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Vitamin D and Isoforms of its Binding Protein, Tissue Biomarkers of Inflammation, and Colorectal Cancer Risk and Survival

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Abstract

Vitamin D and Isoforms of its Binding Protein, Tissue Biomarkers of Inflammation, and Colorectal Cancer Risk and Survival

By David Corley Gibbs

Colorectal cancer (CRC) is a leading cause of cancer mortality in the United States. Vitamin D may influence CRC development and progression in part via reducing inflammation, but the effect of supplemental vitamin D on inflammation in humans and whether certain individuals may particularly benefit from higher vitamin D for CRC prevention and prognosis is unclear.

In the first study, we tested the effects of supplemental vitamin D (1000 I.U./day) and/or calcium (1,200 mg/day) on two inflammation-related biomarkers of risk for CRC (COX-2 and 15-HPGD) in the rectal mucosa of 62 colorectal adenoma patients in a placebo-controlled chemoprevention trial. We found that after one year of treatment the pro-inflammatory ratio of COX-2 /15-HPGD expression in full-length crypts statistically significantly decreased 47% more in the vitamin D group than in the placebo group (95% confidence interval [CI]: 36–76%) .

In the second study, we investigated whether the association of circulating vitamin D (25[OH]D) with CRC risk differed by the missense *GC*-rs4588*A variant (Thr436Lys), encoding the vitamin D-binding protein-2 (DBP2) isoform, among 1,710 incident CRC cases and 1,649 matched controls nested within three prospective cohorts. Multivariable-adjusted relative risks for CRC associated with 25(OH)D concentrations considered sufficient (≥50 nmol/L), relative to deficient (<30 nmol/L), were 0.47 (95% CI: 0.33–0.67) among individuals with DBP2, and 0.88 (95% CI: 0.61–1.27) among individuals without DBP2 ($P_{heterogeneity}$ = 0.01).

In the third study, we investigated whether the association of pre-diagnostic 25(OH)D with mortality among CRC patients differed by the DBP2 isoform among 1,281 CRC cases (635 deaths, 483 from CRC) in two large prospective cohorts. In the pooled analysis, multivariable-adjusted hazard ratios for CRC-specific mortality associated with deficient relative to sufficient 25(OH)D concentrations were 2.24 (95% CI: 1.44–3.49) among those with DBP2, and 0.94 (95% CI: 0.68–1.22) among those without DBP2 ($P_{interaction}$ = 0.0002).

The results of these studies indicate that vitamin D supplementation reduces CRC-promoting inflammation in the gut and that individuals with the inherited DBP2-encoding *GC*-rs4588*A missense variant—linked to vitamin D insufficiency—may particularly benefit from higher vitamin D exposure for CRC prevention and prognosis.

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CHAPTER I

Introduction and Background

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer death among men and women in the United States (US) (1). Despite screening measures and new treatments, deaths from CRC have declined only modestly in recent decades (1), underscoring the importance of primary prevention and identifying factors that may improve survival for CRC patients. In observational epidemiologic studies, higher circulating concentrations of 25-hydroxyvitamin D (25[OH]D)—considered the best marker of total vitamin D exposure—are consistently associated with lower risk of CRC and lower mortality rates among CRC patients (2-6). Additionally, strong experimental evidence supports several anti-neoplastic effects of vitamin D in the colon, including effects on inflammation-related pathways (2, 7, 8). However, several important questions remain, including whether: 1) vitamin D supplementation modulates tissue biomarkers of inflammation linked to colorectal carcinogenesis, 2) the association of circulating vitamin D concentrations with CRC risk differs by inherited genotypes that affect vitamin D status and metabolism, and 3) the latter genotypes modify the association of circulating vitamin D concentrations with survival among CRC patients. The overarching goals of this dissertation research are to answer these three questions. Doing so will help elucidate the putative anti-inflammatory effects of vitamin D supplementation in humans and identify individuals who may particularly benefit from higher vitamin D exposures, thus providing clinically relevant public health information that could help reduce the incidence and mortality of CRC worldwide.

BACKGROUND

Colorectal Cancer

Colorectal cancer (CRC) includes cancers of the colon and rectum. Despite cancer screening and advances in treatment, CRC is one of the most common cancers that affect both men and women, and is the second leading cause of cancer-related deaths among men and women combined in the US and globally (1, 9). In 2018, there were an estimated 1,800,977 new cases of CRC (third behind lung cancer and breast cancer), and 861,663 CRC deaths (second only to lung cancer deaths) worldwide (9). Given current trends, the global burden of CRC is expected to increase by 60% by 2030 with an estimated 2.2 million new cases and 1.1 million deaths attributable to CRC per year (10).

Most, if not all, colorectal cancers (carcinomas) arise from pre-neoplastic colorectal lesions known as colorectal adenomatous polyps, or adenomas (11). Approximately 40% of individuals in Western countries will develop a colorectal adenoma during their lifetime, and approximately 30-35% of patients who have an adenoma removed will develop an additional adenoma within 3-5 years (11). The American Cancer Society recommends screening for colorectal neoplasms among US adults starting at age 45 using direct-visualization methods (i.e., colonoscopy or flexible sigmoidoscopy) or stool-based tests (i.e., guaiac-based fecal occult blood test) (12). These screening recommendations are supported by results from randomized clinical studies that indicate that such screening measures reduce (albeit with variable levels of effectiveness) the incidence of, and mortality attributed to, CRC (13).

Striking differences in CRC incidence rates globally indicate that CRC is a disease strongly linked to Western diet and lifestyle (10). Countries with a high or very high human development index account for over 60% of all CRC cases, and incidence rates vary up to 10-

fold between high and low-risk countries (14). Additionally, immigrants moving from low- to high-risk countries experience approximately the same rate of CRC within one to two generations, supporting a strong environmental component of CRC etiology (14). According to 2012 GLOBOCAN data, age-adjusted incidence rates were highest in Canada, most European countries, Japan, Australia and New Zealand, and were lowest in parts of Africa and the Middle East; although these results may be biased in part due to underreporting in lower income countries (**Figure 1.1**) (10). Although current incidence rates are generally lower in lower-income countries than in higher-income countries, incidence and mortality rates are rising most rapidly in low and middle income countries (10).

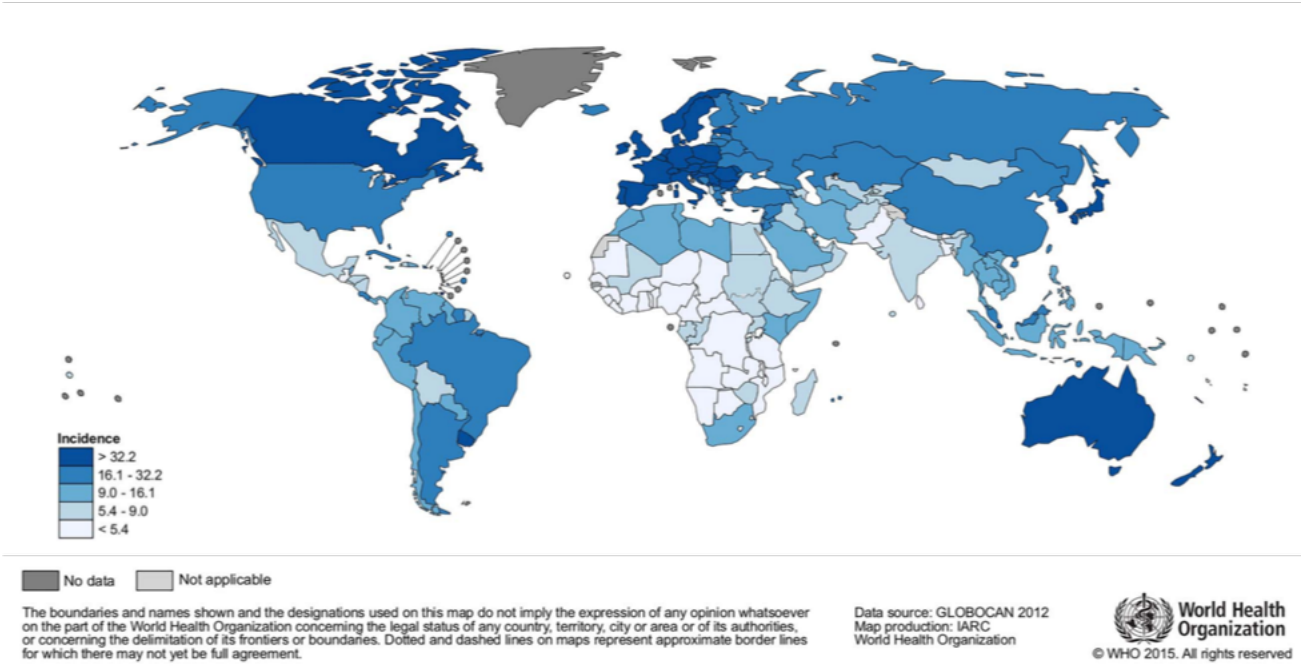

Figure 1.1. Age-adjusted colorectal cancer incidence rates in men worldwide in 2012 (GLOBOCAN 2012 data).

Colorectal Cancer Risk Factors

Several factors are associated with CRC risk. These include non-modifiable risk factors, such as age and inherited genotypes, as well as modifiable risk factors such as diet, lifestyle, and

exercise. CRC risk increases dramatically after the age of 50, and an estimated 90% of CRC cases are diagnosed in individuals who are 50 years of age or older (14). However, in recent decades, CRC incidence rates in the US increased among individuals younger than age 50, while rates generally decreased for those older than 50 (15). Reasons for this rise in CRC incidence among younger US individuals are unclear. Inflammatory bowel diseases resulting in chronic inflammation in the colorectal epithelium also increase the risk of CRC (described in more detail below). Other non-modifiable risk factors include inherited genetic disorders, which account for approximately 5 – 10% of CRC cases (14). These include familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC or Lynch syndrome), which are caused by inherited mutations in the tumor suppressor gene *APC* or in DNA mismatch repair genes (predominantly *MSH2* and *MLH1*), respectively (14).

As evidenced by the striking differences in estimated CRC incidence rates globally, CRC is believed to largely be an "environmental" disease with strong dietary and lifestyle components to its etiology. CRC is consistently associated with higher intakes of red/processed meats and alcohol and lower intakes of milk/dairy products, whole grains and fiber, and vegetables in observational epidemiologic studies (16). Although randomized clinical trials (RCT) of these dietary components in relation to CRC risk are limited, according to the International Agency for Cancer Research (IARC), there is sufficient evidence that higher intakes of red/processed meat and alcohol are causally related to CRC based on consistent findings in observational studies and strong mechanistic evidence (17, 18). According to the 2017 World Cancer Research Fund (WCRF) report, there is also "strong" evidence that intakes of wholegrains, fiber, dairy products, and calcium supplements lower CRC risk, and "some" evidence that intakes of fish, vegetables, multivitamin supplements, and vitamin D supplements lower CRC risk, based on a combination

of epidemiologic study and laboratory study findings (19). According to this same report, dietary factors for which there is "limited or inconclusive" evidence of an association with CRC include poultry, shellfish, garlic, sugar, starch, folates, vitamins A, C, and E, dietary fat, methionine, beta-carotene, alpha-carotene, lycopene, retinol, total energy intake, meal frequency, coffee, and tea (19).

Of non-dietary modifiable risk factors, there is strong evidence that smoking and obesity increase the risk of CRC, according to the WCRF, based on observational epidemiologic and experimental study findings (19). There is also strong evidence that increased physical activity and aspirin use (particularly >5 years) lowers the risk of CRC (19, 20). Low-dose aspirin (81-325 mg/day) was effective at reducing risk of CRC (20) as well as colorectal adenoma (21) in RCTs.

Higher calcium intake and vitamin D exposure are consistently associated with a lower risk of colorectal neoplasms in observational studies (discussed in detail later) (22-24), and strong experimental evidence supports several protective effects of calcium and vitamin D against colorectal carcinogenesis (2, 25-29). Calcium and vitamin D may also underlie, at least in part, the strong and consistent associations of dairy and fortified milk products (the primary dietary source of vitamin D in the US) in epidemiologic studies (19). Proposed mechanisms for calcium's anti-neoplastic effects include binding bile and fatty acids in the gut, which can prevent tumor-promoting inflammatory responses in colorectal epithelia (25, 26, 28). Vitamin D also promotes bile acid degradation and regulates multiple inflammatory carcinogenesis-promoting pathways *via* binding to the vitamin D receptor (VDR) in colorectal tissue (28, 29). These mechanisms and the interrelatedness of vitamin D and calcium metabolism are discussed in detail in the next sections. Importantly, findings from several RCTs support that supplemental

calcium lowers the risk of colorectal adenoma recurrence among colorectal adenoma patients (22); however, evidence of protective effects of supplemental calcium and/or vitamin D against CRC in RCTs is limited (30-32), supporting the need for additional research.

