 concentrations and incident sporadic colorectal adenoma risk: a pooled case-control study.* Am J Epidemiol, 2010. **172**(5): p. 489-500.
190. Benito, E., et al., *Nutritional factors in colorectal cancer risk: a case-control study in Majorca.* Int J Cancer, 1991. **49**(2): p. 161-7.
191. Boutron, M.C., et al., *Calcium, phosphorus, vitamin D, dairy products and colorectal carcinogenesis: a French case--control study.* Br J Cancer, 1996. **74**(1): p. 145-51.
192. De Stefani, E., et al., *Influence of dietary levels of fat, cholesterol, and calcium on colorectal cancer.* Nutr Cancer, 1997. **29**(1): p. 83-9.
193. Ghadirian, P., et al., *Nutritional factors and colon carcinoma: a case-control study involving French Canadians in Montreal, Quebec, Canada.* Cancer, 1997. **80**(5): p. 858-64.
194. Heilbrun, L.K., et al., *Colon cancer and dietary fat, phosphorus, and calcium in Hawaiian-Japanese men.* Am J Clin Nutr, 1986. **43**(2): p. 306-9.
195. Kampman, E., et al., *Calcium, vitamin D, sunshine exposure, dairy products and colon cancer risk (United States).* Cancer Causes Control, 2000. **11**(5): p. 459-66.
196. Kampman, E., et al., *Fermented dairy products, dietary calcium and colon cancer: a case-control study in The Netherlands.* Int J Cancer, 1994. **59**(2): p. 170-6.
197. Kune, G.A., S. Kune, and L.F. Watson, *Dietary sodium and potassium intake and colorectal cancer risk.* Nutr Cancer, 1989. **12**(4): p. 351-9.

198. La Vecchia, C., et al., *Intake of selected micronutrients and risk of colorectal cancer.* Int J Cancer, 1997. **73**(4): p. 525-30.
199. Le Marchand, L., et al., *Independent and joint effects of family history and lifestyle on colorectal cancer risk: implications for prevention.* Cancer Epidemiol Biomarkers Prev, 1999. **8**(1): p. 45-51.
200. Lee, H.P., et al., *Colorectal cancer and diet in an Asian population--a case-control study among Singapore Chinese.* Int J Cancer, 1989. **43**(6): p. 1007-16.
201. Little, J., et al., *Colorectal adenomas and diet: a case-control study of subjects participating in the Nottingham faecal occult blood screening programme.* Br J Cancer, 1993. **67**(1): p. 177-84.
202. Marcus, P.M. and P.A. Newcomb, *The association of calcium and vitamin D, and colon and rectal cancer in Wisconsin women.* Int J Epidemiol, 1998. **27**(5): p. 788-93.
203. Negri, E., et al., *Calcium, dairy products, and colorectal cancer.* Nutr Cancer, 1990. **13**(4): p. 255-62.
204. Peters, R.K., et al., *Diet and colon cancer in Los Angeles County, California.* Cancer Causes Control, 1992. **3**(5): p. 457-73.
205. Pritchard, R.S., J.A. Baron, and M. Gerhardsson de Verdier, *Dietary calcium, vitamin D, and the risk of colorectal cancer in Stockholm, Sweden.* Cancer Epidemiol Biomarkers Prev, 1996. **5**(11): p. 897-900.
206. Slattery, M.L., et al., *Dietary calcium, vitamin D, VDR genotypes and colorectal cancer.* Int J Cancer, 2004. **111**(5): p. 750-6.
207. Slattery, M.L., A.W. Sorenson, and M.H. Ford, *Dietary calcium intake as a mitigating factor in colon cancer.* Am J Epidemiol, 1988. **128**(3): p. 504-14.
208. Tuyns, A.J., M. Haelterman, and R. Kaaks, *Colorectal cancer and the intake of nutrients: oligosaccharides are a risk factor, fats are not. A case-control study in Belgium.* Nutr Cancer, 1987. **10**(4): p. 181-96.
209. Wakai, K., et al., *Dietary risk factors for colon and rectal cancers: a comparative case-control study.* J Epidemiol, 2006. **16**(3): p. 125-35.
210. White, E., J.S. Shannon, and R.E. Patterson, *Relationship between vitamin and calcium supplement use and colon cancer.* Cancer Epidemiol Biomarkers Prev, 1997. **6**(10): p. 769-74.
211. Yang, C.Y., et al., *Calcium and magnesium in drinking water and risk of death from colon cancer.* Jpn J Cancer Res, 1997. **88**(10): p. 928-33.
212. Cho, E., et al., *Dairy foods, calcium, and colorectal cancer: a pooled analysis of 10 cohort studies.* J Natl Cancer Inst, 2004. **96**(13): p. 1015-22.
213. Flood, A., et al., *Calcium from diet and supplements is associated with reduced risk of colorectal cancer in a prospective cohort of women.* Cancer Epidemiol Biomarkers Prev, 2005. **14**(1): p. 126-32.
214. Gaard, M., S. Tretli, and E.B. Loken, *Dietary factors and risk of colon cancer: a prospective study of 50,535 young Norwegian men and women.* Eur J Cancer Prev, 1996. **5**(6): p. 445-54.
215. Jarvinen, R., et al., *Prospective study on milk products, calcium and cancers of the colon and rectum.* Eur J Clin Nutr, 2001. **55**(11): p. 1000-7.
216. Kampman, E., et al., *Fermented dairy products, calcium, and colorectal cancer in The Netherlands Cohort Study.* Cancer Res, 1994. **54**(12): p. 3186-90.
217. Kato, I., et al., *Prospective study of diet and female colorectal cancer: the New York University Women's Health Study.* Nutr Cancer, 1997. **28**(3): p. 276-81.

218. Kesse, E., et al., *Dietary calcium, phosphorus, vitamin D, dairy products and the risk of colorectal adenoma and cancer among French women of the E3N-EPIC prospective study.* Int J Cancer, 2005. **117**(1): p. 137-44.
219. Larsson, S.C., et al., *Calcium and dairy food intakes are inversely associated with colorectal cancer risk in the Cohort of Swedish Men.* Am J Clin Nutr, 2006. **83**(3): p. 667-73; quiz 728-9.
220. Lin, J., et al., *Intakes of calcium and vitamin D and risk of colorectal cancer in women.* Am J Epidemiol, 2005. **161**(8): p. 755-64.
221. McCullough, M.L., et al., *Calcium, vitamin D, dairy products, and risk of colorectal cancer in the Cancer Prevention Study II Nutrition Cohort (United States).* Cancer Causes Control, 2003. **14**(1): p. 1-12.
222. Park, S.Y., et al., *Calcium and vitamin D intake and risk of colorectal cancer: the Multiethnic Cohort Study.* Am J Epidemiol, 2007. **165**(7): p. 784-93.
223. Pietinen, P., et al., *Diet and risk of colorectal cancer in a cohort of Finnish men.* Cancer Causes Control, 1999. **10**(5): p. 387-96.
224. Sellers, T.A., et al., *Diet and risk of colon cancer in a large prospective study of older women: an analysis stratified on family history (Iowa, United States).* Cancer Causes Control, 1998. **9**(4): p. 357-67.
225. Slob, I.C., et al., *Calcium intake and 28-year gastro-intestinal cancer mortality in Dutch civil servants.* Int J Cancer, 1993. **54**(1): p. 20-5.
226. Stemmermann, G.N., A. Nomura, and P.H. Chyou, *The influence of dairy and nondairy calcium on subsite large-bowel cancer risk.* Dis Colon Rectum, 1990. **33**(3): p. 190-4.
227. Terry, P., et al., *Dietary calcium and vitamin D intake and risk of colorectal cancer: a prospective cohort study in women.* Nutr Cancer, 2002. **43**(1): p. 39-46.
228. Wu, A.H., et al., *Alcohol, physical activity and other risk factors for colorectal cancer: a prospective study.* Br J Cancer, 1987. **55**(6): p. 687-94.
229. Wu, K., et al., *Calcium intake and risk of colon cancer in women and men.* J Natl Cancer Inst, 2002. **94**(6): p. 437-46.
230. Zheng, W., et al., *A prospective cohort study of intake of calcium, vitamin D, and other micronutrients in relation to incidence of rectal cancer among postmenopausal women.* Cancer Epidemiol Biomarkers Prev, 1998. **7**(3): p. 221-5.
231. Palacios, C., et al., *Association between calcium intake and colorectal neoplasia in Puerto Rican Hispanics.* Arch Latinoam Nutr, 2010. **60**(4): p. 348-54.
232. Sun, Z., et al., *Calcium and vitamin D and risk of colorectal cancer: results from a large population-based case-control study in Newfoundland and Labrador and Ontario.* Can J Public Health, 2011. **102**(5): p. 382-9.
233. Baron, J.A., et al., *Calcium supplements for the prevention of colorectal adenomas. Calcium Polyp Prevention Study Group.* N Engl J Med, 1999. **340**(2): p. 101-7.
234. Wallace, K., et al., *Effect of calcium supplementation on the risk of large bowel polyps.* J Natl Cancer Inst, 2004. **96**(12): p. 921-5.
235. Grau, M.V., et al., *Vitamin D, calcium supplementation, and colorectal adenomas: results of a randomized trial.* J Natl Cancer Inst, 2003. **95**(23): p. 1765-71.
236. Shaukat, A., N. Scouras, and H.J. Schunemann, *Role of supplemental calcium in the recurrence of colorectal adenomas: a metaanalysis of randomized controlled trials.* Am J Gastroenterol, 2005. **100**(2): p. 390-4.
237. Bolland, M.J., et al., *Calcium and vitamin D supplements and health outcomes: a reanalysis of the Women's Health Initiative (WHI) limited-access data set.* The American Journal of Clinical Nutrition, 2011. **94**(4): p. 1144-1149.

238. Lupton, J.R., et al., *Calcium supplementation modifies the relative amounts of bile acids in bile and affects key aspects of human colon physiology.* The Journal of Nutrition, 1996. **126**(5): p. 1421-8.
239. Wargovich, M.J., V.W.S. Eng, and H.L. Newmark, *Calcium inhibits the damaging and compensatory proliferative effects of fatty acids on mouse colon epithelium.* Cancer Letters, 1984. **23**(3): p. 253-258.
240. Wargovich, M.J., et al., *Calcium ameliorates the toxic effect of deoxycholic acid on colonic epithelium.* Carcinogenesis, 1983. **4**(9): p. 1205-1207.
241. Kallay, E., et al., *Dietary calcium and growth modulation of human colon cancer cells: role of the extracellular calcium-sensing receptor.* Cancer detection and prevention, 2000. **24**(2): p. 127-36.
242. Rey, O., et al., *Negative cross-talk between calcium-sensing receptor and beta-catenin signaling systems in colonic epithelium.* The Journal of biological chemistry, 2012. **287**(2): p. 1158-67.
243. Van der Meer, R., et al., *Mechanisms of the intestinal effects of dietary fats and milk products on colon carcinogenesis.* Cancer Letters, 1997. **114**(1–2): p. 75-83.
244. Umar, S., et al., *Dietary pectin and calcium inhibit colonic proliferation in vivo by differing mechanisms.* Cell Proliferation, 2003. **36**(6): p. 361-375.
245. Kállay, E., et al., *Calcium-Dependent c-mycProto-Oncogene Expression and Proliferation of CACO-2 Cells: A Role for a Luminal Extracellular Calcium-Sensing Receptor.* Biochemical and Biophysical Research Communications, 1997. **232**(1): p. 80-83.
246. Bostick, R.M., et al., *Calcium and colorectal epithelial cell proliferation in sporadic adenoma patients: a randomized, double-blinded, placebo-controlled clinical trial.* J Natl Cancer Inst, 1995. **87**(17): p. 1307-15.
247. Chakrabarty, S., et al., *Extracellular Calcium and Calcium Sensing Receptor Function in Human Colon Carcinomas: Promotion of E-Cadherin Expression and Suppression of {beta}-Catenin/TCF Activation.* Cancer Res, 2003. **63**(1): p. 67-71.
248. Chakrabarty, S., et al., *Calcium Sensing Receptor in Human Colon Carcinoma: Interaction with Ca2+ and 1,25-Dihydroxyvitamin D3.* Cancer Res, 2005. **65**(2): p. 493-498.
249. Bhagavathula, N., et al., *Regulation of E-cadherin and β-catenin by Ca2+ in colon carcinoma is dependent on calcium-sensing receptor expression and function.* International Journal of Cancer, 2007. **121**(7): p. 1455-1462.
250. Hizaki, K., et al., *Epigenetic inactivation of calcium-sensing receptor in colorectal carcinogenesis.* Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc, 2011. **24**(6): p. 876-84.
251. Sheinin, Y., et al., *Immunocytochemical Localization of the Extracellular Calcium-Sensing Receptor in Normal and Malignant Human Large Intestinal Mucosa.* J. Histochem. Cytochem., 2000. **48**(5): p. 595-602.
252. Palmer, H.G., et al., *Vitamin D3 promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of β-catenin signaling.* J. Cell Biol., 2001. **154**(2): p. 369-388.
253. Egan, J.B., et al., *Vitamin D receptor ligands, adenomatous polyposis coli, and the vitamin D receptor FokI polymorphism collectively modulate β-catenin activity in colon cancer cells.* Molecular Carcinogenesis, 2010. **49**(4): p. 337-352.
254. Liu, G., X. Hu, and S. Chakrabarty, *Vitamin D mediates its action in human colon carcinoma cells in a calcium-sensing receptor-dependent manner: Downregulates malignant cell behavior and the expression of thymidylate synthase and survivin and*

promotes cellular sensitivity to 5-FU. International Journal of Cancer, 2010. **126**(3): p. 631-639.
255. Kizildag, S. and H. Ates, *Treatment of K562 cells with 1,25-dihydroxyvitamin D(3) induces distinct alterations in the expression of apoptosis-related genes BCL2, BAX, BCL(XL), and p21.* Ann Hematol, 2009.
256. Diaz, G.D., et al., *Apoptosis is induced by the active metabolite of vitamin D3 and its analogue EB1089 in colorectal adenoma and carcinoma cells: possible implications for prevention and therapy.* Cancer Res, 2000. **60**(8): p. 2304-12.
257. Yang, K., et al., *Dietary Calcium and Cholecalciferol Modulate Cyclin D1 Expression, Apoptosis, and Tumorigenesis in Intestine of adenomatous polyposis coli1638N/+ Mice.* The Journal of Nutrition, 2008. **138**(9): p. 1658-1663.
258. Larriba, M.J., et al., *Vitamin D Receptor Deficiency Enhances Wnt/β-Catenin Signaling and Tumor Burden in Colon Cancer.* PLoS ONE, 2011. **6**(8): p. e23524.
259. Fedirko, V., et al., *Effects of Vitamin D and Calcium Supplementation on Markers of Apoptosis in Normal Colon Mucosa: A Randomized, Double-Blind, Placebo-Controlled Clinical Trial.* Cancer Prev Res, 2009. **2**(3): p. 213-223.
260. Sidelnikov, E., et al., *Effects of calcium and vitamin D on MLH1 and MSH2 expression in rectal mucosa of sporadic colorectal adenoma patients.* Cancer Epidemiol Biomarkers Prev, 2010. **19**(4): p. 1022-32.
261. Peterlik, M., *Role of bile acid secretion in human colorectal cancer.* Wien Med Wochenschr, 2008. **158**(19-20): p. 539-41.
262. KÖSTNER, K., et al., *The Relevance of Vitamin D Receptor (VDR) Gene Polymorphisms for Cancer: A Review of the Literature.* Anticancer Research, 2009. **29**(9): p. 3511-3536.
263. Jurutka, P.W., et al., *Molecular nature of the vitamin D receptor and its role in regulation of gene expression.* Rev Endocr Metab Disord, 2001. **2**(2): p. 203-16.
264. Arai, H., et al., *A Vitamin D Receptor Gene Polymorphism in the Translation Initiation Codon: Effect on Protein Activity and Relation to Bone Mineral Density in Japanese Women.* Journal of Bone and Mineral Research, 1997. **12**(6): p. 915-921.
265. Jurutka, P.W., et al., *The Polymorphic N Terminus in Human Vitamin D Receptor Isoforms Influences Transcriptional Activity by Modulating Interaction with Transcription Factor IIB.* Molecular Endocrinology, 2000. **14**(3): p. 401-420.
266. Ingles, S., et al., *Vitamin D receptor polymorphisms and risk of colorectal adenomas (United States).* Cancer Causes and Control, 2001. **12**(7): p. 607-614.
267. Murtaugh, M.A., et al., *Vitamin D receptor gene polymorphisms, dietary promotion of insulin resistance, and colon and rectal cancer.* Nutr Cancer, 2006. **55**(1): p. 35-43.
268. Peters, U., et al., *Vitamin D, Calcium, and Vitamin D Receptor Polymorphism in Colorectal Adenomas.* Cancer Epidemiology Biomarkers & Prevention, 2001. **10**(12): p. 1267-1274.
269. Slattery, M., et al., *Variants of the VDR gene and Risk of Colon Cancer (United States).* Cancer Causes and Control, 2001. **12**(4): p. 359-364.
270. Slattery, M.L., et al., *Associations between BMI, energy intake, energy expenditure, <i>VDR genotype and colon and rectal cancers (United States).* Cancer Causes and Control, 2004. **15**(9): p. 863-872.
271. Wong, H.-L., et al., *Vitamin D receptor start codon polymorphism and colorectal cancer risk: effect modification by dietary calcium and fat in Singapore Chinese.* Carcinogenesis, 2003. **24**(6): p. 1091-1095.

272. Yaylım-Eraltan, İ., et al., *Investigation of the VDR gene polymorphisms association with susceptibility to colorectal cancer.* Cell Biochemistry and Function, 2007. **25**(6): p. 731-737.
273. Raimondi, S., et al., *Review and meta-analysis on vitamin D receptor polymorphisms and cancer risk.* Carcinogenesis, 2009. **30**(7): p. 1170-1180.
274. Lee, J.E., *Circulating levels of vitamin D, vitamin D receptor polymorphisms, and colorectal adenoma: a meta-analysis.* Nutr Res Pract, 2011. **5**(5): p. 464-470.
275. Hubner, R.A., et al., *Dairy products, polymorphisms in the vitamin D receptor gene and colorectal adenoma recurrence.* Int J Cancer, 2008. **123**(3): p. 586-93.
276. Bises, G., et al., *25-Hydroxyvitamin D3-1α-hydroxylase Expression in Normal and Malignant Human Colon.* J. Histochem. Cytochem., 2004. **52**(7): p. 985-989.
277. Cross, H.S., et al., *25-Hydroxyvitamin D3-1α-hydroxylase and vitamin D receptor gene expression in human colonic mucosa is elevated during early cancerogenesis.* Steroids, 2001. **66**(3-5): p. 287-292.
278. Cross, H.S., et al., *The Vitamin D endocrine system of the gut--Its possible role in colorectal cancer prevention.* The Journal of Steroid Biochemistry and Molecular Biology, 2005. **97**(1-2): p. 121-128.
279. Bareis, P., et al., *25-Hydroxy-Vitamin D Metabolism in Human Colon Cancer Cells during Tumor Progression.* Biochemical and Biophysical Research Communications, 2001. **285**(4): p. 1012-1017.
280. Jemal, A., et al., *Cancer Statistics, 2010.* CA: A Cancer Journal for Clinicians, 2010. **60**(5): p. 277-300.
281. Goss, K.H. and J. Groden, *Biology of the Adenomatous Polyposis Coli Tumor Suppressor.* Journal of Clinical Oncology, 2000. **18**(9): p. 1967-1979.
282. Willert, K. and K.A. Jones, *Wnt signaling: is the party in the nucleus?* Genes & Development, 2006. **20**(11): p. 1394-1404.
283. Daniel, C.R., et al., *TGF-α Expression as a Potential Biomarker of Risk Within the Normal-appearing Colorectal Mucosa of Patients with and without Incident Sporadic Adenoma.* Cancer Epidemiology Biomarkers & Prevention, 2009. **18**(1): p. 65-73.
284. Sidelnikov, E., et al., *MutL-Homolog 1 Expression and Risk of Incident, Sporadic Colorectal Adenoma: Search for Prospective Biomarkers of Risk for Colorectal Cancer.* Cancer Epidemiology Biomarkers & Prevention, 2009. **18**(5): p. 1599-1609.
285. Sidelnikov, E., et al., *Colorectal Mucosal Expression of MSH2 as a Potential Biomarker of Risk for Colorectal Neoplasms.* Cancer Epidemiology Biomarkers & Prevention, 2009. **18**(11): p. 2965-2973.
286. Pereira, M.A., et al., *A collection of Physical Activity Questionnaires for health-related research.* Med Sci Sports Exerc, 1997. **29**(6 Suppl): p. S1-205.
287. Willett, W.C., et al., *The use of a self-administered questionnaire to assess diet four years in the past.* Am J Epidemiol, 1988. **127**(1): p. 188-99.
288. Willett, W.C., et al., *Reproducibility and validity of a semiquantitative food frequency questionnaire.* American Journal of Epidemiology, 1985. **122**(1): p. 51-65.
289. O'Brien, M.J., et al., *The National Polyp Study. Patient and polyp characteristics associated with high-grade dysplasia in colorectal adenomas.* Gastroenterology, 1990. **98**(2): p. 371-9.
290. Bostick, R.M., et al., *Methodological findings and considerations in measuring colorectal epithelial cell proliferation in humans.* Cancer Epidemiology Biomarkers & Prevention, 1997. **6**(11): p. 931-942.

291. Saenger, A.K., et al., *Quantification of Serum 25-Hydroxyvitamin D2 and D3Using HPLC–Tandem Mass Spectrometry and Examination of Reference Intervals for Diagnosis of Vitamin D Deficiency.* American Journal of Clinical Pathology, 2006. **125**(6): p. 914-920.
292. Long, Q., et al., *Robust statistical methods for analysis of biomarkers measured with batch/experiment-specific errors.* Stat Med, 2010. **29**(3): p. 361-70.
293. Willett, W. and M.J. Stampfer, *Total energy intake: implications for epidemiologic analyses.* Am J Epidemiol, 1986. **124**(1): p. 17-27.
294. Tsanou, E., et al., *The E-Cadherin Adhesion Molecule and Colorectal Cancer. A Global Literature Approach.* Anticancer Research, 2008. **28**(6A): p. 3815-3826.
295. Ahearn, T.U., et al., *A randomized clinical trial of the effects of supplemental calcium and vitamin D3 on markers of their metabolism in normal mucosa of colorectal adenoma patients.* Cancer Res, 2011. **71**(2): p. 413-23.
296. Fedirko, V., et al., *Effects of Vitamin D and Calcium on Proliferation and Differentiation In Normal Colon Mucosa: a Randomized Clinical Trial.* Cancer Epidemiology Biomarkers & Prevention, 2009. **18**(11): p. 2933-2941.
297. Fedirko, V., et al., *Effects of supplemental vitamin D and calcium on oxidative DNA damage marker in normal colorectal mucosa: a randomized clinical trial.* Cancer Epidemiol Biomarkers Prev, 2010. **19**(1): p. 280-91.
298. Risio, M., et al., *Immunohistochemical study of epithelial cell proliferation in hyperplastic polyps, adenomas, and adenocarcinomas of the large bowel.* Gastroenterology, 1988. **94**(4): p. 899-906.
299. Bostick, R.M., et al., *Colorectal epithelial cell proliferative kinetics and risk factors for colon cancer in sporadic adenoma patients.* Cancer Epidemiology Biomarkers & Prevention, 1997. **6**(12): p. 1011-1019.
300. Liu, E.S., et al., *Cyclooxygenase-2 in cancer cells and macrophages induces colon cancer cell growth by cigarette smoke extract.* Eur J Pharmacol, 2005. **518**(1): p. 47-55.
301. Simanowski, U.A., et al., *Effect of alcohol on gastrointestinal cell regeneration as a possible mechanism in alcohol-associated carcinogenesis.* Alcohol. **12**(2): p. 111-115.
302. Ruder, E.H., et al., *Adolescent and mid-life diet: risk of colorectal cancer in the NIH-AARP Diet and Health Study.* The American Journal of Clinical Nutrition, 2011. **94**(6): p. 1607-1619.
303. Gorham, E.D., et al., *Vitamin D and prevention of colorectal cancer.* J Steroid Biochem Mol Biol, 2005. **97**(1-2): p. 179-94.
304. Liu, Z., et al., *Mild Depletion of Dietary Folate Combined with Other B Vitamins Alters Multiple Components of the Wnt Pathway in Mouse Colon.* The Journal of Nutrition, 2007. **137**(12): p. 2701-2708.
305. Liu, Z., et al., *Combined inadequacies of multiple B vitamins amplify colonic Wnt signaling and promote intestinal tumorigenesis in BAT-LacZ×Apc1638N mice.* The FASEB Journal, 2011. **25**(9): p. 3136-3145.
306. Brenner, B.M., et al., *The effect of dietary vitamin D3 on the intracellular calcium gradient in mammalian colonic crypts.* Cancer Letters, 1998. **127**(1-2): p. 43-53.
307. Ferlay, J., et al., *Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008.* International Journal of Cancer, 2010. **127**(12): p. 2893-2917.
308. Grau, M.V., et al., *Vitamin D, Calcium Supplementation, and Colorectal Adenomas: Results of a Randomized Trial.* J. Natl. Cancer Inst., 2003. **95**(23): p. 1765-1771.
309. Fedirko, V., et al., *Blood 25-Hydroxyvitamin D3 Concentrations and Incident Sporadic Colorectal Adenoma Risk: A Pooled Case-Control Study.* Am. J. Epidemiol., 2010: p. kwq157.