Molecular Basis of Colorectal Carcinogenesis and the Role of Inflammation

Most colorectal neoplasms primarily arise from one, or a combination of, the following molecular pathways: chromosomal instability (CIN), microsatellite instability (MSI), or CpG island methylator phenotype (CIMP) (33). These pathways are characterized by different genetic alternations that drive colorectal carcinogenesis and are not necessarily mutually exclusive. Most (~85%) of sporadic CRC tumors involve the CIN pathway characterized by acquired mutations in the adenomatous polyposis coli (*APC*) gene (33). *APC* inactivation leads to an increase in Wnt/ß-catenin pathway signaling that promotes tumor growth in the colon and other body sites (33). The MSI pathway involves inactivation of, or genetic alternations in, DNA mismatch repair (MMR) genes (such *MLH1*, *MSH2* and *MSH2*) and is estimated to contribute to ~15% of sporadic CRC cases (33). MMR-gene mutations often lead to aberrant signaling of the *BAX* (Bcl-2) gene, resulting in decreased apoptosis, and the *TGF-ß* gene, resulting in increased cellular proliferation (33). In addition to sporadic CRCs due to acquired mutations in these CIN/MSI-associated genes, inherited mutations in *APC* and in MMR-related genes cause familial adenomatous polyposis and Lynch syndrome, respectively, which greatly increase the risk of CRC as mentioned above. The CIMP pathway is characterized by promoter hypermethylation and inactivation of various tumor suppressor genes including *MLH1*, *MGMT*, and *CDKN2A*; hypermethylation of MMR genes can, in turn, lead to MSI-associated CRCs (33). These initial genetic alterations in the CIN, MSI, and/or CIMP pathways lead to the development of colorectal

adenomas, while additional genetic changes, such as *p53*-inhibition and/or mutations in mitogen-activated protein kinase (MAPK) pathway genes *BRAF* or *KRAS*, appear to be required for the progression of most colorectal adenomas into CRC (33). These molecular pathways are also associated with CRC anatomic site, degree of cellular differentiation, and prognosis among CRC patients (33).

Inflammation appears to play an important role in the etiology of colorectal neoplasms. Individuals with inflammatory bowel diseases (IBD), such as Crohn's disease or ulcerative colitis, have an increased risk of CRC, and the magnitude of this risk increases with greater extent of bowel involved, earlier age at IBD diagnosis, and longer disease duration (34, 35). There are several genetic and environmental risk factors for IBD, the details of which are beyond the scope of this dissertation; however, it is believed that chronic gut inflammation (and not the inherited genetic variants associated with IBD) is what increases CRC risk in these patients (34, 35). Prolonged inflammation in the colon (colitis), due to IBD or other reasons, can cause colitis-associated CRC. Colitis-associated CRCs also have genetic alterations in the CIN, CIMP, and/or MSI-pathways described above. However, compared to non-inflamed colorectal mucosa, inflamed colorectal mucosa is more likely to harbor these genetic/molecular alterations, even before any histologic evidence of neoplasia (34). The mechanisms by which inflammation affects these pathways are not completely elucidated, but appear to involve the generation of reactive oxygen and nitrogen species (i.e., oxidative stress) as well the upregulation of prostaglandins (PGs) and nuclear factor kappa B (NF-κB) signaling (34). PGs are a class of lipid molecules that direct a variety of host inflammatory responses (36). High expression of the PG-synthesizing cyclooxygenase-2 (COX-2, also known as prostaglandin synthase type 2) and low expression of the PG-catabolizing 15-hydroxyprostaglandin dehydrogenase (15-HPGD) are

hallmarks of neoplastic colorectal tissue and promote colorectal tumor growth *in vitro* and in mice (37-39). In APC-knockout mice, COX2+/- mice had a statistically significant 34% reduction and COX2-/- mice had an 86% reduction in number of adenomatous polyps relative to COX2+/+ mice (40). Treatment with the COX-2 inhibitor rofecoxib also significantly reduced the number of size of colonic polyps in APC-knockout mice (41). PGs (especially PGE2) and NF-kB may promote colorectal carcinogenesis, at least in part, via the upregulation of cytokines such as IL-6. IL-6 induction by PGE2 and NF-κB activates STAT3 signaling (part of the JAK/STAT pathway), which can promote cancer development by increasing cellular proliferation and inhibiting apoptosis (34). Increased PG synthesis may also promote colorectal carcinogenesis by upregulating the Wnt/ß-catenin signaling pathway mentioned above (34).

Furthermore, there is compelling evidence from observational and randomized epidemiologic studies to support that pharmacologic interventions targeting these inflammatory pathways can reduce CRC risk. Findings from several RCTs suggest that long-term use (>5 years) of aspirin—which irreversibly inactivates cyclooxygenase enzymes COX-1 and COX-2—may reduce the 20-year risk of CRC by 30-40% (20). Aspirin also reduces the risk of colorectal adenoma recurrence among colorectal adenoma patients (21). The primary anti-cancer mechanism of aspirin appears to be COX-2 inhibition, but other mechanisms, including potential regulation of 15-HPGD and inhibition of NF-κB signaling, have been proposed (42). Long-term use of NSAIDs, which includes aspirin, can cause kidney damage and increase the risk of gastrointestinal bleeding (43, 44). As these side effects appear to be primarily mediated by COX-1 inhibition, selective COX-2 inhibitors were developed and substantially found to reduce colorectal adenoma risk in RCTs (45, 46). However these agents are not commonly used for prevention as they may also increase the risk of adverse cardiovascular events (45). Thus,

identification of other chemopreventive agents that may favorably modulate PG-metabolizing enzymes with fewer side effects is critical. Vitamin D is an attractive candidate as findings from experimental studies suggest that vitamin D may specifically downregulate COX-2 and upregulate 15-HPGD in cancer cell lines (7, 8). Additionally, vitamin D, as well as calcium, may inhibit the NF-κB signaling pathway (47, 48), which, in turn, may affect COX-2 and 15-HPGD expression in colon tissue (49).

The metabolism of vitamin D, its effects on calcium levels in the body, and the mechanisms by which it may prevent colorectal carcinogenesis/progression, alone or in combination with supplemental calcium, are subsequently discussed.

Vitamin D Metabolism

Vitamin D (collective term for vitamin D2 and D3) metabolism is complex. Sources of vitamin D for humans include foods, dietary supplements, and sunlight; however, for individuals not taking vitamin D supplements, the majority (~80-95%) of vitamin D is gained from environmental sun exposure (50-52). Vitamin D2 is synthesized by ultraviolet (UV) radiation of ergosterol in plants and fungi, while vitamin D3 is synthesized by UV radiation of 7-dehydrocholesterol in the skin of humans and other vertebrate animals (**Figure 1.2**) (27). Vitamin D3 is found naturally in fatty fish and in fortified foods, such as milk and cereal in some countries, including the United States; however, individual servings of these foods generally contain less than the recommended daily amount of vitamin D, and thus maintaining adequate amounts of vitamin D from dietary sources alone is difficult (51). Vitamin D2 is found in plants and fungi, and is primarily gained by humans from the dietary intake of mushrooms (51).

Vitamin D supplements can contain either D2 or D3; however, vitamin D3 is more commonly used and may be more effective at increasing circulating vitamin D concentrations (53).

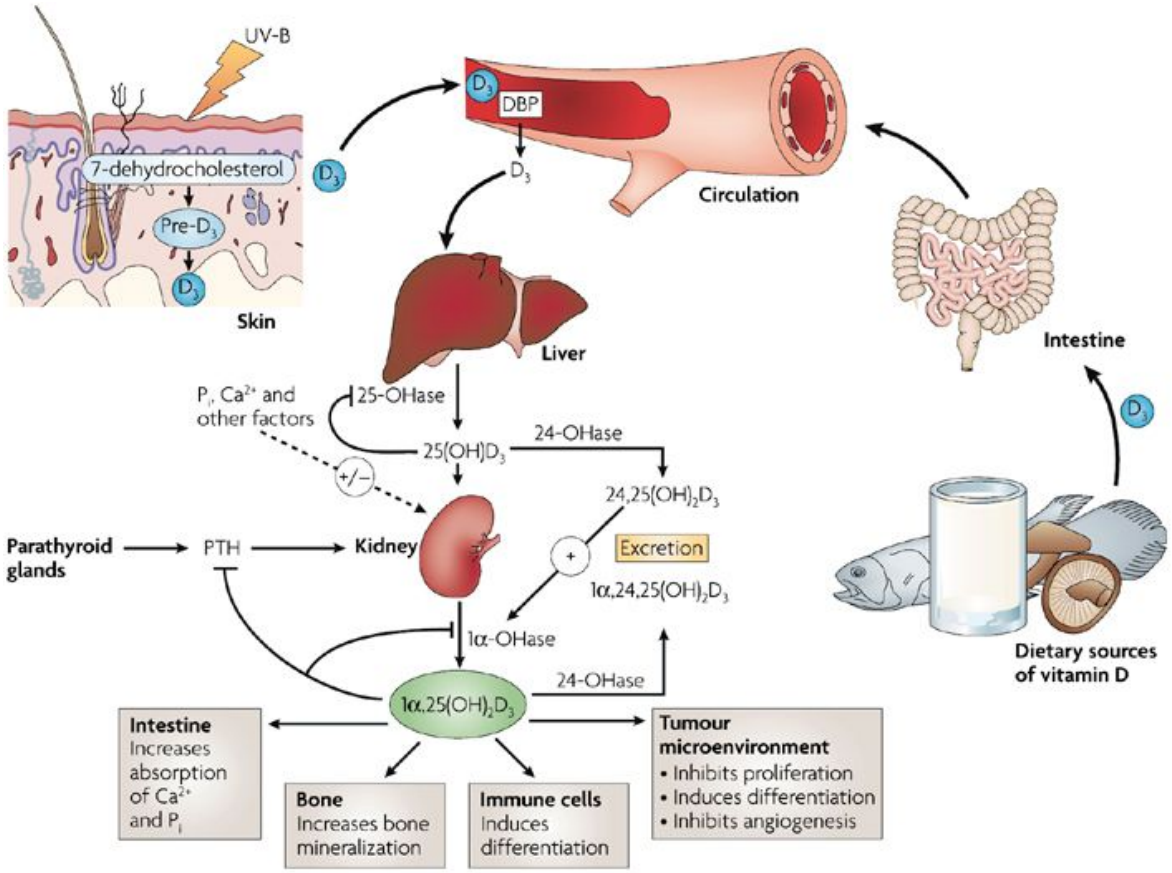

Figure 1.2. Vitamin D metabolism. [Image reproduced from Nature Reviews Cancer. Deeb KK, Trump DL, Johnson CS. Vitamin D signaling pathways in cancer: potential for anticancer therapeutics. *Nat Rev Cancer* 7, 684–700 (2007). Copyright 2007.]

Vitamin D2 and D3 are hydroxylated at the 25 position in the liver into 25-hydroxyvitamin D [25(OH)D, collective term for 25(OH)D_2 and 25(OH)D_3]. The conversion of vitamin D to 25(OH)D is catalyzed by one of the several enzymes with 25-hydroxylase activity: CYP27A1, CYP2D6, CYP2R1, CYP2C11, CYP3A4, CYP2D25, and CYP2J3 (54). CYP2R1 appears to be a key enzyme for this reaction, as CYP2R1 mutations can lead to low 25(OH)D concentrations and vitamin D-dependent rickets in humans (55). Circulating 25(OH)D

concentration is considered the best marker of total vitamin D exposure since it encompasses vitamin D exposure from multiple sources (i.e., foods, supplements and sunlight) and has a longer circulating half-life than other vitamin D metabolites (56). As a result, circulating 25(OH)D concentrations are also used clinically to assess vitamin D status. Note, hereafter, the term "circulating vitamin D concentration(s)" may be used interchangeably with "circulating 25(OH)D concentration(s)". Because $25(OH)D_2$ concentrations are low or undetectable in most individuals, the vast majority of 25(OH)D is in the form of $25(OH)D_3$ (57). Most vitamin D assays, particularly those used clinically, either measure total 25(OH)D without distinguishing $25(OH)D_2$ or $25(OH)D_3$ or measure $25(OH)D_3$ only (57).

25(OH)D is hydroxylated at the C1α position to form 1α,25-$(OH)_2$-vitamin D [$1,25(OH)_2D$; also known as calcitriol)]—a steroid hormone that is the primary activator of the vitamin D-receptor (VDR). This conversion is catalyzed by the cytochrome P450 CYP27B1 enzyme (also known as P450C1α or 1α-OHase) expressed in the kidneys and in extrarenal target tissues including the colon (55, 58). CYP27B1 expression in the kidneys is regulated by parathyroid hormone (PTH) and by $1,25(OH)_2D$ itself, while its expression in extrarenal tissues appears to be principally regulated by 25(OH)D concentrations (2, 58). The 24-hydroxylase enzyme CYP24 (also known as 24-OHase), encoded by the *CYP24A1* gene (27), catalyzes the conversion of 25(OH)D and $1,25(OH)_2D$ to 24,25(OH)D and $1,24,25(OH)_2D$, respectively, which have reduced biologic activities and are subsequently catabolized for excretion (2, 59). *CYP24A1* is expressed in all VDR-expressing cell types and is important for regulating the biologic activity of vitamin D (59). $1,25(OH)_2D$ is a strong inducer of *CYP24A1* gene expression following VDR activation, thereby promoting its own degradation (59). In summary, *CYP27B1* and *CYP24A1* gene expression is associated with increased and decreased vitamin D

pathway-induction, respectively, as the enzymes encoded by these genes are important for regulating the synthesis (by CYP27B1) and degradation (by CYP24) of the VDR-activating $1,25(OH)_2D$ molecule.