310. Yang, K., et al., *Dietary Induction of Colonic Tumors in a Mouse Model of Sporadic Colon Cancer.* Cancer Research, 2008. **68**(19): p. 7803-7810.
311. Hollis, B.W., *Quantitation of 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D by radioimmunoassay using radioiodinated tracers.* Methods Enzymol, 1997. **282**: p. 174-86.
312. Hollis, B.W., et al., *Quantification of circulating 1,25-dihydroxyvitamin D by radioimmunoassay with 125I-labeled tracer.* Clin Chem, 1996. **42**(4): p. 586-92.
313. Elzagheid, A., et al., *E-cadherin expression pattern in primary colorectal carcinomas and their metastases reflects disease outcome.* World J Gastroenterol, 2006. **12**(27): p. 4304-9.
314. TSANOU, E., et al., *The E-Cadherin Adhesion Molecule and Colorectal Cancer. A Global Literature Approach.* Anticancer Research, 2008. **28**(6A): p. 3815-3826.
315. Mi, B., et al., *Beta-catenin expression is altered in dysplastic and nondysplastic aberrant crypt foci of human colon.* Appl Immunohistochem Mol Morphol, 2009. **17**(4): p. 294-300.
316. Ahearn, T.U., et al., *Markers of the APC/β-catenin signaling pathway as potential treatable, pre-neoplastic biomarkers of risk for colorectal neoplasms* In Press.
317. Murillo, G. and R.G. Mehta, *Chemoprevention of chemically-induced mammary and colon carcinogenesis by 1α-hydroxyvitamin D5.* The Journal of Steroid Biochemistry and Molecular Biology, 2005. **97**(1–2): p. 129-136.
318. Yu, W., et al., *Dietary Vitamin D Exposure Prevents Obesity-Induced Increase in Endometrial Cancer in Pten+/− Mice.* Cancer Prevention Research, 2010. **3**(10): p. 1246-1258.
319. Park, J.-I., et al., *Telomerase modulates Wnt signalling by association with target gene chromatin.* Nature, 2009. **460**(7251): p. 66-72.
320. van de Wetering, M., et al., *The β-Catenin/TCF-4 Complex Imposes a Crypt Progenitor Phenotype on Colorectal Cancer Cells.* Cell, 2002. **111**(2): p. 241-250.
321. Lappe, J.M., et al., *Vitamin D and calcium supplementation reduces cancer risk: results of a randomized trial.* The American Journal of Clinical Nutrition, 2007. **85**(6): p. 1586-1591.
322. Pence, B.C. and F. Buddingh, *Inhibition of dietary fat-promoted colon carcinogenesis in rats by supplemental calcium or vitamin D3.* Carcinogenesis, 1988. **9**(1): p. 187-90.
323. Jemal, A., et al., *Cancer Statistics, 2009.* CA A Cancer Journal for Clinicians, 2009. **59**(4): p. 225-249.
324. Peterlik, M., W.B. Grant, and H.S. Cross, *Calcium, vitamin D and cancer.* Anticancer Res, 2009. **29**(9): p. 3687-98.
325. Kallay, E., et al., *Molecular and functional characterization of the extracellular calcium-sensing receptor in human colon cancer cells.* Oncol Res, 2003. **13**(12): p. 551-9.
326. Kallay, E., et al., *Colon-specific regulation of vitamin D hydroxylases--a possible approach for tumor prevention.* Carcinogenesis, 2005. **26**(9): p. 1581-1589.
327. McCullough, M.L. and E.L. Giovannucci, *Diet and cancer prevention.* Oncogene, 2004. **23**(38): p. 6349-6364.
328. Jiménez-Lara, A.M., *Colorectal cancer: Potential therapeutic benefits of Vitamin D.* The International Journal of Biochemistry & Cell Biology, 2007. **39**(4): p. 672-677.
329. Palmer, H.G., et al., *Vitamin D3 promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of {beta}-catenin signaling.* J. Cell Biol., 2001. **154**(2): p. 369-388.

330. Bostick, R.M., M. Goodman, and E. Sidelnikov, *Genetics of Colorectal Cancer*, ed. J.D. Potter and L. NM2009, New York: Springer Science + Business Media, LLC.
331. Whitfield, J.F., *The calcium-sensing receptor--a driver of colon cell differentiation.* Curr Pharm Biotechnol, 2009. **10**(3): p. 311-6.
332. Cross, H.S. and E. Kallay, *Regulation of the colonic vitamin D system for prevention of tumor progression: an update.* Future Oncology, 2009. **5**(4): p. 493-507.
333. Cross, H.S., T. Nittke, and M. Peterlik, *Modulation of Vitamin D Synthesis and Catabolism in Colorectal Mucosa: A New Target for Cancer Prevention.* Anticancer Research, 2009. **29**(9): p. 3705-3712.
334. Willett, W.C., et al., *Reproducibility and validity of a semiquantitative fodd frequency questionnaire* Am. J. Epidemiol., 1985. **122**(1): p. 51-65.
335. Tangrea, J., et al., *Serum levels of vitamin D metabolites and the subsequent risk of colon and rectal cancer in Finnish men.* Cancer Causes and Control, 1997. **8**(4): p. 615-625.
336. Wu, K., et al., *Calcium Intake and Risk of Colon Cancer in Women and Men.* J. Natl. Cancer Inst., 2002. **94**(6): p. 437-446.
337. Whitfield, J.F., *Calcium, calcium-sensing receptor and colon cancer.* Cancer Lett, 2009. **275**(1): p. 9-16.
338. Bhagavathula, N., et al., *Regulation of E-cadherin and beta-catenin by Ca^{2+} in colon carcinomain colon carcinoma is dependent on calcium-sensing receptor expression and function.* International Journal of Cancer, 2007. **121**(7): p. 1455-1462.
339. Kallay, E., et al., *Dietary calcium and growth modulation of human colon cancer cells: role of the extracellular calcium-sensing receptor.* Cancer Detect Prev, 2000. **24**(2): p. 127-36.
340. Cheng, S.X., et al., *Expression of calcium-sensing receptor in rat colonic epithelium: evidence for modulation of fluid secretion.* Am J Physiol Gastrointest Liver Physiol, 2002. **283**(1): p. G240-250.
341. Bhagavathula, N., et al., *Upregulation of calcium-sensing receptor and mitogen-activated protein kinase signalling in the regulation of growth and differentiation in colon carcinoma.* Br J Cancer, 2005. **93**(12): p. 1364-1371.
342. Canaff, L. and G.N. Hendy, *Human Calcium-sensing Receptor Gene.* Journal of Biological Chemistry, 2002. **277**(33): p. 30337-30350.
343. Oh, K., et al., *Calcium and Vitamin D Intakes in Relation to Risk of Distal Colorectal Adenoma in Women.* Am. J. Epidemiol., 2007: p. kwm026.
344. Zheng, W., et al., *A prospective cohort study of intake of calcium, vitamin D, and other micronutrients in relation to incidence of rectal cancer among postmenopausal women.* Cancer Epidemiology Biomarkers & Prevention, 1998. **7**(3): p. 221-225.
345. Lechner, D., E. Kállay, and H.S. Cross, *1α,25-Dihydroxyvitamin D3 downregulates CYP27B1 and induces CYP24A1 in colon cells.* Molecular and Cellular Endocrinology, 2007. **263**(1-2): p. 55-64.
346. Cross, H.S., et al., *Vitamin D metabolism in human colon adenocarcinoma-derived Caco-2 cells: Expression of 25-hydroxyvitamin D3-1α-hydroxylase activity and regulation of sidechain metabolism.* The Journal of Steroid Biochemistry and Molecular Biology, 1997. **62**(1): p. 21-28.
347. Bareis, P., et al., *Clonal Differences in Expression of 25-Hydroxyvitamin D3-1[alpha]-hydroxylase, of 25-Hydroxyvitamin D3-24-hydroxylase, and of the Vitamin D Receptor in Human Colon Carcinoma Cells: Effects of Epidermal Growth Factor and 1[alpha],25-Dihydroxyvitamin D3.* Experimental Cell Research, 2002. **276**(2): p. 320-327.

348. Murillo, G., et al., *Chemopreventive efficacy of 25-hydroxyvitamin D3 in colon cancer.* The Journal of Steroid Biochemistry and Molecular Biology, 2007. **103**(3-5): p. 763-767.
349. Nittke, T., et al., *Nutritional calcium modulates colonic expression of vitamin D receptor and pregnane X receptor target genes.* Molecular Nutrition & Food Research, 2008. **52**(S1): p. S45-S51.
350. Nittke, T., et al., *Parallel Elevation of Colonic 1,25-Dihydroxyvitamin D3 Levels and Apoptosis in Female Mice on a Calcium-deficient Diet.* Anticancer Research, 2009. **29**(9): p. 3727-3732.
351. Liu, Z., et al., *Mild depletion of dietary folate combined with other B vitamins alters multiple components of the Wnt pathway in mouse colon.* J Nutr, 2007. **137**(12): p. 2701-8.
352. Bostick, R.M., et al., *Detecting and quantifying biomarkers of risk for colorectal cancer using quantum dots and novel image analysis algorithms.* Conference proceedings : ... Annual International Conference of the IEEE Engineering in Medicine and Biology Society. IEEE Engineering in Medicine and Biology Society. Conference, 2006. **1**: p. 3313-6.
353. Campbell, P.T., et al., *Prospective study reveals associations between colorectal cancer and type 2 diabetes mellitus or insulin use in men.* Gastroenterology, 2010.
354. Hu, F.B., et al., *Prospective Study of Adult Onset Diabetes Mellitus (Type 2) and Risk of Colorectal Cancer in Women.* J. Natl. Cancer Inst., 1999. **91**(6): p. 542-547.
355. Limburg, P.J., et al., *Clinically Confirmed Type 2 Diabetes Mellitus and Colorectal Cancer Risk: A Population-Based, Retrospective Cohort Study.* Am J Gastroenterol, 2006. **101**(8): p. 1872-1879.
356. Limburg, P.J., et al., *Diabetes mellitus and subsite-specific colorectal cancer risks in the Iowa Women's Health Study.* Cancer Epidemiol Biomarkers Prev, 2005. **14**(1): p. 133-7.
357. Bjork, J., et al., *Growth-regulatory effects of sensory neuropeptides, epidermal growth factor, insulin, and somatostatin on the non-transformed intestinal epithelial cell line IEC-6 and the colon cancer cell line HT 29.* Scand J Gastroenterol, 1993. **28**(10): p. 879-84.
358. Koenuma, M., T. Yamori, and T. Tsuruo, *Insulin and insulin-like growth factor 1 stimulate proliferation of metastatic variants of colon carcinoma 26.* Jpn J Cancer Res, 1989. **80**(1): p. 51-8.
359. Shi, B., et al., *Micro RNA 145 Targets the Insulin Receptor Substrate-1 and Inhibits the Growth of Colon Cancer Cells.* Journal of Biological Chemistry, 2007. **282**(45): p. 32582-32590.
360. Sun, J. and T. Jin, *Both Wnt and mTOR signaling pathways are involved in insulin-stimulated proto-oncogene expression in intestinal cells.* Cellular Signalling, 2008. **20**(1): p. 219-229.
361. Weinstein, D., et al., *Insulin analogues display IGF-I-like mitogenic and anti-apoptotic activities in cultured cancer cells.* Diabetes Metab Res Rev, 2009. **25**(1): p. 41-9.
362. Koohestani, N., et al., *Aberrant crypt focus promotion and glucose intolerance: correlation in the rat across diets differing in fat, n-3 fatty acids and energy.* Carcinogenesis, 1998. **19**(9): p. 1679-84.
363. Tran, T.T., et al., *Direct measure of insulin sensitivity with the hyperinsulinemic-euglycemic clamp and surrogate measures of insulin sensitivity with the oral glucose tolerance test: correlations with aberrant crypt foci promotion in rats.* Cancer Epidemiol Biomarkers Prev, 2003. **12**(1): p. 47-56.

364. Akagi, Y., et al., *Regulation of vascular endothelial growth factor expression in human colon cancer by insulin-like growth factor-I.* Cancer Res, 1998. **58**(17): p. 4008-14.
365. Warren, R.S., et al., *Induction of Vascular Endothelial Growth Factor by Insulin-like Growth Factor 1 in Colorectal Carcinoma.* Journal of Biological Chemistry, 1996. **271**(46): p. 29483-29488.
366. Garrouste, F., et al., *Prevention of cytokine-induced apoptosis by insulin-like growth factor-I is independent of cell adhesion molecules in HT29-D4 colon carcinoma cells-evidence for a NF-kappaB-dependent survival mechanism.* Cell Death Differ, 2002. **9**(7): p. 768-79.
367. Reinmuth, N., et al., *Blockade of Insulin-like Growth Factor I Receptor Function Inhibits Growth and Angiogenesis of Colon Cancer.* Clinical Cancer Research, 2002. **8**(10): p. 3259-3269.
368. Alberobello, A.T., et al., *Selective disruption of insulin-like growth factor-1 (IGF-1) signaling via phosphoinositide-dependent kinase-1 prevents the protective effect of IGF-1 on human cancer cell death.* J Biol Chem, 2010. **285**(9): p. 6563-72.
369. Fenton, J.I., et al., *Leptin, insulin-like growth factor-1, and insulin-like growth factor-2 are mitogens in ApcMin/+ but not Apc+/+ colonic epithelial cell lines.* Cancer Epidemiol Biomarkers Prev, 2005. **14**(7): p. 1646-52.
370. Bauer, T.W., et al., *Targeting of insulin-like growth factor-I receptor with a monoclonal antibody inhibits growth of hepatic metastases from human colon carcinoma in mice.* Ann Surg Oncol, 2007. **14**(10): p. 2838-46.
371. Brierley, G.V., et al., *Silencing of the Insulin Receptor Isoform A Favors Formation of Type 1 Insulin-Like Growth Factor Receptor (IGF-IR) Homodimers and Enhances Ligand-Induced IGF-IR Activation and Viability of Human Colon Carcinoma Cells.* Endocrinology, 2010. **151**(4): p. 1418-1427.
372. Zarrilli, R., et al., *Expression of insulin-like growth factor (IGF)-II and IGF-I receptor during proliferation and differentiation of CaCo-2 human colon carcinoma cells.* Cell Growth Differ, 1994. **5**(10): p. 1085-1091.
373. Lambert, S., et al., *Abnormal expression and structural modification of the insulin-like growth-factor-II gene in human colorectal tumors.* Int J Cancer, 1990. **46**(3): p. 405-10.
374. Tricoli, J.V., et al., *Enhanced levels of insulin-like growth factor messenger RNA in human colon carcinomas and liposarcomas.* Cancer Res, 1986. **46**(12 Pt 1): p. 6169-73.
375. Freier, S., et al., *Expression of the insulin-like growth factors and their receptors in adenocarcinoma of the colon.* Gut, 1999. **44**(5): p. 704-708.

Appendix

Table A1. Differences in E-cadherin expression in normal-appearing rectal mucosa between incident sporadic colorectal adenoma cases and controls, the MAPII study

Batch-adjusted biomarker labeling optical density							
Controls (N=35)	**(SE)**	**Cases (N=32)**	**(SE)**	**Prop diff (%)[a]**	***P*[b]**	**OR[c]**	**95% CI**
1.01	0.03	1.00	0.03	-0.70	0.88	1.18	0.49-2.82

[a] Proportional difference = [(mean of cases - mean of controls)/mean of controls]*100%

[b] Based on F-test for significance in a linear model

[c] The labeling optical density was dichotomized on the mean of the colon site-specific distributions in the controls

Table A2. Differences in expression of E-cadherin in normal-appearing colorectal mucosa according to potential risk factors for colorectal neoplasms, the MAPII study

Characteristic[a,b]	*N*	E-cadherin[c]	SE	Pro. Diff. (%)[d]	*P*[e]
Sex					
Female	44	0.98	0.03		
Male	42	1.03	0.03	4.6	0.35
Age (yrs.)					
< 55	44	0.99	0.03		
≥ 55	42	1.01	0.03	1.9	0.68
Family hx of CRC					
No	45	1.00	0.03		
Yes	11	1.06	0.07	6.3	0.37
Smoking status					
Never	45	1.00	0.03		
Former/Current	11	1.06	0.07	6.3	0.37
Alcohol intake					
Never	12	0.99	0.07		
Former/Current	73	1.01	0.03	1.2	0.86
Physical activity (METS/d)					
Low	36	0.97	0.04		
High	46	1.03	0.03	6.5	0.21
BMI (Kg/m2)					
< 30	43	0.97	0.03		
≥ 30	42	1.04	0.03	7.3	0.13
WHR					
Low	41	0.97	0.03		
High	43	1.02	0.03	5.1	0.30
NSAID - cases					
No	26	1.03	0.04		
Yes	14	0.95	0.06	-7.7	0.27
NSAID - controls					
No	27	0.96	0.04		
Yes	18	1.08	0.05	13.2	0.07
$p_{interaction}$[f]					0.10
Serum 25(OH)D_3 (ng/ml)					
< 27	36	0.99	0.03		
≥ 27	34	0.96	0.04	-2.6	0.61
Total energy intake					
Low	30	1.03	0.04		
High	52	0.99	0.03	-4.4	0.37
Total fat					
Low	40	0.96	0.03		
High	42	1.05	0.03	9.0	0.08

Table A2. Con't

Characteristic[a,b]	*N*	E-cadherin[c]	SE	Pro. Diff. (%)[d]	*P*[e]
Total[g] calcium					
Low	39	1.04	0.04		
High	43	0.98	0.03	-6.0	0.21
Total[g] folate					
Low	40	1.01	0.04		
High	42	1.00	0.04	-1.6	0.77
Processed meat intake					
Low	32	0.99	0.04		
High	50	1.02	0.03	2.5	0.64

[a] Referent category = "Female", the youngest or lowest category, or "No" groups

[b] Abbreviations: Family hx of CRC = family history of colorectal cancer in a first degree relative, BMI = body mass index, WHR = waist to hip ratio, NSAID = take nonsteroidal anti-inflammatory drug ≥ once/week

[c] All estimates adjusted for age and sex; dietary and physical activity covariates also adjusted for energy consumption; and total energy consumption adjusted for physical activity

[d] Proportional difference = [(mean of comparative category - mean of referent category)/mean of referent category]*100%

[e] p-value for comparison of means (analysis of covariance)

[f] p-value of multiplicative interaction term between regular NSAID use and case/control status in ANACOVA models

[g] Total = dietary + supplemental

Table B1. Expression of APC[§] in the normal-appearing colorectal mucosa by sex, in the CaDvMAP study

Treatment Group	Baseline				6-mo follow-up				Absolute Rx effect			Relative Effect[¥]
	n	Mean	Std Err	*P*	*n*	Mean	Std Err	*P*	Rx effect[*]	Std Err	*P*[**]	
APC – Males												
Placebo	15	0.33	0.02		14	0.42	0.02		0.00	.	.	1.00
Calcium	16	0.36	0.02	0.15	14	0.46	0.02	0.06	0.01	0.03	0.69	1.00
Vitamin D	16	0.31	0.02	0.31	16	0.46	0.02	0.86	0.07	0.03	0.04	1.17
Ca + Vit D	16	0.35	0.02	0.40	14	0.44	0.02	0.35	0.00	0.03	0.91	0.99
APC – Females												
Placebo	7	0.38	0.03		7	0.42	0.03		0.00	.	.	1.00
Calcium	7	0.30	0.03	0.02	7	0.44	0.03	0.57	0.11	0.05	0.04	1.33
Vitamin D	7	0.33	0.03	0.17	6	0.46	0.03	0.69	0.09	0.05	0.10	1.26
Ca + Vit D	7	0.37	0.03	0.70	7	0.44	0.03	0.64	0.03	0.05	0.54	1.08

[§] APC = proportion of APC in the upper 40% of the crypt

[*] Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

Table B2. Expression of β-catenin in the normal-appearing colorectal mucosa by sex, in the CaDvMAP study

Treatment Group	Baseline				6-mo follow-up				Absolute Rx effect			Relative Effect[¥]
	n	Mean	Std Err	*P*	*n*	Mean	Std Err	*P*	Rx effect[*]	Std Err	*P*[**]	
β-catenin – Males												
Placebo	14	1.02	0.05		12	0.96	0.06		0.00	.	.	1.00
Calcium	16	1.05	0.05	0.73	13	0.90	0.06	0.48	-0.08	0.11	0.42	0.91
Vitamin D	16	0.98	0.05	0.57	15	0.86	0.06	0.19	-0.06	0.11	0.54	0.93
Ca + Vit D	15	1.01	0.05	0.90	11	0.90	0.06	0.48	-0.05	0.11	0.65	0.95
β-catenin – Females												
Placebo	7	0.92	0.07		6	1.02	0.07		0.00	.	.	1.00
Calcium	6	0.90	0.07	0.88	6	0.74	0.07	0.01	-0.27	0.14	0.07	0.74
Vitamin D	5	0.82	0.07	0.35	6	0.82	0.07	0.06	-0.10	0.14	0.48	0.90
Ca + Vit D	7	1.09	0.07	0.08	6	0.95	0.07	0.04	-0.24	0.14	0.10	0.79

[*] Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

Table B3. Expression of E-cadherin in the normal-appearing colorectal mucosa by sex, in the CaDvMAP study

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	***n***	**Mean**	**Std Err**	***P***	***n***	**Mean**	**Std Err**	***P***	**Rx effect***	**Std Err**	***P*** **	**Relative Effect¥**
E-cadherin – Males												
Placebo	14	1.16	0.16		13	0.98	0.17		0.00	.	.	1.00
Calcium	15	1.14	0.16	0.93	14	1.21	0. 17	0.33	0.25	0.26	0.33	1.26
Vitamin D	15	0.96	0.16	0.39	12	1.32	0. 17	0.16	0.55	0.26	0.04	1.63
Ca + Vit D	15	0.92	0.16	0.31	11	1.11	0. 17	0.60	-0.37	0.26	0.17	1.43
E-cadherin – Females												
Placebo	7	1.14	0.17		6	1.16	0.18		0.00	.	.	1.00
Calcium	6	1.53	0.18	0.13	7	0.83	0.17	0.19	-0.72	0.35	0.06	0.53
Vitamin D	5	0.79	0.20	0.18	6	1.17	0.18	0.98	0.36	0.37	0.98	1.46
Ca + Vit D	7	0.85	0.17	0.23	7	1.16	0.17	0.97	0.28	0.35	0.81	1.34

* Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

Table B4. APC/β-catenin score[§] in the normal-appearing colorectal mucosa by sex, in the CaDvMAP study

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	***n***	**Mean**	**Std Err**	***P***	***n***	**Mean**	**Std Err**	***P***	**Rx effect[*]**	**Std Err**	***P*[**]**	**Relative Effect[¥]**
APC/β-catenin – Males												
Placebo	14	0.34	0.04		12	0.44	0.04		0.00	.	.	1.00
Calcium	16	0.35	0.04	0.91	12	0.56	0.04	0.05	0.11	0.08	0.15	1.24
Vitamin D	16	0.34	0.04	0.97	14	0.56	0.04	0.03	0.12	0.08	0.10	1.27
Ca + Vit D	15	0.37	0.04	0.62	11	0.53	0.04	0.15	0.06	0.08	0.45	1.11
APC/β-catenin – Females												
Placebo	7	0.43	0.04		6	0.45	0.04		0.00	.	.	1.00
Calcium	6	0.34	0.04	0.12	6	0.62	0.04	0.01	0.26	0.07	0.002	1.74
Vitamin D	5	0.44	0.04	0.87	6	0.58	0.04	0.03	0.12	0.07	0.11	1.26
Ca + Vit D	7	0.35	0.04	0.18	6	0.46	0.04	0.86	0.08	0.07	0.25	1.26

[§] APC/β-catenin score = proportion of APC in the upper 40% of the crypt/β-catenin

[*] Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

Table B5. Expression of APC[§] in the normal-appearing colorectal mucosa by age, in the CaDvMAP study

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	***n***	**Mean**	**Std Err**	***P***	***n***	**Mean**	**Std Err**	***P***	**Rx effect[*]**	**Std Err**	***P*[**]**	**Relative Effect[¥]**
APC – >55 years												
Placebo	9	0.35	0.05		8	0.45	0.05		0.00	.	.	1.00
Calcium	7	0.31	0. 05	0.62	7	0.59	0.06	0.004	0.17	0.09	0.09	1.48
Vitamin D	5	0.48	0.06	0.09	5	0.58	0.06	0.14	-0.02	0.10	0.88	0.94
Ca + Vit D	5	0.29	0.06	0.50	5	0.51	0.08	0.53	0.11	0.11	0.33	1.37
APC – ≤55 years												
Placebo	13	0.39	0.03		13	0.43	0.04		0.00	.	.	1.00
Calcium	16	0.36	0.03	0.54	14	0.57	0.04	0.01	0.17	0.07	0.02	1.44
Vitamin D	18	0.33	0.03	0.17	17	0.57	0.04	0.01	0.20	0.07	0.01	1.57
Ca + Vit D	18	0.39	0.03	0.90	16	0.50	0.04	0.19	0.07	0.07	0.30	1.16

[§] APC = proportion of APC in the upper 40% of the crypt

[*] Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

Table B6. Expression of β-catenin in the normal-appearing colorectal mucosa by age, in the CaDvMAP study

Treatment Group	Baseline				6-mo follow-up				Absolute Rx effect			Relative Effect[¥]
	n	Mean	Std Err	*P*	*n*	Mean	Std Err	*P*	Rx effect[*]	Std Err	*P*[**]	
β-catenin – <55 years												
Placebo	9	1.02	0.06		8	0.93	0.07		0.00	.	.	1.00
Calcium	7	0.95	0. 07	0.47	6	0.77	0.08	0.15	-0.08	0.14	0.55	0.89
Vitamin D	5	0.96	0.09	0.56	5	0.81	0.09	0.28	-0.06	0.15	0.70	0.93
Ca + Vit D	5	1.20	0.09	0.11	3	0.94	0.11	0.91	-0.17	0.16	0.33	0.86
β-catenin – ≥55 years												
Placebo	12	0.96	0.05		10	1.03	0.06		0.00	.	.	1.00
Calcium	15	1.03	0.05	0.34	13	0.89	0.06	0.10	-0.21	0.10	0.05	0.81
Vitamin D	16	0.93	0.05	0.73	16	0.86	0.06	0.04	-0.14	0.10	0.18	0.86
Ca + Vit D	17	0.99	0.05	0.70	14	0.92	0.06	0.18	-0.14	0.10	0.19	0.87