The VDR is ubiquitously expressed in tissues throughout the body and has myriad functions (27, 59). VDR acts in large part as a nuclear transcription factor and is principally activated by $1,25(OH)_2D$, which can enter cells via simple diffusion (60). Important and well-established functions of the VDR are to regulate calcium homeostasis and, in turn, promote skeletal health (27, 59). Low serum calcium concentrations stimulate the release of PTH from the parathyroid, which increases CYP27B1 expression and subsequent conversion of 25(OH)D to $1,25(OH)_2D$ in the kidneys (61). $1,25(OH)_2D$ can increase serum calcium concentrations by stimulating calcium absorption in the gut, reabsorption in the kidneys, and resorption from bone via VDR-mediated mechanisms (61). The importance of vitamin D and VDR function for skeletal health has been clearly demonstrated in gene-knockout studies of mice. *VDR*-null mice develop hypocalcemia (low serum calcium) and osteomalacia unless they are supplemented with high intakes of dietary calcium (55). Deletions or mutations of *CYP27B1,* resulting in inadequate production of $1,25(OH)_2D$ and thus VDR activation, can also result in hypocalcemia and vitamin D-dependent rickets in mice and humans (55). Inadequate vitamin D blood concentrations due to other reasons (e.g., inadequate dietary intake, lack of sun exposure etc.) can also cause rickets in children and osteomalacia in adults (61).

VDR activation affects non-skeletal physiologic pathways, including several pathways involved in cancer development and progression (2). Extra-skeletal functions of vitamin D are highlighted by the fact that VDR is expressed in almost all tissues of the body, many of which are not (at least directly) involved in calcium homeostasis (27). CYP27B1 and CY24 enzymes

are also expressed in many of these tissues, thereby allowing $1,25(OH)_2D$ to be locally metabolized and exert its effects in target tissues as an autocrine and paracrine factor (27, 59). The importance of VDR in extra-skeletal functions is also evident in *VDR*-deficient mice, which are more susceptible to developing several diseases, including autoimmune diseases such as inflammatory bowel disease and type 1 diabetes, and various cancers, including CRC (55). These findings are supported by epidemiologic studies that reported associations of vitamin D exposure and/or polymorphisms in the *VDR* gene with risk of these same health outcomes in human populations (54, 55, 62).

Vitamin D may affect colorectal carcinogenesis via several genomic and non-genomic mechanisms mediated, at least in part, by the VDR (60). Overlapping "genomic" and "alternative" ligand binding pockets of the VDR have been described that appear to direct these genomic (e.g., gene transcription) and non-genomic actions (e.g., rapid cellular responses), respectively (60). Rapid cellular responses to vitamin D may also involve a distinct membrane-bound VDR that can activate intracellular signaling molecules such as mitogen-activated protein (MAP) kinases and protein kinase C (PKC) (63). Binding of $1,25(OH)_2D$ to the nuclear VDR causes its dimerization with the retinoid X receptor (RXR); the VDR/RXR heterodimeric complex regulates the transcription of numerous genes by interacting with co-regulatory proteins and binding to vitamin D responsive elements (VDREs) in promoter regions or distal regulatory sites of target genes (27). The VDR is estimated to regulate up to 3-5% of the human genome, and over 200 genes have been identified with VDREs involved in cancer-related pathways including cell proliferation, differentiation, angiogenesis, apoptosis, inflammation, and metastasis (2, 7). VDR activation also induces expression *in vivo* of CYP3A4, a cytochrome p450 enzyme that detoxifies lithocholic acid in the intestines, which may prevent bile acid-

induced DNA mutations and compensatory hyperproliferation responses by colorectal epithelial cells (29).

Importantly, normal and neoplastic colorectal epithelial cells, as well as cells in the CRC microenvironment, can express the VDR and vitamin D-metabolizing enzymes such as CYP24 and CYP27B1 (58, 64). This highlights the relevance of vitamin D-signaling pathways in the maintenance of normal colorectal epithelial cell function in addition to their relevance in colorectal neoplasm development and progression. The regulation of VDR and vitamin D-metabolizing enzymes during CRC development and progression is complex and appears to be influenced (and affected by) tumor type, stage, and degree of cellular differentiation (2). Additionally, loss of VDR expression in several cancer cell types, including breast, melanoma, and colorectal cancers, is associated with more aggressive cancers and increased risk of cancer-related mortality.

Vitamin D Recommendations

The optimal dietary vitamin D intakes and circulating 25(OH)D concentrations are a matter of some debate. The Recommended Dietary Allowance, which aims to cover the requirements of ≥97.5% of the population, of vitamin D are 600 international units (IU) per day for individuals who are 1-70 years of age and 800 IU/day for individuals who are 71 years of age or older (65). These recommendations by the IOM are based primarily on skeletal health research, (65) and, generally, correspond to 25(OH)D blood concentrations of at least 20 ng/mL (50 nmol/L), which the IOM considers to be sufficient for bone health (65). However, various other lines of evidence—including the effects of vitamin D on PTH suppression, calcium absorption, and the V_{max} of 25-hydroxylase enzymes, in addition to paleolithic evidence—

suggest that blood levels of 30-100 ng/mL (75-250 nmol/L) are more likely to reflect levels needed for optimal health (50, 66). Additionally, these blood levels are consistent with vitamin D exposures of 1000-4000 IU/day, which are more comparable to the daily vitamin D exposures that humans likely experienced throughout most of our evolutionary history (52). In contrast to IOM recommendations, the Endocrine Society recommends 1500-2000 IU/day of vitamin D and blood 25(OH)D concentrations of at least 30 ng/mL (75 nmol/L) for adults (56, 67). Further, Endocrine Society guidelines postulate that individuals with darker skin or who are obese, sun-deprived, and/or living in more northern latitudes may require between 3000-6000 IU/day to sustain recommended 25(OH)D blood concentrations of at least 75 nmol/L (56).

Vitamin D and Calcium

As highlighted above, vitamin D and calcium metabolism in the human body are intricately linked. Thus, many RCTs related to colorectal neoplasm prevention have randomized participants to receive vitamin D and calcium alone and in combination. Experimental and epidemiologic study findings suggest that vitamin D and calcium may interact biologically and in relation to CRC risk. For example, combined calcium and vitamin D supplementation may act synergistically to lower PTH concentrations, thereby reducing $1,25(OH)_2D$ synthesis in the kidneys and calcium absorption in the gut, compared to the use of either supplement alone (68, 69). In addition to the anti-neoplastic effects of vitamin D discussed above, calcium may prevent colorectal carcinogenesis via distinct and overlapping cellular mechanisms (28). A key role of calcium in preventing CRC appears to be its effects on reducing inflammation in the gut. For example, calcium can bind to bile acids and fatty acids in the gut lumen, thereby sequestering them and preventing their pro-inflammatory toxicity to colonocytes (25, 26). Calcium may also

have direct effects on proliferation, differentiation, and apoptotic pathways in normal and neoplastic colonic cells, mediated in part via activation of the calcium-sensing receptors on colonocytes (28). On the one hand, since vitamin D promotes calcium reabsorption in the gut, increased concentrations of circulating vitamin D could theoretically antagonize the effects of supplemental calcium by reducing the amount of calcium able to bind to bile and/or fatty acids (and prevent inflammation) in the gut lumen. On the other hand, since vitamin D and calcium may also act on distinct and overlapping intracellular signaling pathways involved in colorectal carcinogenesis, increasing intake or exposure to both calcium and vitamin D could be important for lowering the risk of colorectal neoplasms. Among 803 individuals in an RCT of calcium supplementation for the prevention of colorectal adenomas, calcium supplementation reduced adenoma recurrence risk but only among participants with 25(OH)D concentrations above the median (29.1 ng/mL) (RR = 0.71, 95% CI = 0.57 to 0.89) (70). Also, higher baseline 25(OH)D concentration was associated with lower adenoma risk during trial follow-up but only among subjects who received calcium supplements (RR per 12 ng/mL increase in 25(OH)D = 0.88, 95% CI = 0.77 to 0.99). These findings suggest that calcium and vitamin D may act together, rather than separately, to lower colorectal neoplasm risk in humans.

Vitamin D, Calcium, and Biomarkers of Risk for Colorectal Neoplasms

There are currently no accepted modifiable biomarkers of risk for colorectal neoplasms that would be analogous to lipid biomarkers (e.g., cholesterol) of risk for ischemic heart disease. After the advent of these cardiovascular risk biomarkers, lifestyle and pharmacologic interventions could be more readily investigated and responses to preventive treatment could be more easily monitored. Subsequent clinical monitoring and control of these biomarkers have

helped reduce the number of cardiac deaths in the US by over 60% in the past few decades (71). Based on the molecular mechanisms underlying colorectal carcinogenesis (as described above), Bostick and colleagues developed a panel of plausible tissue biomarkers of risk for CRC, investigated the validity of these biomarkers in case-control studies of colorectal adenomas, and estimated the effects of vitamin D +/- calcium supplementation on biomarkers in pilot chemoprevention trials (72-76). To reliably quantify levels of biomarkers in colorectal tissue, Bostick *et al.* developed automated immunohistochemistry and quantitative image analysis methods (the only ones that have been validated in this context) to "score" colorectal crypts for biomarker expression (**Figure 1.3**). Significant case-control differences in biomarker expression were observed in the colorectal mucosa, supporting their validity as biomarkers of risk for colorectal neoplasms. Additionally, case-control proportional differences in the rectal mucosa were consistent with those in the upper (sigmoid and ascending) colon, supporting the appropriateness of using rectal biopsies, which offer the advantage of being less invasive, for future investigations. Findings from subsequent chemoprevention trials among patients with a previous colorectal adenoma strongly indicate that supplemental vitamin D, alone or in combination with calcium, can favorably modulate the expression of multiple biomarkers of CRC risk (e.g., APC, p21, bax) in the normal-appearing rectal mucosa (72, 76-82). However, the effects of vitamin D on tissue biomarkers of inflammation linked to CRC risk are unknown.

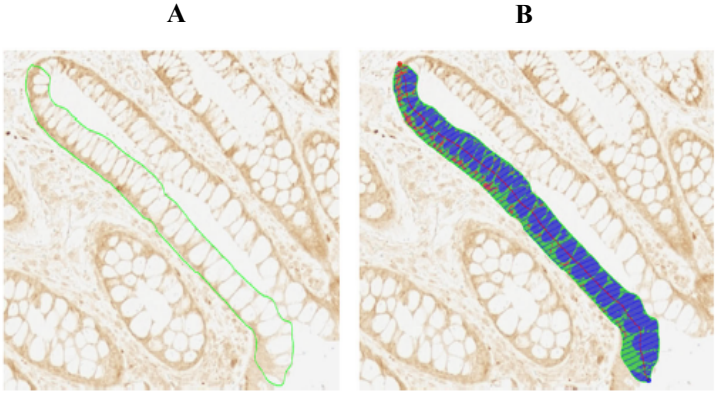

Figure 1.3. Measurement of biomarker in crypts of normal mucosa using quantitative image analysis software. **(A)** Tracing of crypt. **(B)** Automated sectioning and quantification of biomarker density.

As mentioned previously, laboratory studies indicate that vitamin D mediates key inflammation processes, such prostaglandin (PG) metabolism, involved in colorectal carcinogenesis (83). Upregulation of the PG-synthesizing COX-2 enzyme and downregulation of the PG-degrading 15-HPGD are hallmark features of dysplastic colorectal epithelia (39, 84). $1,25(OH)_2D$ also decreases COX-2 and increases 15-HPGD expression in VDR-expressing cancer cell lines (7, 8), but the effect of vitamin D supplementation on COX-2 and 15-HPGD expression in humans is unknown. Low levels of COX-2 expression in normal colorectal tissue have been an obstacle for investigating COX-2 in previous studies, but newer validated antibodies and superior staining kits have been developed demonstrating accurate and quantifiable detection of COX-2 in normal colorectal tissue (85-87). In comparison, 15-HPGD is widely expressed in normal colorectal tissue but has not been investigated in previous chemoprevention trials. These biomarkers may ultimately be used clinically for preventative treatment recommendations (i.e., prescribed vitamin D supplementation) or patient management (e.g., colonoscopies based on biomarker risk profile).