[*] Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

Table B7. Expression of E-cadherin in the normal-appearing colorectal mucosa by age, in the CaDvMAP study

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	***n***	**Mean**	**Std Err**	***P***	***n***	**Mean**	**Std Err**	***P***	**Rx effect***	**Std Err**	***P*** **	**Relative Effect¥**
E-cadherin – <55 years												
Placebo	9	1.14	0.13		8	1.03	0.20		0.00	.	.	1.00
Calcium	7	1.03	0.15	0.59	7	0.89	0. 18	0.49	-0.03	0.26	0.90	0.96
Vitamin D	5	0.77	0.18	0.12	5	0.89	0. 15	0.49	0.21	0.28	0.47	1.28
Ca + Vit D	5	0.84	0.18	0.19	4	0.99	0. 14	0.87	0.26	0.30	0.38	1.30
E-cadherin – ≥55 years												
Placebo	11	1.12	0.19		11	1.05	0.19		0.00	.	.	1.00
Calcium	14	1.31	0.16	0.45	14	1.20	0.16	0.55	-0.03	0.42	0.93	0.98
Vitamin D	15	0.71	0.16	0.10	13	1.44	0.17	0.12	0.80	0.42	0.06	2.16
Ca + Vit D	17	0.91	0.15	0.39	14	1.16	0.16	0.65	0.32	0.41	0.44	1.36

* Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

Table B8. APC/β-catenin[§] score in the normal-appearing colorectal mucosa by age, in the CaDvMAP study

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	***n***	**Mean**	**Std Err**	***P***	***n***	**Mean**	**Std Err**	***P***	**Rx effect[*]**	**Std Err**	***P*[**]**	**Relative Effect[¥]**
APC/β-catenin – <55 years												
Placebo	9	0.35	0.05		8	0.45	0.05		0.00	.	.	1.00
Calcium	7	0.31	0.05	0.62	6	0.59	0.06	0.10	0.16	0.09	0.09	1.48
Vitamin D	5	0.48	0.05	0.09	5	0.58	0.06	0.14	-0.02	0.10	0.88	0.94
Ca + Vit D	5	0.29	0.05	0.50	3	0.51	0.08	0.53	0.11	0.11	0.33	1.37
APC/β-catenin – ≥55 years												
Placebo	12	0.39	0.04		10	0.43	0.04		0.00	.	.	1.00
Calcium	15	0.36	0.03	0.54	12	0.57	0.04	0.01	0.17	0.07	0.02	1.44
Vitamin D	16	0.33	0.03	0.17	15	0.57	0.03	0.01	0.20	0.07	0.01	1.57
Ca + Vit D	17	0.39	0.03	0.90	14	0.50	0.03	0.19	0.07	0.07	0.30	1.16

[§] APC/β-catenin score = proportion of APC in the upper 40% of the crypt/β-catenin

[*] Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

Table B9. Expression of APC[§] in the normal-appearing colorectal mucosa by obesity, in the CaDvMAP study

Treatment Group	Baseline				6-mo follow-up				Absolute Rx effect			Relative Effect[¥]
	n	Mean	Std Err	*P*	*n*	Mean	Std Err	*P*	Rx effect[*]	Std Err	*P*[**]	
APC – BMI <30												
Placebo	11	0.33	0.02		11	0.42	0.02		0.00	.	.	1.00
Calcium	12	0.35	0.02	0.53	11	0.47	0.02	0.11	0.03	0.04	0.43	1.06
Vitamin D	16	0.32	0.02	0.83	15	0.46	0.02	0.14	0.05	0.03	0.18	1.13
Ca + Vit D	11	0.35	0.02	0.52	11	0.44	0.02	0.56	0.00	0.04	0.97	0.99
APC – BMI ≥30												
Placebo	11	0.36	0.02		10	0.42	0.02		0.00	.	.	1.00
Calcium	11	0.34	0.02	0.36	10	0.44	0.02	0.35	0.05	0.04	0.21	1.11
Vitamin D	7	0.29	0.02	0.03	7	0.45	0.02	0.25	0.11	0.05	0.03	1.33
Ca + Vit D	12	0.36	0.02	0.94	10	0.44	0.02	0.44	0.02	0.4	0.55	1.05

[§] APC = proportion of APC in the upper 40% of the crypt

[*] Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

Table B10. Expression of β-catenin in the normal-appearing colorectal mucosa by obesity, in the CaDvMAP study

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	***n***	**Mean**	**Std Err**	***P***	***n***	**Mean**	**Std Err**	***P***	**Rx effect***	**Std Err**	***P*** **	**Relative Effect¥**
β-catenin – BMI <30												
Placebo	10	1.04	0.06		10	0.94	0.06		0.00	.	.	1.00
Calcium	12	1.01	0.05	0.72	10	0.83	0.06	0.20	-0.08	0.11	0.47	0.91
Vitamin D	14	0.92	0.05	0.15	14	0.80	0.05	0.08	-0.03	0.10	0.80	0.96
Ca + Vit D	10	1.04	0.06	0.54	7	0.86	0.07	0.39	-0.03	0.12	0.82	0.91
β-catenin – BMI ≥30												
Placebo	11	0.94	0.06		8	1.04	0.07		0.00	.	.	1.00
Calcium	10	1.01	0.06	0.47	8	0.87	0.07	0.11	-0.23	0.13	0.09	0.78
Vitamin D	7	0.98	0.08	0.73	7	0.96	0.08	0.47	-0.11	0.14	0.43	0.89
Ca + Vit D	12	1.08	0.06	0.11	10	0.97	0.06	0.48	-0.21	0.12	0.10	0.81

* Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

Table B11. Expression of E-cadherin in the normal-appearing colorectal mucosa by obesity, in the CaDvMAP study

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	*n*	Mean	Std Err	*P*	*n*	Mean	Std Err	*P*	Rx effect*	Std Err	*P***	Relative Effect¥
E-cadherin – BMI >30												
Placebo	9	1.25	0.21		10	1.06	0.21		0.00	.	.	1.00
Calcium	12	0.95	0.20	0.32	11	0.98	0.20	0.52	0.21	0.23	0.36	1.22
Vitamin D	13	1.00	0.18	0.38	12	1.26	0.19	0.48	0.45	0.23	0.06	1.49
Ca + Vit D	10	0.79	0.21	0.14	8	1.13	0.22	0.81	0.53	0.25	0.04	1.69
E-cadherin – BMI ≤30												
Placebo	11	1.08	0.14		9	1.01	0.16		0.00	.	.	1.00
Calcium	9	1.58	0.16	0.03	10	1.21	0.15	0.37	-0.30	0.32	0.36	0.82
Vitamin D	7	0.91	0.18	0.46	6	1.29	0.19	0.28	0.44	0.36	0.22	1.52
Ca + Vit D	12	0.99	0.14	0.65	10	1.10	0.15	0.69	0.18	0.31	0.58	1.19

* Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

Table B12. APC[§]/β-catenin score[§] in the normal-appearing colorectal mucosa by obesity, in the CaDvMAP study

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	***n***	**Mean**	**Std Err**	***P***	***n***	**Mean**	**Std Err**	***P***	**Rx effect[*]**	**Std Err**	***P*[**]**	**Relative Effect[¥]**
APC/β-catenin – BMI >30												
Placebo	10	0.33	0.04		10	0.45	0.04		0.00	.	.	1.00
Calcium	12	0.35	0.04	0.82	10	0.59	0.04	0.03	0.13	0.08	0.13	1.24
Vitamin D	14	0.39	0.04	0.34	14	0.60	0.04	0.02	0.09	0.08	0.26	1.13
Ca + Vit D	10	0.39	0.04	0.47	7	0.53	0.05	0.26	0.02	0.09	0.82	1.00
APC/β-catenin – BMI ≤30												
Placebo	11	0.41	0.03		8	0.43	0.04		0.00	.	.	1.00
Calcium	10	0.34	0.04	0.20	8	0.56	0.04	0.03	0.19	0.08	0.02	1.57
Vitamin D	7	0.32	0.04	0.11	6	0.50	0.05	0.27	0.16	0.09	0.08	1.49
Ca + Vit D	12	0.34	0.03	0.19	10	0.48	0.04	0.39	0.11	0.08	0.16	1.35

[§] APC/β-catenin score = proportion of APC in the upper 40% of the crypt/β-catenin

[*] Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

Table B13. Expression of APC[§] in the normal-appearing colorectal mucosa by 25(OH)D concentrations, in the CaDvMAP study

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	***n***	**Mean**	**Std Err**	***P***	***n***	**Mean**	**Std Err**	***P***	**Rx effect[*]**	**Std Err**	***P*[**]**	**Relative Effect[¥]**
APC – Baseline 25(OH)D <22 ng/ml												
Placebo	13	0.40	0.04		13	0.40	0.04		0.00	.	.	1.00
Calcium	7	0.49	0.06	0.24	7	0.41	0.06	0.86	-0.08	0.09	0.39	0.84
Vitamin D	11	0.30	0.05	0.13	11	0.44	0.05	0.49	0.14	0.08	0.07	1.47
Ca + Vit D	13	0.30	0.04	0.10	12	0.42	0.05	0.72	0.13	0.07	0.10	1.40
APC – Baseline 25(OH)D ≥22 ng/ml												
Placebo	9	0.38	0.06		8	0.42	0.06		0.00	.	.	1.00
Calcium	16	0.36	0.05	0.79	14	0.46	0.05	0.66	0.06	0.10	0.56	1.16
Vitamin D	11	0.31	0.06	0.38	11	0.48	0.06	0.49	0.13	0.10	0.21	1.40
Ca + Vit D	10	0.43	0.06	0.54	9	0.39	0.06	0.71	-0.09	0.11	0.43	0.82

[§] APC = proportion of APC in the upper 40% of the crypt

[*] Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

Table B14. Expression of β-catenin in the normal-appearing colorectal mucosa by 25(OH)D concentrations, in the CaDvMAP study

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	*n*	Mean	Std Err	*P*	*n*	Mean	Std Err	*P*	Rx effect*	Std Err	*P***	Relative Effect¥
β-catenin – Baseline 25(OH)D <22 ng/ml												
Placebo	12	0.97	0.06		11	0.94	0.06		0.00	.	.	1.00
Calcium	6	1.12	0.09	0.16	5	0.70	0.10	0.05	-0.39	0.15	0.01	0.64
Vitamin D	11	0.91	0.06	0.51	11	0.81	0.06	0.17	-0.07	0.12	0.56	0.92
Ca + Vit D	13	1.01	0.06	0.64	10	0.90	0.07	0.63	-0.09	0.12	0.48	0.92
β-catenin – Baseline 25(OH)D ≥22 ng/ml												
Placebo	9	1.01	0.06		7	1.05	0.07		0.00	.	.	1.00
Calcium	16	0.97	0.04	0.53	14	0.90	0.05	0.09	-0.10	0.10	0.36	0.89
Vitamin D	10	0.97	0.06	0.64	10	0.83	0.06	0.02	-0.18	0.11	0.13	0.82
Ca + Vit D	9	1.08	0.06	0.91	7	0.96	0.07	0.38	-0.15	0.12	0.21	0.86

* Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

Table B15. Expression of E-cadherin in the normal-appearing colorectal mucosa by 25(OH)D concentrations, in the CaDvMAP study

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	*n*	Mean	Std Err	*P*	*n*	Mean	Std Err	*P*	Rx effect*	Std Err	*P***	Relative Effect¥
E-cadherin – Baseline 25(OH)D <22 ng/ml												
Placebo	12	1.16	0.15		12	0.97	0.15		0.00	.	.	1.00
Calcium	5	1.56	0.23	0.17	7	0.96	0.20	1.00	-0.39	0.37	0.30	0.74
Vitamin D	11	0.71	0.16	0.04	9	1.09	0.17	0.61	0.58	0.32	0.08	1.84
Ca + Vit D	13	0.83	0.14	0.12	11	1.35	0.16	0.09	0.71	0.30	0.02	1.95
E-cadherin – Baseline 25(OH)D ≥22 ng/ml												
Placebo	9	1.08	0.21		7	1.16	0.24		0.00	.	.	1.00
Calcium	16	1.12	0.16	0.88	14	1.15	0.17	0.97	-0.05	0.39	0.90	0.96
Vitamin D	10	0.95	0.20	0.64	9	1.49	0.21	0.29	0.47	0.42	0.27	1.46
Ca + Vit D	9	0.99	0.21	0.75	8	1.15	0.22	0.99	0.09	0.44	0.84	1.08

* Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

Table B16. APC/β-catenin score[§] in the normal-appearing colorectal mucosa by 25(OH)D concentrations, in the CaDvMAP study

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	*n*	Mean	Std Err	*P*	*n*	Mean	Std Err	*P*	Rx effect[*]	Std Err	*P*[**]	Relative Effect[¥]
APC/β-catenin score – Baseline 25(OH)D <22 ng/ml												
Placebo	12	0.39	0.05		11	0.46	0.05		0.00	.	.	1.00
Calcium	6	0.33	0.07	0.47	5	0.65	0.07	0.04	0.25	0.12	0.05	1.67
Vitamin D	11	0.39	0.05	0.97	11	0.64	0.05	0.02	0.18	0.10	0.08	1.39
Ca + Vit D	13	0.36	0.05	0.60	13	0.52	0.05	0.39	0.10	0.10	0.32	1.22
APC/β-catenin score – Baseline 25(OH)D <22 ng/ml												
Placebo	9	0.34	0.04		7	0.42	0.04		0.00	.	.	1.00
Calcium	16	0.35	0.03	0.87	14	0.55	0.03	0.01	0.12	0.07	0.08	1.27
Vitamin D	10	0.33	0.03	0.84	10	0.56	0.03	0.01	0.16	0.07	0.04	1.37
Ca + Vit D	9	0.37	0.04	0.55	7	0.47	0.04	0.34	0.02	0.4	0.75	1.03

[§] APC/β-catenin score = proportion of APC in the upper 40% of the crypt/β-catenin

[*] Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

Table B17. Expression of APC[§] in the normal-appearing colorectal mucosa by *BsmI* genotype, in the CaDvMAP study

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	***n***	**Mean**	**Std Err**	***P***	***n***	**Mean**	**Std Err**	***P***	**Rx effect[*]**	**Std Err**	***P*[**]**	**Relative Effect[¥]**
APC – *BsmI BB/Bb*												
Placebo	15	0.36	0.02		13	0.41	0.02		0.00	.	.	1.00
Calcium	14	0.34	0.02	0.38	13	0.45	0.02	0.17	0.06	0.03	0.08	1.16
Vitamin D	11	0.31	0.02	0.06	11	0.47	0.02	0.04	0.11	0.03	0.003	1.33
Ca + Vit D	16	0.36	0.02	0.87	14	0.43	0.02	0.35	0.03	0.03	0.38	1.05
APC – *BsmI bb*												
Placebo	7	0.32	02		8	0.43	0.02		0.00	.	.	1.00
Calcium	9	0.35	0.02	0.30	8	0.47	0.02	0.19	0.01	0.04	0.84	1.00
Vitamin D	11	0.32	0.02	0.92	11	0.45	0.02	0.50	0.02	0.04	0.69	1.05
Ca + Vit D	7	0.35	0.02	0.31	7	0.45	0.02	0.48	-0.01	0.05	0.80	0.96

[§] APC = proportion of APC in the upper 40% of the crypt

[*] Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

Table B18. Expression of β-catenin in the normal-appearing colorectal mucosa by *BsmI* genotype, in the CaDvMAP study

Treatment Group	Baseline				6-mo follow-up				Absolute Rx effect			Relative Effect[¥]
	n	Mean	Std Err	*P*	*n*	Mean	Std Err	*P*	Rx effect[*]	Std Err	*P*[**]	
β-catenin – *BsmI BB/Bb*												
Placebo	15	0.99	0.04		12	0.98	0.05		0.00	.	.	1.00
Calcium	14	0.97	0.05	0.75	12	0.81	0.05	0.02	-0.15	0.09	0.11	0.84
Vitamin D	11	1.00	0.05	0.93	11	0.83	0.05	0.03	-0.16	0.10	0.09	0.84
Ca + Vit D	15	0.96	0.04	0.67	10	0.88	0.05	0.19	-0.07	0.09	0.45	0.93
β-catenin – *BsmI bb*												
Placebo	6	0.97	0.09		6	0.99	0.09		0.00	.	.	1.00
Calcium	8	1.07	0.08	0.44	7	0.92	0.09	0.57	-0.17	0.18	0.35	0.84
Vitamin D	10	0.88	0.07	0.44	10	0.82	0.07	0.16	-0.08	0.17	0.64	0.91
Ca + Vit D	7	1.19	0.09	0.10	7	0.99	0.09	0.97	-0.23	0.18	0.22	0.82

[*] Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

Table B19. Expression of E-cadherin in the normal-appearing colorectal mucosa by *BsmI* genotype, in the CaDvMAP study

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	***n***	**Mean**	**Std Err**	***P***	***n***	**Mean**	**Std Err**	***P***	**Rx effect***	**Std Err**	***P*** **	**Relative Effect¥**
E-cadherin – *BsmI BB/Bb*												
Placebo	15	1.10	0.12		12	1.02	0.13		0.00	.	.	1.00
Calcium	14	1.20	0.12	0.58	13	0.97	0.13	0.81	-0.14	0.25	0.58	0.87
Vitamin D	11	0.91	0.14	0.30	9	0.98	0.15	0.86	0.16	0.27	0.57	1.16
Ca + Vit D	15	0.88	0.12	0.20	12	1.34	0.13	0.10	0.54	0.25	0.04	1.64
E-cadherin – *BsmI bb*												
Placebo	6	1.21	0.30		7	1.06	0.28		0.00	.	.	1.00
Calcium	7	1.30	0.28	0.83	8	1.27	0.26	0.61	0.11	0.54	0.84	1.12
Vitamin D	10	0.73	0.23	0.22	9	1.60	0.25	0.16	1.02	0.52	0.06	2.50
Ca + Vit D	7	0.93	0.28	0.51	7	1.14	0.28	0.86	0.35	0.55	0.53	1.40

* Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

Table B20. APC/β-catenin score[§] in the normal-appearing colorectal mucosa by *BsmI* genotype, in the CaDvMAP study

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	*n*	Mean	Std Err	*P*	*n*	Mean	Std Err	*P*	Rx effect[*]	Std Err	*P*[**]	Relative Effect[¥]
APC/β-catenin score – *BsmI BB/Bb*												
Placebo	15	0.38	0.03		12	0.44	0.03		0.00	.	.	1.00
Calcium	14	0.35	0.03	0.48	12	0.58	0.03	0.004	0.17	0.06	0.01	1.43
Vitamin D	11	0.31	0.03	0.14	11	0.59	0.03	0.002	0.22	0.06	0.001	1.64
Ca + Vit D	15	0.39	0.03	0.82	10	0.51	0.04	0.18	0.06	0.06	0.36	1.13
APC/β-catenin score – *BsmI bb*												
Placebo	6	0.35	0.07		6	0.44	0.07		0.00	.	.	1.00
Calcium	8	0.34	0.06	0.90	7	0.56	0.07	0.26	0.13	0.14	0.36	1.31
Vitamin D	10	0.42	0.06	0.47	10	0.61	0.06	0.08	0.10	0.13	0.45	1.16
Ca + Vit D	7	0.31	0.07	0.68	7	0.49	0.07	0.63	0.09	0.14	0.53	1.26

[§] APC/β-catenin score = proportion of APC in the upper 40% of the crypt/β-catenin

[*] Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

Table C1. Expression of calcium receptor in the normal-appearing colorectal mucosa during the clinical trial

Treatment Group	Baseline				6-mo follow-up				Absolute Rx effect			Relative Effect[¥]
	n	Mean	Std Err	*P*	*n*	Mean	Std Err	*P*	Rx effect[*]	Std Err	*P*[**]	
CaR[§] **Φh**[‡]												
Placebo	20	0.30	0.01		19	0.30	0.01		0.00	.	.	1.00
Calcium	22	0.30	0.01	0.73	20	0.30	0.01	0.81	0.001	0.02	0.95	1.00
Vitamin D	23	0.28	0.01	0.13	19	0.29	0.01	0.41	0.001	0.02	0.64	1.04
Ca + Vit D	22	0.28	0.01	0.19	20	0.29	0.01	0.43	0.001	0.02	0.73	1.04

[*] Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

[§] CaR = Calcium receptor

[‡] proportion of CaR in the upper 40% of the crypt

Table C2. Expression of vitamin D receptor in the normal-appearing colorectal mucosa during the clinical trial

Treatment Group	Baseline				6-mo follow-up				Absolute Rx effect			Relative Effect[¥]
	n	Mean	Std Err	*P*	*n*	Mean	Std Err	*P*	Rx effect[*]	Std Err	*P*[**]	
VDR[§] ΦH[‡]												
Placebo	21	0.56	0.02		18	0.59	0.02		0.00	.	.	1.00
Calcium	21	0.55	0.02	0.57	21	0.59	0.02	0.96	0.01	0.03	0.67	1.02
Vitamin D	22	0.53	0.01	0.14	17	0.56	0.02	0.19	0.00	0.03	0.98	1.00
Ca + Vit D	21	0.55	0.02	0.66	18	0.57	0.02	0.26	-0.02	0.03	0.59	0.98

[*] Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

[§] VDR = Vitamin D receptor

[‡] proportion of VDR in the upper 40% of the crypt

Table C3. Expression of CYP27B1[§] in the normal-appearing colorectal mucosa during the clinical trial.

Treatment Group	Baseline				6-mo follow-up				Absolute Rx effect			Relative Effect[¥]
	n	Mean	Std Err	*P*	*n*	Mean	Std Err	*P*	Rx effect[*]	Std Err	*P*[**]	
CYP27B1 Φh[‡]												
Placebo	20	0.37	0.02		20	0.45	0.02		0.00	.	.	1.00
Calcium	21	0.40	0.02	0.31	16	0.45	0.02	0.99	-0.03	0.04	0.48	0.93
Vitamin D	22	0.37	0.02	0.77	18	0.49	0.02	0.28	0.03	0.04	0.54	1.09
Ca + Vit D	22	0.39	0.02	0.49	19	0.47	0.02	0.53	-0.002	0.04	0.97	0.99

[*] Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

[§] CYP27B1 = Cytochrome P450 family 27B1

[‡] proportion of CYP27B1 in the upper 40% of the crypt

Table C4. Expression of CYP24A1[§] in the normal-appearing colorectal mucosa during the clinical trial.

Treatment Group	Baseline				6-mo follow-up				Absolute Rx effect			Relative Effect[¥]
	n	Mean	Std Err	*P*	*n*	Mean	Std Err	*P*	Rx effect[*]	Std Err	*P*[**]	
CYP24A1 ΦH[‡]												
Placebo	20	0.30	0.03		19	0.38	0.03		0.00	.	.	1.00
Calcium	22	0.33	0.03	0.56	20	0.32	0.03	0.16	-0.08	0.06	0.14	0.77
Vitamin D	23	0.32	0.03	0.60	19	0.37	0.03	0.84	-0.03	0.06	0.60	0.91
Ca + Vit D	22	0.39	0.03	0.03	20	0.43	0.03	0.23	-0.03	0.06	0.55	0.87

[*] Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

[§] CYP24A1 = Cytochrome P450 family 24A1

[‡] proportion of CYP24A1 in the upper 40% of the crypt

Table C5. Expression of calcium receptor in the normal-appearing colorectal mucosa by sex, in the CaDvMAP study

Treatment Group	Baseline				6-mo follow-up				Absolute Rx effect			Relative Effect[¥]
	n	Mean	Std Err	*P*	*n*	Mean	Std Err	*P*	Rx effect[*]	Std Err	*P*[**]	
***CaR*[§] – Males**												
Placebo	15	1.11	0.08		13	0.97	0.08		0.00	.	.	1.00
Calcium	15	1.10	0.08	0.94	13	1.23	0.08	0.03	0.27	0.13	0.05	1.28
Vitamin D	16	0.90	0.07	0.05	15	1.04	0.08	0.09	0.29	0.13	0.03	1.32
Ca + Vit D	15	1.05	0.08	0.54	13	1.15	0.08	0.11	0.25	0.13	0.07	1.25
***CaR*[§] – Females**												
Placebo	5	1.06	0.13		6	1.08	0.12		0.00	.	.	1.00
Calcium	7	0.81	0.11	0.14	7	1.03	0.11	0.74	0.20	0.23	0.39	1.25
Vitamin D	7	0.79	0.11	0.12	4	1.28	0.14	0.30	0.47	0.25	0.08	1.59
Ca + Vit D	7	1.02	0.11	0.81	7	1.17	0.11	0.57	0.13	0.23	0.57	1.13

[*] Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

[§] CaR = Calcium receptor

Table C6. Expression of vitamin D receptor in the normal-appearing colorectal mucosa by sex, in the CaDvMAP study

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	***n***	**Mean**	**Std Err**	***P***	***n***	**Mean**	**Std Err**	***P***	**Rx effect***	**Std Err**	***P*** **	**Relative Effect¥**
VDR§ – Males												
Placebo	14	1.09	0.08		12	0.98	0.08		0.00	.	.	1.00
Calcium	16	0.94	0.07	0.16	14	0.96	0.08	0.83	0.13	0.14	0.36	1.14
Vitamin D	16	0.96	0.07	0.22	11	0.94	0.09	0.70	0.08	0.14	0.55	1.09
Ca + Vit D	14	1.10	0.08	0.96	12	1.13	0.08	0.20	0.15	0.14	0.31	1.14
VDR§ – Females												
Placebo	6	0.97	0.13		6	1.05	0.13		0.00	.	.	1.00
Calcium	5	1.00	0.14	0.86	7	0.88	0.12	0.34	-0.20	0.24	0.42	0.81
Vitamin D	5	0.94	0.14	0.88	6	1.03	0.13	0.93	0.01	0.25	0.96	1.01
Ca + Vit D	7	0.99	0.12	0.88	6	1.26	0.13	0.25	0.18	0.24	0.45	1.18

* Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

§ VDR = Vitamin D receptor

Table C7. Expression of CYP27B1[§] in the normal-appearing colorectal mucosa by sex, in the CaDvMAP study

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	***n***	**Mean**	**Std Err**	***P***	***n***	**Mean**	**Std Err**	***P***	**Rx effect[*]**	**Std Err**	***P*[**]**	**Relative Effect[¥]**
CYP27B1 – Males												
Placebo	15	1.29	0.35		14	0.95	0.36		0.00	.	.	1.00
Calcium	15	0.91	0.35	0.45	10	1.23	0.43	0.62	0.66	0.74	0.37	1.84
Vitamin D	16	0.89	0.34	0.42	14	2.15	0.36	0.02	1.61	0.70	0.03	3.28
Ca + Vit D	15	1.16	0.35	0.79	13	1.18	0.38	0.66	0.37	0.71	0.61	1.38
CYP27B1 – Females												
Placebo	5	0.37	0.47		6	1.12	0.43		0.00	.	.	1.00
Calcium	6	0.81	0.43	0.50	6	0.92	0.43	0.76	-0.63	0.89	0.49	0.38
Vitamin D	5	0.74	0.47	0.59	4	1.74	0.53	0.37	0.26	0.97	0.79	0.78
Ca + Vit D	7	1.29	0.40	0.16	6	2.06	0.43	0.14	0.03	0.88	0.98	0.53

[*] Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

[§] CYP27B1 = Cytochrome P450 family 27B1

Table C8. Expression of CYP24A1[§] in the normal-appearing colorectal mucosa by sex, in the CaDvMAP study

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	***n***	**Mean**	**Std Err**	***P***	***n***	**Mean**	**Std Err**	***P***	**Rx effect[*]**	**Std Err**	***P*[**]**	**Relative Effect[¥]**
CYP24A1 – Males												
Placebo	14	1.08	0.24		13	0.93	0.25		0.00	.	.	1.00
Calcium	15	1.22	0.23	0.68	13	0.79	0.25	0.69	-0.28	0.48	0.56	0.75
Vitamin D	16	0.90	0.22	0.58	13	1.05	0.25	0.73	0.30	0.48	0.53	1.35
Ca + Vit D	15	1.22	0.23	0.67	11	1.42	0.27	0.19	0.35	0.50	0.48	1.35
CYP24A1 – Females												
Placebo	7	0.96	0.41		5	1.16	0.49		0.00	.	.	1.00
Calcium	5	0.54	0.49	0.52	7	0.83	0.41	0.61	0.08	0.85	0.92	1.27
Vitamin D	5	0.64	0.49	0.63	6	1.97	0.45	0.24	1.12	0.87	0.21	2.55
Ca + Vit D	7	0.71	0.41	0.68	6	1.76	0.45	0.38	0.85	0.83	0.32	2.05

[*] Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

[§] CYP24A1 = Cytochrome P450 family 24A1

Table C9. Expression of calcium receptor in the normal-appearing colorectal mucosa by age, in the CaDvMAP study

Treatment Group	Baseline				6-mo follow-up				Absolute Rx effect			
	n	Mean	Std Err	*P*	*n*	Mean	Std Err	*P*	Rx effect*	Std Err	*P***	Relative Effect¥
***CaR*§ – <55 years of age**												
Placebo	7	1.15	0.11		6	1.12	0.12		0.00	.	.	1.00
Calcium	7	0.85	0.11	0.08	7	1.09	0.11	0.86	0.27	0.23	0.26	1.32
Vitamin D	5	0.84	0.13	0.09	4	1.02	0.15	0.59	0.20	0.26	0.44	1.25
Ca + Vit D	4	1.14	0.15	0.94	5	1.05	0.13	0.70	-0.05	0.26	0.84	0.95
***CaR*§ – ≥55 years of age**												
Placebo	13	1.08	0.08		13	0.94	0.08		0.00	.	.	1.00
Calcium	15	1.08	0.08	0.99	13	1.20	0.08	0.03	0.25	0.13	0.07	1.28
Vitamin D	18	0.87	0.07	0.06	15	1.11	0.08	0.14	0.37	0.13	0.01	1.47
Ca + Vit D	18	1.02	0.07	0.56	15	1.20	0.08	0.03	0.31	0.13	0.02	1.35

* Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

§ CaR = Calcium receptor

Table C10. Expression of vitamin D receptor in the normal-appearing colorectal mucosa by age, in the CaDvMAP study

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	***n***	**Mean**	**Std Err**	***P***	***n***	**Mean**	**Std Err**	***P***	**Rx effect***	**Std Err**	***P*** **	**Relative Effect¥**
VDR§ – <55 years of age												
Placebo	8	0.92	0.09		7	1.02	0.09		0.00	.	.	1.00
Calcium	7	1.14	0.09	0.10	7	1.03	0.09	0.97	-0.21	0.15	0.18	0.81
Vitamin D	5	0.85	0.11	0.59	4	0.83	0.12	0.22	-0.12	0.18	0.50	0.88
Ca + Vit D	5	1.10	0.11	0.23	3	1.04	0.14	0.94	-0.16	0.19	0.40	0.85
VDR§ – ≥55 years of age												
Placebo	12	1.14	0.09		11	1.00	0.09		0.00	.	.	1.00
Calcium	14	0.86	0.08	0.02	14	0.89	0.08	0.38	0.17	0.16	0.28	1.18
Vitamin D	16	0.99	0.08	0.20	13	1.01	0.08	0.91	0.16	0.16	0.30	1.16
Ca + Vit D	16	1.05	0.08	0.42	15	1.21	0.08	0.08	0.31	0.15	0.05	1.31

* Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

§ VDR = Vitamin D receptor

Table C11. Expression of CYP27B1[§] in the normal-appearing colorectal mucosa by age, in the CaDvMAP study

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	***n***	**Mean**	**Std Err**	***P***	***n***	**Mean**	**Std Err**	***P***	**Rx effect[*]**	**Std Err**	***P*[**]**	**Relative Effect[¥]**
CYP27B1 – <55 years of age												
Placebo	8	1.06	0.34		8	0.97	0.34		0.00	.	.	1.00
Calcium	7	0.78	0.36	0.58	6	1.10	0.39	0.81	0.40	0.69	0.57	1.54
Vitamin D	5	0.60	0.42	0.41	5	2.40	0.42	0.02	1.89	0.74	0.02	4.37
Ca + Vit D	5	0.97	0.34	0.46	5	0.55	0.42	0.45	-0.83	0.74	0.28	0.62
CYP27B1 – ≥55 years of age												
Placebo	12	1.08	0.40		12	1.02	0.40		0.00	.	.	1.00
Calcium	14	0.94	0.37	0.80	10	1.13	0.44	0.85	0.25	0.80	0.75	1.27
Vitamin D	16	0.94	0.35	0.80	13	1.93	0.39	0.11	1.05	0.76	0.17	2.17
Ca + Vit D	17	1.12	0.34	0.93	14	1.79	0.37	0.17	0.72	0.75	0.34	1.69

[*] Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

[§] CYP27B1 = Cytochrome P450 family 27B1

Table C12. Expression of CYP24A1[§] in the normal-appearing colorectal mucosa by age, in the CaDvMAP study

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	***n***	**Mean**	**Std Err**	***P***	***n***	**Mean**	**Std Err**	***P***	**Rx effect[*]**	**Std Err**	***P*[**]**	**Relative Effect[¥]**
CYP24A1 – <55 years of age												
Placebo	8	0.83	0.04		8	0.68	0.09		0.00	.	.	1.00
Calcium	7	1.11	0.01	0.62	7	0.95	0.40	0.84	-0.01	0.81	0.99	1.04
Vitamin D	5	0.52	0.29	0.51	5	1.94	0.48	0.05	1.58	0.90	0.09	4.55
Ca + Vit D	5	1.00	0.05	0.79	4	1.49	0.53	0.23	0.65	0.93	0.49	1.82
CYP24A1 – ≥55 years of age												
Placebo	13	1.16	0.25		10	1.25	0.29		0.00	.	.	1.00
Calcium	13	1.02	0.25	0.70	13	0.71	0.25	0.17	-0.40	0.51	0.44	0.65
Vitamin D	16	0.94	0.23	0.53	14	1.13	0.25	0.76	0.10	0.49	0.84	1.12
Ca + Vit D	17	1.07	0.22	0.80	13	1.54	0.25	0.46	0.38	0.49	0.45	1.34

[*] Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

[§] CYP24A1 = Cytochrome P450 family 24A1

Table C13. Expression of calcium receptor in the normal-appearing colorectal mucosa by obesity, in the CaDvMAP study

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	***n***	**Mean**	**Std Err**	***P***	***n***	**Mean**	**Std Err**	***P***	**Rx effect***	**Std Err**	***P*** **	**Relative Effect¥**
***CaR*§ – BMI <30**												
Placebo	11	1.08	0.09		10	1.02	0.10		0.00	.	.	1.00
Calcium	11	0.99	0.09	0.51	10	1.29	0.10	0.06	0.36	0.16	0.03	1.38
Vitamin D	16	0.95	0.08	0.31	12	1.10	0.09	0.52	0.21	0.15	0.18	1.23
Ca + Vit D	11	0.96	0.09	0.39	10	1.18	0.10	0.24	0.28	0.16	0.09	1.30
***CaR*§ – BMI ≥30**												
Placebo	9	1.14	0.09		9	0.98	0.09		0.00	.	.	1.00
Calcium	11	1.03	0.08	0.34	10	1.04	0.08	0.66	0.17	0.15	0.28	1.17
Vitamin D	7	0.67	0.10	0.002	7	1.04	0.10	0.65	0.53	0.17	0.004	1.81
Ca + Vit D	11	1.11	0.08	0.81	10	1.14	0.08	0.22	0.18	0.15	0.25	1.19

* Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

§ CaR = Calcium receptor

Table C14. Expression of vitamin D receptor in the normal-appearing colorectal mucosa by obesity, in the CaDvMAP study

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	***n***	**Mean**	**Std Err**	***P***	***n***	**Mean**	**Std Err**	***P***	**Rx effect***	**Std Err**	***P*** **	**Relative Effect¥**
VDR§ – BMI <30												
Placebo	10	1.01	0.09		9	0.92	0.09		0.00	.	.	1.00
Calcium	12	0.95	0.08	0.58	11	0.94	0.08	0.90	0.08	0.17	0.63	1.09
Vitamin D	14	0.98	0.08	0.74	12	0.94	0.08	0.87	0.06	0.16	0.72	1.05
Ca + Vit D	10	0.96	0.09	0.65	9	1.26	0.09	0.02	0.39	0.18	0.03	1.44
VDR§ – BMI ≥30												
Placebo	10	1.10	0.10		9	1.08	0.10		0.00	.	.	1.00
Calcium	9	0.95	0.10	0.29	10	0.92	0.10	0.27	-0.004	0.16	0.98	0.99
Vitamin D	7	0.92	0.12	0.23	5	1.02	0.13	0.75	0.13	0.18	0.48	1.13
Ca + Vit D	11	1.15	0.10	0.71	9	1.09	0.10	0.94	-0.04	0.16	0.80	0.97

* Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

§ VDR = Vitamin D receptor

Table C15. Expression of CYP27B1[§] in the normal-appearing colorectal mucosa by obesity, in the CaDvMAP study

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	***n***	**Mean**	**Std Err**	***P***	***n***	**Mean**	**Std Err**	***P***	**Rx effect[*]**	**Std Err**	***P*[**]**	**Relative Effect[¥]**
CYP27B1 – BMI <30												
Placebo	11	1.30	0.47		11	0.93	0.47		0.00	.	.	1.00
Calcium	11	0.89	0.47	0.55	10	1.20	0.50	0.69	0.68	0.93	0.47	1.88
Vitamin D	14	0.93	0.42	0.56	13	2.30	0.43	0.04	1.75	0.88	0.05	3.46
Ca + Vit D	11	0.97	0.47	0.62	11	1.85	0.47	0.18	1.25	0.92	0.18	2.67
CYP27B1 – BMI ≥30												
Placebo	9	0.76	0.21		9	1.08	0.21		0.00	.	.	1.00
Calcium	10	0.87	0.20	0.71	6	0.94	0.26	0.67	-0.26	0.48	0.60	0.76
Vitamin D	7	0.72	0.24	0.91	5	1.45	0.28	0.32	0.40	0.52	0.44	1.42
Ca + Vit D	11	1.42	0.19	0.03	8	0.93	0.22	0.63	-0.82	0.45	0.09	0.46

[*] Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

[§] CYP27B1 = Cytochrome P450 family 27B1

Table C16. Expression of CYP24A1[§] in the normal-appearing colorectal mucosa by obesity, in the CaDvMAP study