Epidemiologic Studies of Vitamin D and Colorectal Neoplasms

Observational Studies

There is strong epidemiologic evidence from observational studies supporting an association of circulating vitamin D concentrations with colorectal neoplasm risk as well as survival among CRC patients. Higher circulating 25(OH)D concentrations are consistently inversely associated with colorectal adenoma risk and CRC risk in both case-control and prospective cohort studies (5). The units of circulating 25(OH)D is generally presented either in ng/mL or nmol/L; concentrations subsequently presented as nmol/L can be converted into ng/mL

by dividing the nmol/L concentration by 2.5. In a meta-analysis of five early observational studies (conducted between 1989 and 2005), highest versus lowest quintile 25(OH)D concentrations were associated with a 54% lower CRC risk ($P_{trend} < 0.0001$), while individuals with 25(OH)D concentrations of at least 33 ng/mL relative to <12 ng/mL had an approximately 50% lower risk of CRC ($P < 0.01$) (88). In the largest pooled nested case-control study to date, including participant level data from 5,706 CRC cases and 7,107 matched controls within 17 prospective cohort studies, relative to 25(OH)D concentrations of 50–<62.5 nmol/L, considered by the IOM to be in the lower range of sufficiency for bone health, 25(OH)D concentrations considered deficient (<30 nmol/L) were associated with a 31% higher CRC risk (24). Additionally, compared to 25(OH)D concentrations of 50–<62.5 nmol/L, higher 25(OH)D concentrations of 75–<87.5 nmol/L or 87.5–<100 ng/mL (considered by the IOM to be "beyond sufficient" for bone health) were associated with statistically significant 19% and 27% lower CRC risk, respectively (24). This study also found that the association of per 25 nmol/L increase in 25(OH)D concentrations with CRC risk was stronger among women (RR = 0.81, 95% CI = 0.75 to 0.87) than among men (RR = 0.93, 95% CI = 0.86 to 1.00), but it did not statistically significantly differ according to colorectal subsite, geographic location, or season of blood collection.

Higher circulating 25(OH)D concentrations are also inversely associated with risk of mortality among CRC patients (4, 89-92). This association has been found in prospective cohort studies in which 25(OH)D was measured prior to diagnosis (89, 90) or around the time of diagnosis (4, 91, 92). Findings from some studies also suggest that 25(OH)D concentrations add value to predictive survival models for CRC patients and could be clinically useful as a prognostic biomarker among CRC patients (91, 93). In a recent meta-analysis of 11 studies with

7,718 total CRC cases, the highest versus lowest study-specific 25(OH)D concentrations were associated with a statistically significant 32% lower overall mortality risk and 33% lower CRC-specific mortality risk (4). In this meta-analysis, the association of 25(OH)D with mortality among CRC patients was slightly stronger in studies conducted in Europe (highest vs. lowest 25(OH)D category HR = 0.59, 95% CI.= 0.48 to 0.72) compared to those in the US or Asia (highest vs. lowest 25(OH)D category HR = 0.82, 95% CI = 0.58 to 1.12); but the association did not meaningfully differ according to median follow-up (< vs. ≥ 5 years) or year of study publication (< vs. ≥ 2013) (4). Also, among 16,818 US individuals followed from 1988-1994 through 2000 in the Third National Health and Nutrition Examination Survey (NHANES), 25(OH)D concentrations ≥ 80 nmol/L relative to <50 nmol/L were associated with statistically significant 72% lower risk of CRC-specific mortality (HR = 0.28, 95% CI = 0.32 to 0.89) (94).

Randomized Clinical Trials

In contrast to findings from observational studies, the results from randomized clinical trials (RCTs) that tested the effects of vitamin D supplementation on colorectal neoplasm (adenoma and carcinoma) risk were largely null. In the Women's Health Initiative study of postmenopausal women, 400 I.U. of vitamin D per day and 1.0 g of calcium per day did not reduce the risk of breast or CRC (pre-specified secondary outcomes) relative to placebo (95); however, in a subgroup analysis of participants not taking calcium or vitamin D supplements at randomization, calcium and vitamin D statistically significantly lowered total cancer and breast cancer risk by 14–18%, and non-statistically significantly lowered CRC risk by an estimated 17% (HR = 0.83, 95% CI = 0.60–1.15) (96). Limitations of the Women's Health Initiative trial were the low dose of vitamin D supplementation (below the IOM Recommended Daily

Allowance), high levels of treatment drop-in/out, treatment duration, and timing of intervention in relation to disease onset. In a recently completed, multicenter, randomized placebo-controlled clinical trial of 2,239 US participants diagnosed with a recent colorectal adenoma, a higher dose of vitamin D supplementation (1,000 I.U./day), either alone or in combination with calcium (1,200 mg/day), did not lower the risk of subsequent colorectal adenoma after 3-5 years of follow-up (97). The largest trial to date that tested the effects of vitamin D supplementation on total cancer risk and CRC risk was the VITamin D and OmegA-3 TriaL (VITAL)—a randomized, placebo-controlled, 2 × 2 factorial trial of daily vitamin D_3 (2,000 I.U.) and marine omega-3 fatty acids (1 g) among 25,871 US men aged ≥50 and women aged ≥55 (98). In this trial, vitamin D did not statistically significantly reduce the primary endpoint of total invasive cancer incidence (HR = 0.96; 95% CI: 0.88–1.06) or the pre-specified secondary endpoint of CRC incidence (HR = 1.09; 95% CI: 0.73–1.62); however, despite the trial's large sample size, less than 100 people were diagnosed with CRC during follow-up.

Although the VITAL and WHI studies did not assess the effects of vitamin D supplementation on CRC-specific survival, their results did suggest protective effects of supplementation on overall cancer survival. In the VITAL study, those randomized to vitamin D had an estimated 17% lower risk of mortality from all invasive cancers relative to those randomized to placebo (HR = 0.83, 95% CI = 0.67-1.02) (98). The Women's Health Initiative study also found a borderline statistically significant protective effect of vitamin D supplementation against total cancer mortality (HR = 0.90, 95% CI = 0.77–1.05) (95).

To our knowledge, only one randomized controlled trial (RCT) of vitamin D supplementation among CRC patients has been reported, but the results were promising

In a phase-II, US multicenter RCT of 139 advanced or metastatic CRC patients, those randomized to high-dose (4,000 IU/day) relative to low-dose (400 IU/day) vitamin D supplementation had longer progression-free survival (HR = 0.64; 1-sided 95% CI, 0 to 0.90; $P = 0.02$), which was the primary outcome (99).

Importantly, the effects of vitamin D supplementation on circulating 25(OH)D concentrations and vitamin D-related health outcomes may differ by functional genetic variants, such as those in the vitamin D-binding protein (DBP) gene, formerly known as group component (*GC*) (100, 101). The DBP protein, its common genetically determined isoforms, and the potential effect modification of the associations of 25(OH)D with, and effects of vitamin D supplementation on, colorectal neoplasm risk by DBP isoform are discussed in the following sections.

Vitamin D-Binding Protein

Structure and Regulation

The DBP, a 52-58 kDa serum ∝2-globulin protein, is a member of the albumin family of binding proteins (102). It was initially named "group-specific component" (Gc) following its discovery in 1959 (103). Shortly after its discovery, three common DBP phenotypes or isoforms were described based on unique gel electrophoresis patterns: DBP1s and DBP1f, characterized by two bands with "slow" (1s) and "fast" (1f) migration patterns, respectively, and DBP2 characterized by a single band (104). These isoforms were later discovered to be the result of two inherited missense polymorphisms at *GC* rs4588 and *GC* rs7041 that alter the amino acid sequence, post-translational glycosylation pattern, and charge of the DBP protein (105).

DBP is encoded by the *GC* gene on chromosome 4q12-13 (105). The gene is 35 kb in length with 13 exons that encode 474 amino acids, including a 16 amino acid leader sequence that is cleaved after translation (105). DBP has three distinct protein domains and multiple binding domains including an actin-binding domain between residues 373 and 409, a vitamin D-binding domain between residues 35 and 49, and two cell-binding domains between residues 150-172 and 379-402 (102).

DBP is primarily synthesized in the liver, and circulating DBP concentrations in healthy subjects generally range from 200 to 600 μg/mL (102). These concentrations are much higher than those of 25(OH)D (10-100 ng/mL) and, thus, only 5-10% of DBP is normally bound to 25(OH)D in the circulation (102). Synthesis of DBP is influenced by estrogen, inflammatory cytokines, and liver function, but does not appear to be regulated by 25(OH)D or $1,25(OH)_2D$ (106). Various clinical conditions such as liver disease, diabetes, primary hyperparathyroidism, shock, trauma, and pregnancy can also affect DBP concentrations (106). DBP concentrations are relatively stable throughout the year and do not appear to be affected by UV exposure (107).

DBP Functions

The principal role of the DBP is the binding and transport of vitamin D metabolites in the circulation. Approximately 88% of circulating 25(OH)D and 85% of circulating $1,25(OH)_2D$ are bound by the DBP in the circulation (6, 10). An important role of DBP is to maintain adequate circulating stores of vitamin D, particularly when vitamin D sources are low, by protecting vitamin D from excretion and by prolonging its half-life in the circulation (10, 11). DBP-knockout mice have extremely low circulating levels of 25(OH)D and $1,25(OH)_2D$ and develop vitamin D-deficient phenotypes (e.g., bone loss) more rapidly, compared to wild-type mice,

when placed on a vitamin D deficient diet (108). DBP is required for megalin-mediated reabsorption of 25(OH)D bound to the DBP in the proximal tubule cells of the kidneys (109). DBP can also facilitate the entry of 25(OH)D and $1,25(OH)_2D$ into target tissue cells via megalin-receptor-mediated endocytosis (109).

Apart from its role in vitamin D transport, DBP can also be converted into a potent macrophage-activating factor (GcMAF), which stimulates macrophage phagocytosis and may inhibit tumor growth (110, 111). DBP is converted into GcMAF following its partial deglycosylation by B and T-cells that express ß-galactosidase and sialidase (112). Experimental studies indicate that GcMAF can activate tumoricidal macrophages and inhibit cell proliferation in breast and prostate cancer cell lines (111, 113).

DBP may further influence immune and inflammatory processes via its role in actin scavenging, fatty acid binding, and complement-mediated immune cell chemotaxis (109). DBP binds actin monomers to prevent polymerization, which, in turn, helps prevent endothelial injury by actin polymers following tissue damage or cell lysis (109). DBP can also bind saturated and unsaturated free fatty acids, and concentrations of unsaturated fatty acids may reduce the affinity of DBP for vitamin D metabolites in the circulation (109). However, the exact role(s) of DBP in fatty acid metabolism and transport is (are) unclear. Last, DBP binds to complement component 5a (C5a) and can enhance C5a-chemotactic activity involved in innate immunity and inflammatory responses (114, 115).

DBP isoforms

Over 120 variants of the DBP have been described by isoelectric focusing, making it the most polymorphic of the major plasma proteins (102, 116). The common DBP1s, DBP1f and

DBP2 isoforms are encoded by the combined genotypes (i.e., haplotype) at *GC* rs4588 C>A (Thr to Lys substitution at position 436) and *GC* rs7041 T>G (Asp to Glu substitution at position 432) (116). The genotype and unique amino acid sequence of each DBP isoform is shown in **Figure 1.4**. DBP1f is the ancestral isoform encoded by *GC* rs4588*C (Thr) and *GC* rs7041*A (Asp), and is the most common isoform in populations with African ancestry (116). The DBP1s isoform is distinguished from the 1f ancestral isoform by the *GC* rs7041 T>G transition (Asp → Glu), while the DBP2 isoform is distinguished from the 1f isoform by the *GC* rs4588 C>A transition (Thr → Lys) (105). Thus, the DBP1f isoform differs from the other two variants by a single amino acid substitution, while the DBP1s and DBP2 isoforms differ by two amino acid substitutions.

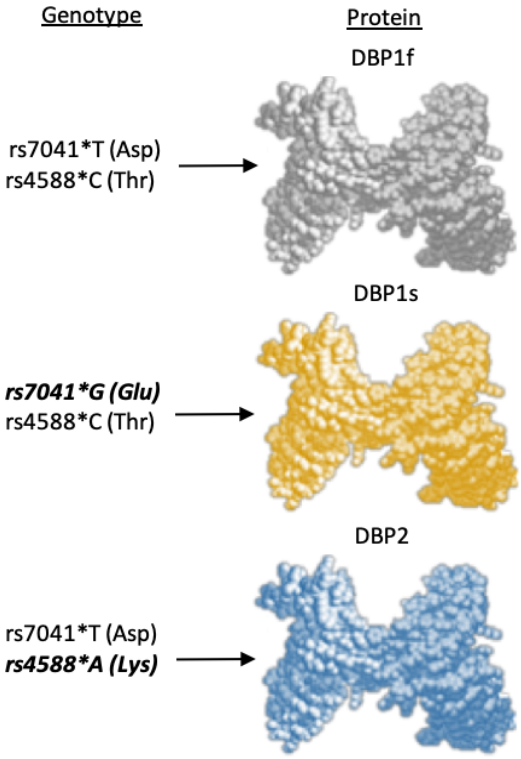

Figure 1.4. Common vitamin D-binding protein isoforms and their encoding genotypes.

Isoform Effects on Vitamin D Metabolism

Although the exact physiologic consequences of these isoforms have not been fully elucidated, their association with 25(OH)D concentration, in both healthy and diseased populations and in different ethnic groups, has been consistently reported (117-123). 25(OH)D concentrations are similar among individuals with the DBP1f- and DBP1s-encoding genotypes; however, 25(OH)D concentrations are approximately 20-30% lower among those with the

DBP2-encoding genotype relative to those with only DBP1 (1f and 1s) isoforms (118, 120). In a pooled case-control study of incident sporadic colorectal adenoma with 942 white US participants, mean 25(OH)D concentrations were 27.1 ng/mL (95% CI: 26.2-28.0) among those with only DBP1 isoforms (DBP1-1) and 20.7 ng/mL (95% CI: 18.6-22.7) among those with only DBP2 isoforms (DBP2-2), adjusted for age, sex, study, and case-control status (118). Supporting these findings are results from recent genome-wide association studies of participants with European ancestry in which tagging SNPs *GC* rs3755967 and rs2282679, in near-perfect LD (r^2>0.99) with the DBP2-encoding rs4588 SNP in European populations, were the polymorphisms most strongly associated with lower 25(OH)D concentrations (124-126).

The putative effect of these *GC* genotypes on circulating 25(OH)D concentrations may be mediated by isoform differences in DBP concentrations since DBP mediates 25(OH)D renal reabsorption and prolongs its circulating half-life (109). DBP-/- and DBP-/+ mice have very low levels of circulating 25(OH)D and 1,25$(OH)_2$D relative to wild-type, DBP+/+, mice (108). DBP concentrations are estimated to be 15-30% lower among DBP2 individuals relative to DBP1 individuals in studies that did not use the isoform-biased monoclonal DBP ELISA (127-131). Studies that used the common R&D monoclonal DBP ELISA reported even more pronounced differences in DBP concentrations by isoform (>80% of the variability in DBP concentrations were explained by DBP isoform), although there is concern that the monoclonal antibody may not have equal affinity for the protein isoforms and may lead to falsely low DBP measurements for those with the DBP1f phenotype (132). Individuals with the DBP2 isoform may be predisposed to lower DBP concentrations since DBP2 may have a faster metabolic rate, and thus shorter half-life, than do the DBP1 isoforms (133).

Findings from some (134, 135), but not all (136, 137), studies indicate that the DBP isoforms also have different binding affinities to vitamin D ligands. Arnaud and Constans reported the following gradient in the affinity of DBP to $25(OH)D_3$ and $1,25(OH)_2D_3$: DBP1f > DBP1s > DBP2 (135). The reported binding constants (Ka) of the isoforms for both $25(OH)D_3$ and $1,25(OH)_2D_3$ were significantly different ($P < 0.001$) and were approximately four times greater for the highest-affinity DBP1f relative to those for the lowest-affinity DBP2 isoform. In this study, the Ka's for $25(OH)D_3$ were 1.12 ± 0.13 for DBP1f, 0.60 ± 0.15 for DBP1s, and 0.36 ± 0.10 ($\times 10^9$ M^{-1}) for DBP2 (135). These affinity constants are the most commonly cited in the literature and are usually used to calculate free and bioavailable 25(OH)D based on DBP isoform genotype (109, 138). These reported differences in binding affinity (as well as differences in DBP concentrations) by isoform may underlie the higher induction of vitamin D target genes by 25(OH)D in monocytes and colon cancer cell lines cultured with DBP2 compared to cells cultured with DBP1 isoforms (119, 139). Some earlier studies reported no significant differences in binding affinity by isoform (136, 137, 140), although it has been argued that these null reports may be due to differences in buffering systems and buffer pH that can alter the protein's binding affinity constants (134). Findings by Constans *et al.* also indicated that the observed 25(OH)D affinity differences by isoform were related to their isoelectric points (pIs), such that isoforms with lower pIs had stronger binding affinities (134). It is established that the common DBP isoforms differ by pI with the following gradient: DBP1F (pI 4.94-4.84) < DBP1S (pI 4.95-4.85) < DBP2 (pI 5.1), which supports the same putative gradient in binding affinity reported in other studies (105, 134, 135). These differences in isoelectric points are the result of different amino acid sequences and post-translational glycosylation patterns (e.g., *O*-

linked glycosylation to Thr at position 436 is found on DBP1f and DBP1s, but not on DBP2 due to the Thr→Lys substitution at position 436) (105, 135).

Free Hormone Hypothesis

According to the free hormone hypothesis, only free ligands can act biologically. Thus, changes in binding proteins concentrations and/or differences in binding affinities can affect the biologic activity of hormones by influencing relative free hormone concentrations. The clinical relevance of measuring binding protein concentrations in order to calculate free hormone concentrations is established for sex hormones and thyroid hormones, but not for vitamin D. Nevertheless, differences in DBP concentrations as well as potential isoform-specific binding affinities may influence bioavailable and free vitamin D concentrations (107, 108, 141). Additionally, some studies suggest that serum free or bioavailable 25(OH)D may better correlate with skeletal health outcomes (142, 143), indicating the potential clinically utility of measuring DBP concentrations and/or DBP isoform along with 25(OH)D.

Free vitamin D concentrations can either be directly measured or calculated based on concentrations and binding affinity constants (Ka) of DBP and albumin. The following formula can be used to calculate free vitamin D: $free\ vitamin\ D = \ (total\ vitamin\ D\ )/(1 + (Ka\_alb * alb) + (Ka\_DBP * DBP))$ (138). Based on isoform-specific DBP concentrations and binding affinities, Chun *et al.* modeled the amount of predicted free 25(OH)D and 1,25$(OH)_2$D expected at a given total concentration *in vivo* (138) (**Figure 1.5**). Since the affinity of 25(OH)D for albumin is very

small relative to its affinity for DBP, some argue that the albumin-bound portion of bioavailable 25(OH)D is essentially free and may have a biologic activity similar to that of truly free 25(OH)D (144). This could be important since differences in bioavailable 25(OH)D by DBP isoform are much more pronounced than differences in free 25(OH)D, given the very small percentage of free 25(OH)D in the circulation (108, 142). For example, in a study of 256 postmenopausal women, Johnsen *et al.* found that the absolute difference in the percent bioavailable 25(OH)D for DBP2-2 (lowest affinity) vs. DBP1f-1f (highest affinity) individuals was 20 (i.e., an estimated 28% of total 25(OH)D was bioavailable among DBP2-2 vs. 8% among DBP1f-1f individuals) whereas the absolute differences in the percent calculated free 25(OH)D between DBP2-2 and DBP1-1 was only 0.05 (i.e., an estimated 0.07% of total 25(OH)D was free among DBP2-2 vs. 0.02% among DBP1f-1f individuals) (142). Thus, despite lower levels of total 25(OH)D associated with DBP2-2 (mean = 62.2 nmol/L) vs. DBP1f-1f (mean = 70.4 nmol/L), DBP2-2 individuals had higher levels of bioavailable 25(OH)D (mean = 17.3 nmol/L) and free 25(OH)D (mean = 0.04 nmol/L) compared to DBP1f-1f individuals

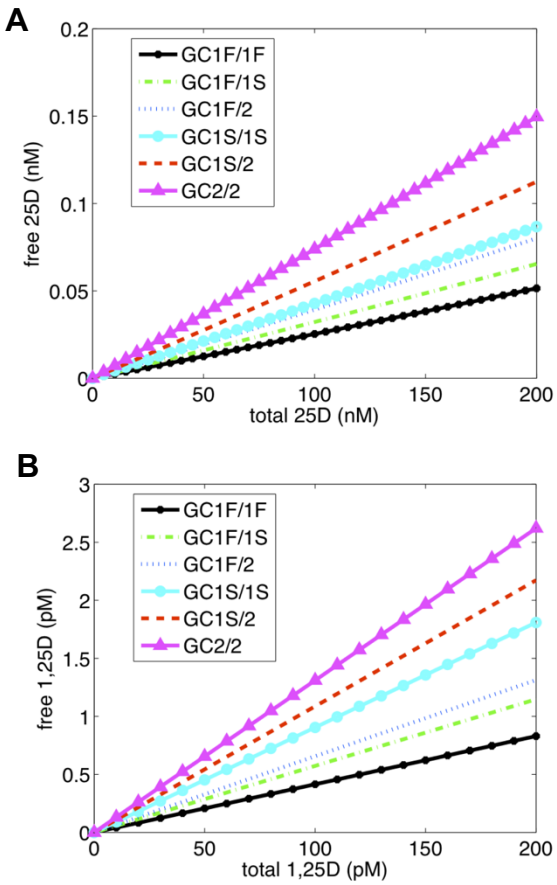

Figure 1.5. Predicted concentrations of free **(A)** 25(OH)D, and **(B)** 1,25(OH)$_2$D relative to the total circulating concentrations of these metabolites *in vivo* (100% serum) according to DBP isoform genotype. [Image reproduced from Plos one: Chun RF et al. Vitamin D binding protein and monocyte response to 25-hydroxyvitamin D and 1, 25-dihydroxyvitamin D: analysis by mathematical modeling. *PloS one* 24;7(1):e30773 (2012). Copyright 2012.]

(bioavailable mean = 5.3 nmol/L, free mean = 0.01 nmol/L) (142). An important caveat, however, is that circulating vitamin D concentrations may not necessarily reflect the amounts of total, bioavailable, and free 25(OH)D (or $1,25(OH)_2D$) available at the tissue level. As a result, the influence of the DBP isoforms on levels of free 25(OH)D and $1,25(OH)_2D$ available to target VDR-expressing cells *in vivo* is still unclear.

Ethnic Variation in DBP Isoform Frequencies

The occurrence and maintenance of DBP variants throughout the course of our evolutionary history is believed to be the result of selective advantages of variant phenotypes in different environments (116). There are striking differences in the frequencies of the common DBP1s, DBP1f and DBP2 variants globally and across different ethnic populations, as shown in **Figure 1.6.** The ancestral DBP1f variant is the most common isoform in African populations and its frequency is higher in Central and West African populations (70-90%) than in Northeast African populations (40-60%) (116). East Asian

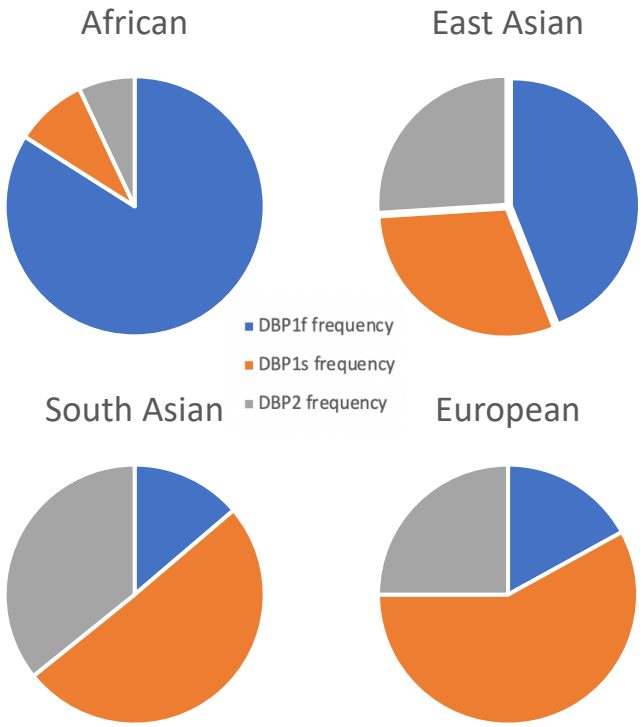

Figure 1.6. DBP isoform frequencies in different populations of the 1000 Genomes Project. **African** includes populations from Nigeria, Kenya, Gambia, Nigeria, and Americans of African ancestry in the southwest US; **East Asian** includes populations from China, Japan and Vietnam; **South Asian** includes populations from India, Pakistan, Bangladesh, and UK residents with Indian and Sri Lankan ancestry; **European** includes populations from Italy, Finland, the UK, and Spain. Data from the NCI "LD Link" tool, accessed online 3 Jan 2020 at: https://ldlink.nci.nih.gov

populations have a relatively equal distribution of DBP1f, DBP1s, and DBP2 isoform frequencies (30-40% for each), while in European populations the most common isoform is DBP1s (50-60%), followed by DBP2 (20-30%), and then DBP1f (10-20%).

It is hypothesized that the DBP1s and DBP2 isoforms may be more common in non-African populations as a lower binding affinity associated with these variants could offer a selective advantage for populations migrating out of Africa into regions with less UV exposure (and thus less total 25(OH)D) by increasing relative amounts of free and bioavailable 25(OH)D. There is a clear geographic cline in the frequency of DBP1f and DBP1s variants moving from the equator to more Northern latitudes, such that as you move away from the equator, populations are less likely to harbor the DBP1f variant and more likely to harbor the DBP1s variant. This cline follows that of lighter skin pigmentation in populations living further from the equator, which is believed to have been important for the maintenance of adequate UV-induced cutaneous synthesis of vitamin D as human populations migrated into regions with less UV radiation.