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	***n***	**Mean**	**Std Err**	***P***	***n***	**Mean**	**Std Err**	***P***	**Rx effect[*]**	**Std Err**	***P*[**]**	**Relative Effect[¥]**
CYP24A1 – BMI >30												
Placebo	10	0.99	0.23		10	0.66	0.23		0.00	.	.	1.00
Calcium	11	1.10	0.22	0.72	11	0.68	0.22	0.96	-0.10	0.40	0.81	0.93
Vitamin D	14	0.87	0.19	0.70	13	1.09	0.20	0.17	0.54	0.38	0.16	1.88
Ca + Vit D	10	1.02	0.23	0.92	7	1.48	0.27	0.03	0.78	0.43	0.08	2.18
CYP24A1 – BMI ≥30												
Placebo	11	1.08	0.36		8	1.48	0.42		0.00	.	.	1.00
Calcium	9	1.01	0.39	0.88	9	0.96	0.39	0.67	-0.44	0.81	0.59	0.69
Vitamin D	7	0.77	0.45	0.58	6	1.94	0.48	0.47	0.78	0.89	0.39	1.84
Ca + Vit D	12	1.08	0.34	0.99	10	1.59	0.37	0.85	0.11	0.77	0.89	1.07

[*] Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

[§] CYP24A1 = Cytochrome P450 family 24A1

Table C17. Expression of calcium receptor in the normal-appearing colorectal mucosa by 25(OH)D concentrations, in the CaDvMAP study

Treatment Group	Baseline				6-mo follow-up				Absolute Rx effect			Relative Effect[¥]
	n	Mean	Std Err	*P*	*n*	Mean	Std Err	*P*	Rx effect[*]	Std Err	*P*[**]	
***CaR*[§]– Baseline 25(OH)D <22 ng/ml**												
Placebo	11	1.09	0.08		11	1.06	0.08		0.00	.	.	1.00
Calcium	7	0.98	0.10	0.38	7	1.04	0.10	0.88	0.09	0.16	0.57	1.09
Vitamin D	11	0.80	0.08	0.01	10	1.00	0.08	0.65	0.23	0.15	0.12	1.29
Ca + Vit D	12	1.01	0.07	0.47	11	1.16	0.08	0.35	0.18	0.14	0.22	1.18
***CaR*[§]– Baseline 25(OH)D ≥22 ng/ml**												
Placebo	9	1.13	0.11		8	0.93	0.12		0.00	.	.	1.00
Calcium	15	1.02	0.09	0.45	13	1.23	0.09	0.05	0.41	0.18	0.03	1.47
Vitamin D	11	0.92	0.10	0.17	9	1.17	0.11	0.13	0.46	0.19	0.02	1.55
Ca + Vit D	10	1.07	0.10	0.71	9	1.16	0.11	0.15	0.30	0.19	0.13	1.32

[*] Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

[§] CaR = Calcium receptor

Table C18. Expression of vitamin D receptor in the normal-appearing colorectal mucosa by 25(OH)D concentrations, in the CaDvMAP study

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	***n***	**Mean**	**Std Err**	***P***	***n***	**Mean**	**Std Err**	***P***	**Rx effect***	**Std Err**	***P*** **	**Relative Effect¥**
***VDR*§ – Baseline 25(OH)D <22 ng/ml**												
Placebo	12	0.99	0.09		12	1.03	0.09		0.00	.	.	1.00
Calcium	5	1.15	0.13	0.31	7	0.82	0.11	0.15	-0.37	0.17	0.04	0.69
Vitamin D	11	0.93	0.09	0.62	9	0.89	0.10	0.30	-0.08	0.15	0.61	0.92
Ca + Vit D	13	1.08	0.08	0.48	10	1.09	0.09	0.65	-0.03	0.14	0.84	0.97
***VDR*§ – Baseline 25(OH)D ≥22 ng/ml**												
Placebo	8	1.16	0.10		6	0.95	0.11		0.00	.	.	1.00
Calcium	16	0.89	0.07	0.03	14	1.00	0.07	0.70	0.32	0.17	0.07	1.37
Vitamin D	10	0.99	0.09	0.18	8	1.05	0.10	0.47	0.28	0.19	0.15	1.30
Ca + Vit D	8	1.03	0.10	0.32	8	1.29	0.10	0.02	0.48	0.19	0.02	1.53

* Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

§ VDR = vitamin D receptor

Table C19. Expression of CYP27B1[§] in the normal-appearing colorectal mucosa by 25(OH)D concentrations, in the CaDvMAP study

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	***n***	**Mean**	**Std Err**	***P***	***n***	**Mean**	**Std Err**	***P***	**Rx effect[*]**	**Std Err**	***P*[**]**	**Relative Effect[¥]**
CYP27B1[§]– Baseline 25(OH)D <22 ng/ml												
Placebo	11	1.04	0.31		13	1.01	0.29		0.00	.	.	1.00
Calcium	6	0.71	0.43	0.54	6	0.82	0.43	0.72	0.14	0.71	0.85	1.19
Vitamin D	11	0.61	0.31	0.34	9	1.58	0.35	0.21	1.00	0.61	0.11	2.67
Ca + Vit D	13	1.25	0.29	0.62	10	1.29	0.32	0.53	0.07	0.59	0.91	1.06
CYP27B1[§]– Baseline 25(OH)D ≥22 ng/ml												
Placebo	9	1.11	0.50		7	0.98	0.56		0.00	.	.	1.00
Calcium	15	0.96	0.38	0.81	10	1.29	0.47	0.68	0.46	0.97	0.64	1.52
Vitamin D	10	1.13	0.47	0.98	9	2.54	0.50	0.05	1.54	1.02	0.14	2.55
Ca + Vit D	9	1.13	0.50	0.98	9	1.66	0.50	0.37	0.66	1.03	0.53	1.66

[*] Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

[§] CYP27B1 = Cytochrome P450 family 27B1

Table C20. Expression of CYP24A1[§] in the normal-appearing colorectal mucosa by 25(OH)D concentrations, in the CaDvMAP study

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	*n*	Mean	Std Err	*P*	*n*	Mean	Std Err	*P*	Rx effect[*]	Std Err	*P*[**]	Relative Effect[¥]
CYP24A1[§]– Baseline 25(OH)D <22 ng/ml												
Placebo	12	1.31	0.31		12	0.98	0.31		0.00	.	.	1.00
Calcium	5	1.10	0.48	0.72	6	1.27	0.43	0.60	0.49	0.78	0.53	1.54
Vitamin D	11	0.68	0.32	0.17	10	1.54	0.34	0.62	1.19	0.64	0.07	3.03
Ca + Vit D	13	0.86	0.29	0.30	10	1.55	0.34	0.22	1.02	0.62	0.11	2.41
CYP24A1[§]– Baseline 25(OH)D ≥22 ng/ml												
Placebo	9	0.67	0.28		6	1.07	0.34		0.00	.	.	1.00
Calcium	15	1.04	0.22	0.31	14	0.59	0.23	0.25	-0.84	0.51	0.11	0.36
Vitamin D	10	1.02	0.27	0.38	9	1.14	0.28	0.87	-0.27	0.55	0.62	0.70
Ca + Vit D	9	1.34	0.28	0.11	7	1.49	0.32	0.38	-0.25	0.57	0.67	0.70

[*] Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

[§] CYP24A1 = Cytochrome P450 family 24A1

Table C21. Expression of calcium receptor in the normal-appearing colorectal mucosa by *BsmI* genotype, in the CaDvMAP study

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	***n***	**Mean**	**Std Err**	***P***	***n***	**Mean**	**Std Err**	***P***	**Rx effect***	**Std Err**	***P*** **	**Relative Effect¥**
CaR*§ – *BsmI BB/Bb												
Placebo	12	1.17	0.08		12	1.04	0.08		0.00	.	.	1.00
Calcium	13	0.86	0.08	0.01	13	1.07	0.08	0.84	0.34	0.13	0.01	1.40
Vitamin D	11	0.99	0.08	0.11	10	1.05	0.09	0.93	0.19	0.14	0.17	1.19
Ca + Vit D	16	0.97	0.07	0.05	13	1.24	0.07	0.07	0.40	0.13	0.004	1.44
CaR*§ – *BsmI bb												
Placebo	8	1.01	0.10		7	0.93	0.11		0.00	.	.	1.00
Calcium	9	1.23	0.10	0.14	7	1.32	0.11	0.02	0.18	0.19	0.35	1.17
Vitamin D	11	0.74	0.09	0.05	9	1.12	0.10	0.19	0.47	0.18	0.01	1.64
Ca + Vit D	6	1.24	0.12	0.17	7	1.02	0.11	0.57	-0.13	0.20	0.50	0.89

* Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

§ CaR = Calcium receptor

Table C22. Expression of vitamin D receptor in the normal-appearing colorectal mucosa by *BsmI* genotype, in the CaDvMAP study

Treatment Group	Baseline				6-mo follow-up				Absolute Rx effect			Relative Effect[¥]
	n	Mean	Std Err	*P*	*n*	Mean	Std Err	*P*	Rx effect[*]	Std Err	*P*[**]	
VDR*[§] *– BsmI BB/Bb												
Placebo	13	1.05	0.08		11	0.98	0.09		0.00	.	.	1.00
Calcium	13	1.01	0.08	0.75	13	0.92	0.08	0.62	-0.02	0.16	0.89	0.98
Vitamin D	11	0.97	0.09	0.53	9	0.92	0.10	0.62	0.01	0.17	0.96	1.02
Ca + Vit D	15	0.98	0.08	0.51	12	1.24	0.09	0.05	0.33	0.16	0.05	1.36
VDR*[§] *– BsmI bb												
Placebo	7	1.07	0.10		7	1.03	0.10		0.00	.	.	1.00
Calcium	8	0.86	0.10	0.15	8	0.94	0.10	0.54	0.12	0.15	0.42	1.14
Vitamin D	10	0.93	0.09	0.32	8	1.04	0.09	0.96	0.14	0.14	0.02	1.16
Ca + Vit D	6	1.27	0.11	0.19	6	1.07	0.11	0.82	-0.17	0.16	0.29	0.88

[*] Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

[§] VDR = vitamin D receptor

Table C23. Expression of CYP27B1[§] in the normal-appearing colorectal mucosa by *BsmI* genotype, in the CaDvMAP study

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	***n***	**Mean**	**Std Err**	***P***	***n***	**Mean**	**Std Err**	***P***	**Rx effect[*]**	**Std Err**	***P*[**]**	**Relative Effect[¥]**
CYP27B1[§]– *BsmI BB/Bb*												
Placebo	14	1.11	0.33		12	1.01	0.35		0.00	.	.	1.00
Calcium	13	0.83	0.34	0.56	12	1.18	0.35	0.74	0.44	0.67	0.52	1.56
Vitamin D	11	0.95	0.37	0.76	11	1.67	0.37	0.20	0.81	0.69	0.25	1.93
Ca + Vit D	16	1.05	0.31	0.90	13	1.78	0.34	0.12	0.83	0.65	0.21	1.86
CYP27B1[§]– *BsmI bb*												
Placebo	6	0.98	0.56		8	0.98	0.49		0.00	.	.	1.00
Calcium	8	0.97	0.49	0.99	4	0.93	0.69	0.96	-0.03	1.09	0.98	0.96
Vitamin D	10	0.75	0.44	0.75	7	2.67	0.52	0.03	1.92	0.98	0.07	3.56
Ca + Vit D	6	1.61	0.56	0.42	6	0.76	0.56	0.78	-0.85	1.06	0.43	0.47

[*] Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

[§] CYP27B1 = Cytochrome P450 family 27B1

Table C24. Expression of CYP24A1[§] in the normal-appearing colorectal mucosa by *BsmI* genotype, in the CaDvMAP study

	Baseline				6-mo follow-up				Absolute Rx effect			
Treatment Group	***n***	**Mean**	**Std Err**	***P***	***n***	**Mean**	**Std Err**	***P***	**Rx effect[*]**	**Std Err**	***P*[**]**	**Relative Effect[¥]**
CYP24A1[§]– *BsmI BB/Bb*												
Placebo	14	1.06	0.24		11	1.02	0.27		0.00	.	.	1.00
Calcium	12	1.22	0.26	0.66	13	0.85	0.25	0.64	-0.33	0.49	0.50	0.72
Vitamin D	11	0.78	0.27	0.44	11	1.37	0.27	0.36	0.63	0.50	0.21	1.83
Ca + Vit D	15	0.76	0.23	0.38	10	1.33	0.28	0.43	0.60	0.49	0.23	1.82
CYP24A1[§]– *BsmI bb*												
Placebo	7	1.00	0.40		7	0.96	0.40		0.00	.	.	1.00
Calcium	8	0.81	0.38	0.74	7	0.71	0.40	0.67	-0.06	0.80	0.94	0.91
Vitamin D	10	0.92	0.34	0.49	8	1.29	0.38	0.55	0.41	0.76	0.60	1.46
Ca + Vit D	7	1.68	0.40	0.24	7	1.84	0.40	0.14	0.20	0.81	0.81	1.14

[*] Rx 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 70% proportional increase in the treatment group relative to that in the placebo group).

[§] CYP24A1 = Cytochrome P450 family 24A1