The geographic variability in DBP2 frequency is somewhat less pronounced than that of the DBP1s, and the selective pressures that could have influenced its maintenance throughout evolution, particularly in non-African populations, are not as evident. On the one hand, it is associated with lower DBP, and thus lower 25(OH)D, concentrations relative to the DBP1s and DBP1f isoforms, which would be seemingly disadvantageous in areas with lower UV exposure. On the other hand, lower DBP coupled with a potentially lower binding affinity may also increase the relative amounts of free 25(OH)D and vitamin D-pathway induction, especially when vitamin D exposure is adequate (as supported by mathematical modeling shown in **Figure 1.5**). This advantage of the DBP2 isoform—conditional on adequate total vitamin D exposure—

may explain why it is most prevalent in non-Black populations living in sunnier climates such as those in South Asia (**Figure 1.6**).

These evolutionary hypotheses related to ethnic variation in DBP isoforms may be difficult to test directly, but nevertheless may be important when considering potential interactions between 25(OH)D concentrations (i.e., total vitamin D exposure) and DBP isoforms in relation to vitamin D-associated health outcomes in population-based studies.

Interaction between Vitamin D and Vitamin D-Binding Protein Isoforms in Epidemiologic Studies

Genetic variants or polymorphisms are important in the field of cancer epidemiology, serving not only as independent risk factors for disease (e.g., BRCA1), but as modifiers of the effects of environmental risk factors (e.g., NAT2 genotypes and smoking (145)). While several studies investigated associations of polymorphisms in vitamin D-related genes with CRC risk and survival (54, 146), few investigated gene-environment interactions between SNPs and vitamin D exposure (147). Findings from recent studies suggest that higher serum 25(OH)D concentrations are more strongly inversely associated with CRC risk among persons with lower DBP concentrations (148), and more strongly inversely associated with diabetes risk among persons with the DBP2 isoform-encoding genotype (149). Given DBP2's strong association with lower DBP concentrations, higher circulating 25(OH)D concentrations may be more beneficial among individuals with DBP2 isoforms, as this may be needed to compensate for their lower DBP-related capacity to otherwise maintain adequate vitamin D concentrations.

We recently investigated whether DBP2 modified the association of 25(OH)D_3 incident, sporadic colorectal adenoma risk (Gibbs *et al.*, Am J Epidemiol, 2018) (118). We hypothesized

that the inverse association of 25(OH)D_3 with incident, sporadic colorectal adenoma risk would be stronger among persons with the DBP2 isoform and weaker among persons with only DBP1 isoforms. To investigate this hypothesis, we pooled data from three US colonoscopy-based case-control studies (418 adenoma cases, 524 polyp-free controls). The pooled case-control study data were used previously by Fedirko *et al.* to investigate the association of 25(OH)D_3 with incident, sporadic colorectal adenoma risk (150).

Table 1.1. Associations of Seasonally-Adjusted Circulating 25(OH)D_3 Concentrations With Incident, Sporadic Colorectal Adenoma Stratified by Vitamin D Binding Protein (GC, Group Component) Isoforms in Pooled CPRU and MAP Case-Control Studies (United States, 1991-2002)

	Among DBP1-1 Individuals[a]			Among DBP1-2/DBP2-2 Individuals[b]			
Variable	**No. Cases / No. Controls**	**OR[c]**	**95% CI**	**No. Cases / No. Controls**	**OR[c]**	**95% CI**	**$P_{interaction}$**
25(OH)D_3 concentration (per 10 ng/mL)[d]	209/257	1.07	0.87, 1.32	208/264	0.71	0.56, 0.90	0.03
25(OH)D_3 quartiles							
1 (< 17.9 ng/mL)	39/46	Referent		69/79	Referent		
2 (17.9 - < 24.3 ng/mL)	52/60	1.10	0.58, 2.08	57/66	0.80	0.47, 1.38	
3 (24.3 - < 31.5 ng/mL)	53/71	0.98	0.52, 1.85	47/65	0.55	0.31, 0.97	
4 (> 31.5 ng/mL)	65/80	1.02	0.55, 1.91	35/54	0.46	0.24, 0.88	0.04
P_{trend}[e]		0.94			0.008		
Clinical 25(OH)D_3 cutoffs[f]							
Deficient (< 20 ng/mL)	51/62	Referent		91/97	Referent		
Non-deficient (≥ 20 ng/mL)	158/195	1.11	0.68, 1.82	117/167	0.51	0.33, 0.80	0.05
Deficient (< 20 ng/mL)	51/62	Referent		91/97	Referent		
Insufficient (20 - 30 ng/mL)	86/101	1.13	0.66, 1.93	71/102	0.52	0.32, 0.85	
Sufficient (≥ 30 ng/mL)	72/94	1.08	0.62, 1.89	46/65	0.48	0.27, 0.87	0.09
P_{trend}[e]		0.83			0.01		

Abbreviations: 25(OH)D_3, 25-hydroxyvitamin D_3; CI, Confidence Interval; CPRU, Cancer Prevention Research Unit; *GC,* group component (encoding vitamin D binding protein); MAP, Markers of Adenomatous Polyps; OR, odds ratio.
[a] *Gc1-1:* combined *Gc1s-1s, Gc1s-1f, Gc1f-1f*
[b] *Gc1-2:* combined *Gc2-1s, Gc2-1f*
[c] Odds ratios adjusted for age (continuous), sex, study (CPRU, MAP), regular use of aspirin or nonsteroidal anti-inflammatory drugs, family history of colorectal cancer in a first-degree relative, smoking status (current, ever, never), alcohol intake (continuous), total calcium intake from diet and supplements (continuous), body mass index (continuous), and physical activity (continuous).
[d] Coded as a continuous variable in the model.
[e] Where denoted, P_{trend} values calculated by including the 25(OH)D_3 predictor variable as a continuous variable in the model.
[f] Categories commonly used in clinical practice.

The associations of $25(OH)D_3$ with incident, sporadic colorectal adenoma according to DBP2 isoform from this study are presented in **Table 1.1.** $25(OH)D_3$ concentration was associated with a statistically significant, approximately 29% lower risk of adenoma per 10 ng/mL higher $25(OH)D_3$ concentration among participants with the DBP2 isoforms (DBP1-2 and DBP2-2 genotypes combined), but the estimated association among those with the DBP1-1 genotype was close to null (*P* for interaction = 0.03). Among participants with the DBP2 isoform, those in the highest (>31.5 ng/mL) relative to those in the lowest (<17.9 ng/mL) quartile of $25(OH)D_3$ were statistically significantly less likely (by >50%) to have an adenoma, but the corresponding estimated associations among participants with only DBP1 isoforms were close to null. Using commonly applied clinical cutoffs for vitamin D deficiency (20 ng/mL (29) or 30 ng/mL (30), depending on professional society), among participants with the DBP2 isoform, a $25(OH)D_3$ concentration of at least 20 ng/mL relative to less than 20 ng/mL was associated with a 49% lower risk of adenoma, whereas concentrations of 20–30 ng/mL and greater than 30 ng/mL, relative to less than 20 ng/mL, were associated with statistically significant 48% and 52% lower risks of adenoma, respectively; however, the estimated associations among participants with only DBP1 isoforms were close to null. $25(OH)D_3$ was used in these analyses because of the poor reliability of our $25(OH)D_2$ measurements. Most participants (96%) in this study had undetectable or very low (< 10 ng/mL) $25(OH)D_2$ concentrations, consistent with other studies (151), and substitution of total 25(OH)D ($D_2 + D_3$) for $25(OH)D_3$ in sensitivity analyses did not materially affect the results.

These findings suggest that circulating $25(OH)D_3$ concentrations may be inversely associated with incident, sporadic colorectal adenoma, but only among those who have inherited the DBP2-encoding genotype, which was previously associated with lower DBP and 25(OH)D

concentrations (6, 13, 15). We hypothesize that higher 25(OH)D_3 blood concentrations, the best indicator of total vitamin D exposure, may be particularly beneficial for adenoma prevention among individuals with the DBP2 isoform as they may have a lower DBP-related capacity to otherwise maintain adequate vitamin D concentrations. In contrast, higher 25(OH)D_3 concentrations may not be associated with adenoma risk among individuals with only DBP1 isoforms, because higher DBP concentrations associated with this isoform may be able to "compensate" and maintain adequate circulating vitamin D concentrations even when vitamin D exposure is low.

Results from two RCTs also suggest that the DBP2 isoform may modify the effects of vitamin D supplementation on 1) increasing 25(OH)D concentrations, and 2) colorectal adenoma recurrence (100, 152). In these trials, vitamin D supplementation increased 25(OH)D concentrations more among those with the DBP2-encoding *GC* rs4588 variant (101, 152). In the smaller of these trials, 98 adults were randomized to receive 600 or 4000 IU/day vitamin D over 1 year; among those randomized to 4000 IU/day, 25(OH)D concentrations increased by 136% (SD=16%) among participants with the DBP1-1 genotype (rs4588*CC), 256% (SD=58%) among those with the DBP1-2 genotype (rs4588*CA), and 416% (SD=52%) among those with the DBP2-2 genotype (rs4588*AA). 25(OH)D changes did not significantly differ by genotype among those randomized to receive 600 IU/day (mean 25(OH)D increase = 50–75%) (152). Additionally, in the larger RCT that tested the effects of 1000 I.U./day vitamin D on colorectal adenoma recurrence among 2,259 participants with a prior adenoma (100), the 'interaction relative risk'—ratio of the vitamin D supplementation RR per *GC* rs4588 minor A allele divided by that for the major C allele—was 0.82 (95% CI: 0.69 to 0.98), indicating that the effect of

vitamin D supplementation on reducing adenoma recurrence was significantly stronger with each DBP2-encoding variant inherited ($P_{interaction}$ = 0.03).

Summary and Rationale for the Proposed Research

As noted above, the results from our previous trials indicate that vitamin D supplementation can favorably modify levels of tissue biomarkers of risk for CRC; however, tissue biomarkers of prostaglandin-mediated inflammation, which appears to play a crucial role in CRC etiology, have not been investigated. Laboratory studies indicate that vitamin D inhibits the tumor-promoting prostaglandin pathway by decreasing expression of COX-2 (which synthesizes prostaglandins) and increasing expression of 15-PGDH (which degrades prostaglandins) (8, 153). COX-2 and 15-PGDH are important biomarkers of risk for CRC that are, respectively, upregulated and downregulated in colorectal neoplasms relative to normal colorectal tissue (39, 84); however, the effects of vitamin D supplementation on COX-2/15-PGDH expression in the normal colorectal mucosa (Aim 1) are unknown.

Additionally, previous epidemiological studies indicate that higher circulating 25(OH)D concentration is associated with lower CRC risk and mortality; however, whether these associations differ by functional polymorphisms known to affect vitamin D metabolism and its circulating concentrations is unknown. Results from our recent study suggest that the association of circulating 25(OH)D concentrations with colorectal adenoma risk differs by vitamin D binding protein (DBP) isoforms, warranting future investigations of 25(OH)D-DBP isoform interactions in relation to CRC risk (Aim 2) and outcomes (Aim 3).

CHAPTER II

Research Aims and Approach

Specific Aims

The overarching goals of this dissertation project are to assess the effects of supplemental vitamin D on inflammation-related tissue biomarkers of risk for CRC (Aim 1), and to investigate the interactions between circulating vitamin D and DBP isoforms in relation to CRC risk (Aim 2) and survival among CRC patients (Aim 3).

Specific Aim 1: Estimate the effects of vitamin D supplementation (1,000 IU/day) on COX-2 (pro-inflammatory) and 15-HPGD (anti-inflammatory) expression in the normal-appearing rectal mucosa of a subset of colorectal adenoma patients (n = 104) in a recently completed randomized, double-blind, placebo-controlled clinical trial (NCT00153816).

Hypothesis: Vitamin D supplementation decreases COX-2 expression and increases 15-HPGD expression in the normal-appearing rectal mucosa.

Specific Aim 2: Investigate whether associations of circulating 25(OH)D_3 concentrations with CRC risk differ by DBP isoform in a pooled prospective case-control study (1,710 CRC cases, 1,649 controls) nested within three large cohorts: the Cancer Prevention Study-II (CPS-II), European Prospective Investigation into Cancer and Nutrition (EPIC), and the Nurses' Health Study (NHS).

Hypothesis: Higher 25(OH)D_3 blood concentrations are associated with lower CRC risk among individuals who have inherited the DBP2 isoform, but not among individuals without the DBP2 isoform.

Specific Aim 3: Investigate whether associations of circulating 25(OH)D_3 concentrations with overall and CRC-specific mortality among individuals with CRC differ by DBP isoform in a pooled prospective cohort study using CPS-II and EPIC data (*n* =1,352 CRC cases; 492 deaths from CRC).

Hypothesis: Higher 25(OH)D_3 blood concentrations are associated lower mortality among CRC patients who have inherited the DBP2 isoform, but not among individuals without the DBP2 isoform.

Research Approach

Aim 1 Study Population

Details of the study population, inclusion and exclusion criteria, recruitment, and protocols were published previously for the parent trial (100, 154) and the nested biomarker study (155). Briefly, eligible participants in the larger trial were 45-75 years of age, in good health, and had a histologically confirmed adenomatous polyp from a colonoscopy within the past four months. Exclusion criteria included colorectal carcinoma, inflammatory bowel disease, serum 25(OH)D_3 <12 ng/mL or >90 ng/mL, and medical conditions for which vitamin D/calcium is required or contraindicated (see (100, 154) for detailed information). For the nested biomarker study, biopsies of normal-appearing rectal mucosa at baseline and 1-year follow-up were collected from 104 participants. Of these 104 participants, the mean age was 59 years, 46% were men, and 79% were white. During the first year after randomization, 76% of participants reported taking 80% or more of their study tablets. There was a mean increase in serum 25(OH)D_3 of 10.87 (SD = 9.57) ng/mL at year 1 in subjects randomized to vitamin D relative to those who were not (155).

Aim 1 Analysis Plan

The distributions of participant characteristics (age, sex, etc.) will be compared across treatment groups using chi-square tests for categorical variables and ANOVA or *t*-tests for continuous variables. The effect of vitamin D on biomarker expression over a one-year period will be assessed by comparing the change in biomarker expression from baseline to one-year follow-up in the treatment groups relative to the placebo group using mixed linear regression models. In exploratory analyses, stratified mixed linear models will be used to estimate whether the effect of vitamin D supplementation on biomarker expression differs by patient characteristics (DBP isoform, age, sex, use of non-steroidal anti-inflammatory drugs [NSAIDs], BMI, adenoma location, race etc.). All statistical analyses will be conducted using SAS 9.4, and a p-value ≤ 0.05 (two-sided) will be considered statistically significant.

Aim 2 & 3 Study Populations

For Aim 2, I will use data collected from participants with available $25(OH)D_3$ and genotyping information in three cohort studies: *EPIC* (1,106 CRC cases, 719 controls), *CPS-II* (246 cases, 217 controls), and *NHS* (358 cases, 713 controls). For Aim 3, I will use data collected from these same 1,106 CRC cases in EPIC and 246 cases in CPS-II for whom follow-up data are available. Details of the study populations, recruitment, and data collection in CPS-II (156, 157), EPIC (158, 159), and NHS (148) were published previously. Briefly, EPIC and CPS-II recruited men and women from 10 Western European countries and 21 US states, respectively; the NHS recruited female nurses from the US. Blood samples were collected from 1998-2001 in CPS-II, 1992-1998 in EPIC, and 1989-1991 in the NHS. $25(OH)D_3$ concentrations were measured for 2,214 incident CRC cases and 2,249 controls, matched on age, sex, and date of

blood draw using incidence density sampling in EPIC (160), CPS-II (24), and NHS (148), as described previously. Of these, 1,710 (77.2%) CRC cases and 1,649 (73.3%) controls have relevant genotyping data and will be included in the analyses. There are no material differences in the distributions of 25(OH)D concentrations or other CRC risk factors between participants with and without genotyping.

Aim 2 & 3 Analysis Plan

For Aim 2, I will investigate associations of common DBP isoforms with serum $25(OH)D_3$ concentrations among CRC cases and matched controls using multivariable general linear models adjusted for age, sex, case-control status and study. $25(OH)D_3$ concentrations will be seasonally-adjusted using a previously-validated method (161). We will investigate the association of $25(OH)D_3$ blood concentrations (per 10 ng/mL) with CRC risk among those who inherited at least one DBP2 isoform and, separately, among those who inherited only DBP1 isoforms. I will also examine these associations with $25(OH)D_3$ coded as a binary categorical variable based on common clinical cut-offs for vitamin D deficiency. $25(OH)D_3$-CRC risk associations will be estimated using conditional logistic regression in minimally adjusted (controlling for matching factors and study) and fully-adjusted models (controlling for other potential confounding variables). Examples of potential confounders include body mass index, physical activity, smoking status, and various dietary intakes; inclusion in the final model will be based on consideration of biological plausibility, previous literature, and the effect of inclusion/exclusion of the variable, singly or grouped with other variables, on the odds ratio (OR) for the $25(OH)D_3$-CRC risk association. Multiplicative interaction between DBP isoforms and $25(OH)D_3$ will be investigated by comparing multivariable logistic regression models with

and without the interaction term using the log-likelihood ratio test. Interaction on the additive scale will be assessed with the attributable proportion due to interaction and interaction contrast ratio (162). In exploratory analyses, we will also stratify our models by selected participant and tumor characteristics that may be plausible effect modifiers.

For Aim 3, I will estimate the association of seasonally-adjusted 25(OH)D_3 blood concentrations with CRC-specific survival among EPIC and CPS-II CRC cases in minimally-adjusted (age, sex, study) and fully-adjusted multivariable Cox proportional hazard models among those who inherited at least one DBP2 isoform and, separately, among those who inherited only DBP1 isoforms. Examples of potential covariates in the fully adjusted model include physical activity, smoking status, body mass index, dietary intakes, tumor location, and tumor stage. The criteria for inclusion and 25(OH)D_3 coding will be the same as that described in Aim 2. Potential interactions between 25(OH)D_3 and DBP isoforms will be estimated by comparing Cox models with and without the interaction terms using the log likelihood ratio test. In exploratory analyses, we will also stratify our models by selected participant and tumor characteristics.

For Aim 2, based on the results of our previous study (163), and the estimated frequency of the DBP2 isoform (~0.3) in whites, we will need a sample size of 1,513 individuals (757 CRC cases, 756 controls) to detect an estimated OR of 0.70 for a per 10 ng/mL increase in serum 25(OH)D_3 levels among individuals with the DBP2 isoform (PASS Version 15.0.3) (164), and a sample size of 1,768 (884 CRC cases / 884 controls) to detect a 25(OH)D_3 x DBP isoform interaction in our logistic regression models with a power of 0.80 and alpha of 0.05 (PASS version 15.0.3) (165). Our sample size exceeds this number.

For Aim 3, given the estimated prevalence of the DBP2 isoform and deficient 25(OH)D_3 levels in our study population, to detect the minimum estimated HR of 1.60 (estimated by previous study (166)) for those with 'deficient' (<30 ng/mL) relative to 'non-deficient' (≥ 30 ng/mL) serum 25(OH)D_3 levels among participants with the DBP2 isoform (power=0.80, alpha=0.05), we will need a sample size of 1,096 CRC cases and 383 colorectal-cancer specific deaths (R version 3.2.0) (167). Our sample size exceeds this number.

Innovation and Significance

The proposed studies will be the first to 1) estimate the effects of vitamin D supplementation on COX-2 and 15-HPGD expression in colorectal adenoma patients (within one of the largest chemoprevention trials of vitamin D/calcium to date), and 2) investigate whether the highly publicized associations of vitamin D status with CRC risk and survival may differ by common, inherited genotypes associated with differences in vitamin D metabolism using participant-level data from some of the largest prospective cohort studies ever conducted in the US and Europe. This research will help elucidate the anti-neoplastic effects of vitamin D, the development of treatable biomarkers of risk for CRC, and the development of personalized vitamin D recommendations, based on one's inherited vitamin D-related handling capacity, ultimately reducing CRC incidence and mortality worldwide.

CHAPTER III

Modulation of inflammation by vitamin D and calcium in morphologically normal colorectal mucosa of colorectal adenoma patients in a randomized chemoprevention trial

Manuscript Information

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ABSTRACT

Increased cyclooxygenase-2 (COX-2) and decreased 15-hydroxyprostaglandin dehydrogenase (15-HPGD) expression promote prostaglandin-mediated inflammation and colorectal carcinogenesis. Experimental studies suggest that vitamin D and calcium may inhibit these inflammatory pathways, but their effects on COX-2 and 15-HPGD colorectal tissue expression in humans is unknown. We tested the effects of vitamin D (1,000 I.U./day) and/or calcium (1,200 mg/day) supplementation on COX-2 and 15-HPGD expression in the morphologically-normal rectal mucosa from 62 colorectal adenoma patients in a placebo-controlled chemoprevention trial. We measured biomarker expression using automated immunohistochemistry and quantitative image analysis at baseline and 1-year follow-up, and assessed treatment effects using mixed linear models. The primary outcome was the COX-2/15-HPGD expression ratio, since these enzymes function as physiologic antagonists. After 1 year of treatment, the mean COX-2/15-HPGD expression ratio in full-length crypts proportionately decreased 47% in the vitamin D group ($P = 0.001$), 46% in the calcium group ($P = 0.002$), and 34% in the calcium + vitamin D group ($P = 0.03$), relative to the placebo group. Among individuals with the functional vitamin D-binding protein isoform DBP2 (*GC* rs4588*A), the COX-2/15-HPDG ratio decreased 70% ($P = 0.0006$), 75% ($P = 0.0002$), and 60% ($P = 0.006$) in the vitamin D, calcium, and combined supplementation groups, respectively, relative to placebo. These results show that vitamin D and calcium favorably modulate the balance of COX-2 and 15-HPGD expression in the normal-appearing colorectal mucosa of colorectal adenoma patients (perhaps especially those with the DBP2-genotype) and support an important anti-inflammatory/anti-colorectal carcinogenesis mechanism for these agents.

INTRODUCTION

Despite advances in screening and treatment, colorectal cancer (CRC) is the third most common type of cancer and the second leading cause of cancer death among men and women in the United States (US) (168). Currently, there are no accepted chemopreventive agents against or modifiable biomarkers of risk for CRC analogous to statins and cholesterol, respectively, for ischemic heart disease, which have helped reduce cardiovascular disease mortality in the US by over 70% in recent decades (169). Prospective epidemiologic study findings indicate that higher calcium intake and vitamin D exposure are associated with lower risk of colorectal neoplasms (23, 24), and strong experimental evidence supports protective effects of calcium and vitamin D against colorectal carcinogenesis (2, 25-29). Proposed mechanisms for calcium's anti-neoplastic effects include binding bile and fatty acids in the gut, thereby preventing their toxicity and thus tumor-promoting inflammatory responses in colorectal epithelia (25, 26, 28). Vitamin D also promotes bile acid degradation and regulates multiple inflammatory carcinogenesis-promoting pathways *via* binding to the vitamin D receptor (VDR) in colorectal tissue (28, 29). However, the effects of supplemental calcium and vitamin D on inflammation-related tissue biomarkers of risk for CRC in humans are unknown.

Prostaglandins (PGs) are a class of lipid molecules that direct inflammatory responses and play an important role in colorectal carcinogenesis (36). PGs are synthesized by cyclooxygenase-2 (COX-2, also known as prostaglandin synthase type 2) and catabolized by 15-hydroxyprostaglandin dehydrogenase (15-HPGD) (36). Increased expression of COX-2 and decreased expression of 15-HPGD are hallmarks of neoplastic colorectal tissue and promote colorectal tumor growth (37-39). Since 15-HPGD physiologically antagonizes COX-2 (38), the ratio of COX-2 to 15-HPGD expression may be a particularly informative biomarker of risk for

CRC, analogous to the ratio of high-density lipoprotein (HDL) to low-density lipoprotein (LDL) ratio for assessing cardiovascular risk. Findings from randomized clinical trials (RCTs) suggest that long-term use (>5 years) of aspirin—a non-steroidal anti-inflammatory drug (NSAID) that directly inhibits cyclooxygenase enzymes COX-1 and COX-2—may reduce the 20-year risk of CRC by 30–40% (20). The primary anti-cancer mechanism of NSAIDs appears to be COX-2 inhibition, but other mechanisms, including potential regulation of 15-HPGD and inhibition of the pro-inflammatory nuclear factor kappa B (NF-κB) pathway, have been proposed (42). However, long-term use of NSAIDs, including aspirin, can cause kidney damage and increase the risk of gastrointestinal bleeding (43, 44). As these side effects appear to be primarily mediated by COX-1 inhibition, selective COX-2 inhibitors were developed and substantially reduced colorectal adenoma risk in RCTs (45, 46). However, these agents also increase the risk of adverse cardiovascular events (45). Thus, identifying other chemopreventive agents that may favorably modulate PG-metabolizing enzymes with fewer side effects is critical. Calcium and vitamin D are attractive candidates as, in addition to their other potential chemopreventive effects, findings from experimental studies suggest that vitamin D may downregulate COX-2 and upregulate 15-HPGD in cancer cell lines (7, 8). Additionally, both vitamin D and calcium may inhibit the NF-κB signaling pathway (47, 48), which may affect COX-2 and 15-HPGD expression in colon tissue (49).

Despite these promising laboratory studies' findings, there are no reported investigations of calcium and vitamin D supplementation effects on COX-2 and 15-HPGD expression in the normal colorectal epithelium in humans. We tested the effects of supplemental vitamin D and/or calcium over 1 year on expression of these markers in the normal-appearing rectal mucosa of colorectal adenoma patients enrolled in a randomized, double-blind, placebo-controlled clinical

trial. We hypothesized that supplemental vitamin D and calcium, alone or in combination, would decrease COX-2 and increase 15-HPGD expression, and decrease the COX-2/15-HPGD expression ratio in the normal colorectal epithelium.

METHODS

Study Participants and Trial Protocol

Participants in this study ("adjunct biomarker study") were recruited from participants enrolled in an 11-center, partial 2x2 factorial, randomized placebo-controlled trial ("parent study") testing the efficacy of supplemental vitamin D and/or calcium on colorectal adenoma recurrence over 3–5 years. The adjunct biomarker study was designed to assess the effects of vitamin D and calcium on putative biomarkers of risk for colorectal neoplasms. Details of the parent study, including eligibility criteria and the study protocol, were previously published (97). Briefly, eligible participants were 45 to 75 years old, in generally good health, and had a histologically-verified colorectal adenoma diagnosed within 4 months of study entry. Participants who adhered to the study protocol during a 3-month placebo run-in trial were randomized (n = 2,259). Most subjects were randomized to one of the following treatment groups in a 4-arm study with full-factorial randomization: placebo, calcium (600 mg of elemental calcium *via* calcium carbonate twice daily), vitamin D (500 I.U. vitamin D_3 twice daily), or calcium plus vitamin D. The parent trial also included a 2-arm study of women who elected to receive calcium and were randomized to also receive either vitamin D or vitamin D placebo pills. Only participants in the 4-arm study with full-factor

ial randomization were included in the present biomarker study. All participants and study staff were blinded to treatment assignments.

Details of the adjunct biomarker study protocol and eligibility criteria were previously published (22). Exclusion criteria included history of a bleeding disorder, unable to forgo aspirin use for 7 days, and current use of an anticoagulant medication. Participants were recruited at 2 of the 11 clinical centers (South Carolina and Georgia) between May 2004 and July 2008, and agreed to undergo rectal biopsies at baseline and 1-year follow-up. Participants were enrolled near the end of their 3-month run-in period in the parent study, prior to randomization (155). Sixty-two participants in the 4-arm (full-factorial randomization) study of the parent trial met final eligibility criteria and had sufficient rectal biopsy tissue taken for biomarker measurements. The Institutional Review Boards at both clinical centers approved the adjunct biomarker study protocol, and all participants provided signed informed consent.

Baseline information was collected from each participant at enrollment and included medical history, medication/supplement use, demographics, lifestyle, and diet (using the Block Brief 2000 food frequency questionnaire [NutritionQuest]) (97). Blood concentrations of 25-hydroxyvitamin D (25[OH]D) and calcium were measured at baseline, as described previously (97). 25(OH)D was also measured at 1-year and end-of-treatment follow-up (97). During the trial, participants agreed to refrain from taking additional vitamin D or calcium supplements, although personal daily supplements up to 1,000 I.U. vitamin D and/or 400 mg elemental calcium were permitted starting in April 2008. Telephone interviews were conducted every 6 months regarding participant adherence to the study treatment, illnesses, use of medications and supplements, and colorectal endoscopic or surgical procedures.

Biopsy Procurement and Immunohistochemistry Protocol for Biomarker Detection

Biopsies were collected from normal-appearing rectal mucosa at baseline and 1-year follow-up without any preceding bowel-cleansing preparation, as described previously (155).

Six ~1-mm-thick biopsies were taken from the rectal mucosa 10 cm above the external anal aperture and at least 4 cm from any polypoid lesions. Biopsies were placed onto a strip of bibulous paper and immediately put in normal saline, oriented, transferred to 10% normal-buffered formalin for 24 hours, and then transferred to 70% ethanol. Within a week, biopsies were processed and embedded in paraffin blocks. For each biomarker, five slides with three levels of 3-μm-thick biopsy sections taken 40 μm apart were prepared for each participant, yielding a total of 15 levels. Dewaxing and heat-induced antigen retrieval was performed in a Lab Vision PT Module device (Lab Vision Corp.) in 1:100x citrate buffer with a pH of 6.0 (ThermoScientific, TH 250-Premix). Slides were immunohistochemically processed in a DakoCytomation Autostainer Plus System (Agilent Dako) using a labeled streptavidin-biotin kit (ThermoScientific UltraVisionKit, TP-125-HL). Slides were processed separately for COX-2 and 15-HPGD using an anti-COX-2 mouse monoclonal antibody (clone COX229, dilution 1:600, ThermoFisher Cat.# 35-8200) and an anti-15-HPGD rabbit polyclonal antibody (dilution 1:600, Sigma-Aldrich Cat.# HPA004919). Specificity validations of these two antibodies were previously published (38, 85, 170). Baseline and 1-year follow-up biopsy slides for each participant were included in the same immunohistochemistry batch. Each batch was balanced by treatment group, and included positive and negative controls. The slides were coverslipped with a Leica CV5000 Coverslipper (Leica Microsystems, Inc.), and images of them acquired and digitized using a PannoramicScan 150-slide scanner (3DHISTECH). Representative images of histologic sections of biopsies of normal-appearing rectal mucosa immunohistochemically processed for COX-2 and 15-HPGD are shown in Supplementary Figure S1.

Quantification of Detected Biomarkers ('Scoring' Protocol)

Biomarker labeling density ("expression") was quantified using custom-developed quantitative image analysis software, CellularEyes (DivEyes LLC), and a validated scoring protocol described previously (155). Briefly, a technician blinded to treatment assignment systematically reviewed each slide and selected only "scorable" hemicrypts, defined as one side of a crypt bisected from base to colon lumen and extending intact from the muscularis mucosa to the colon lumen (Fig. 1A). Each section on the slide was viewed sequentially with the aim of identifying and analyzing at least 16, but no more than 40, scorable hemicrypts per patient per biomarker. The image-analysis program divided the outlined area into 50 equally-spaced segments of approximately average normal colonocyte width and measured the background-corrected biomarker labeling optical density within the entire hemicrypt and within each segment (Fig 1B). Resulting data were automatically transferred into a MySQL database (Sun Microsystems, Inc.).

To measure biomarker expression in the inter-crypt stroma adjacent to a previously scored hemicrypt, a technician located the previously scored hemicrypt and visually inspected whether the inter-crypt width was sufficient for stroma scoring. If suitable, the inter-crypt stroma was outlined, excluding epithelial cells, muscle tissue, and staining artifacts, and biomarker labeling optical density was quantified as described above (Fig. 1C). The protocol was continued with the aim of analyzing at least 16, but no more than 40, stromal regions per patient per biomarker. Using this protocol, we outlined 2,220 hemicrypts and 2,714 stromal regions for COX-2 measurements (a mean of 18 and 22, respectively, per patient-visit), and 3,240 hemicrypts and 2,699 stromal regions for 15-HPGD (a mean of 32 and 22, respectively, per patient-visit).

To assess intrareader scoring reliability, slides previously analyzed by the technician were re-scored during the course of the study. Separate technicians scored the crypts and stroma for COX-2 with intraclass coefficients of 0.97 and 0.96, respectively. One technician scored the crypts and stroma for 15-HPGD with intraclass correlation coefficients of 0.98 and 0.93, respectively.

Statistical Analyses

Baseline characteristics of study participants were compared across treatment groups using the chi-square test for categorical variables and ANOVA for continuous variables. Treatment effects were assessed by comparing changes in biomarker expression from baseline to 1-year follow-up in each treatment group relative to placebo using generalized linear mixed models with an unstructured correlation matrix (PROC MIXED, SAS 9.4). Models included a random intercept, and fixed effects for visit (baseline and 1-year follow-up), treatment group, and a treatment-by-visit interaction term. In a sensitivity analysis, inclusion of physical activity (metabolic equivalents of task [MET]-minutes /week) and total dietary fiber (g/day)—selected as potential confounding variables based on statistically significant imbalances across treatment group at baseline (Table 1)—did not materially affect the estimates and, thus, were not included in the final models. Participants were retained in their assigned treatment group regardless of adherence or missing data (intent-to-treat analysis).

We estimated treatment effects on changes in biomarker expression within: (i) whole crypts, (ii) the lower 60% of crypts (the canonical 'proliferation zone'), (iii) the upper 40% of crypts (the canonical 'differentiation zone'), and (iv) the stromal region adjacent to crypts. In each of these regions, we estimated treatment effects on the expression of COX-2, 15-HPGD, and the ratio of COX-2 to 15-HPGD (COX-2 divided by 15-HPGD). The primary outcome was

the COX-2/15-HPGD ratio in the full-length of crypts since these enzymes function as antagonists in PG metabolism (38).

Relative treatment effects (on the ratio scale) and absolute treatment effects (on the absolute scale) were calculated as follows: relative effect = [(treatment group follow-up) / (treatment group baseline)] / [(placebo group follow-up) / (placebo group baseline)]; absolute effect = [(treatment group follow-up) - (treatment group baseline)] - [(placebo group follow-up) - (placebo group baseline)]. The interpretation of the relative effect is similar to that of an odds ratio; for example, a value of 1.50 could be interpreted as a 50% increase in biomarker expression in the treatment group relative to the placebo group after 1 year. The mixed linear model estimates relative effects when the outcome variable is log-transformed, and absolute effects when the outcome variable is not transformed. Serum 25(OH)D concentrations, COX-2 and 15-HPGD expression in crypts, and the COX-2/15-HPGD ratio in the stroma were normalized by natural log-transformation. Thus, for these variables, the relative effects, and corresponding 95% confidence intervals (CIs) and *P*-values, were taken directly from the mixed linear models' output, while the absolute effects were hand-calculated from the geometric means. The expression of COX-2 and 15-HPGD in the stroma were normally distributed. Thus, for these variables, the absolute effects were taken directly from the mixed linear models' output, while the relative effects were hand-calculated from the crude means, and their corresponding 95% CIs and *P*-values were calculated using the Delta method (171).

Potential effect modification by *a priori*-selected factors was assessed by stratifying the above models according to body mass index (BMI) (< *vs*. ≥ 30 kg/m^2 [obesity threshold]), non-aspirin NSAID use (< *vs.* ≥ once a week), pill-taking adherence (< *vs*. ≥ 99% [median]), baseline 25(OH)D concentrations (< *vs.* ≥ 21.3 ng/mL [median]), and *GC* rs4588 genotype (CC *vs.*

AC/AA; A allele encodes the functional vitamin D-binding protein 2 [DBP2] isoform). *GC* rs4588 was chosen *a priori* for these analyses based on consistent effect-modification findings by this genotype in observational studies of associations of 25(OH)D with risk of colorectal neoplasms (100, 118, 172).

All statistical analyses were conducted using SAS 9.4 (SAS Institute Inc., Cary, NC). A two-sided P-value ≤ 0.05 was considered statistically significant.

RESULTS

Participant Characteristics at Baseline

Selected baseline characteristics of the adjunct biomarker study participants, by treatment group, are summarized in Table 1. The mean age of participants was 59 years, 77% were male, 82% were white, and 33% reported taking a non-aspirin NSAID at least once a week. Baseline physical activity and dietary fiber intakes statistically significantly differed across treatment groups, and were, on average, lowest in the placebo group and highest in the calcium + vitamin D group. Mean baseline serum 25(OH)D concentrations were similar across treatment groups. After 1-year of treatment, 25(OH)D increased by 33% in the vitamin D group ($P = 0.03$), an estimated 0.05% in the calcium group ($P = 0.97$), and 54% in the vitamin D + calcium group ($P = 0.001$), relative to the placebo group.

Distributions of COX-2 and 15-HPGD Expression in Crypts and Stroma

The distributions of COX-2 and 15-HPGD labeling optical densities from the base of the crypt to the colon lumen, and in the adjacent stroma, by treatment group after 1-year are shown in Figure 2. Both COX-2 and 15-HPGD expression throughout the full length of crypts were

similar across treatment groups at baseline (Supplementary Figure S2). However, after 1 year, in each active treatment group, COX-2 expression appeared to be lower and 15-HPGD expression appeared to be higher than in the placebo group fairly uniformly along the full length of crypts (Fig. 2A, 2C). In the adjacent stroma, COX-2 and 15-HPGD expression were weaker, and treatment group differences appeared less pronounced than in the crypts (Fig. 2B, 2D).

Treatment Effects on COX-2 and 15-HPGD

The estimated relative and absolute treatment effects on COX-2, 15-HPGD, and the COX-2/15-HPGD expression ratio in crypts and stroma are presented in Table 2 and described below. Treatment effects on each biomarker were, in general, stronger in the crypt epithelia than in the adjacent stroma.

COX-2

As shown in Table 2, among participants in the vitamin D group, mean COX-2 expression decreased proportionately by 44% in whole crypts ($P = 0.008$) and an estimated 20% ($P = 0.10$) in the adjacent stroma relative to the placebo group. COX-2 expression in whole crypts non-statistically significantly decreased an estimated 10% in the calcium group and 7% in the calcium + vitamin D group, relative to the placebo group. Relative treatment effects on COX-2 were stronger in the upper 40% ('differentiation zone') of crypts where COX-2 expression decreased by 60% in the vitamin D group ($P = 0.0002$), 37% in the calcium group ($P = 0.05$), and an estimated 21% in the calcium + vitamin D group ($P = 0.29$), relative to placebo.

15-HPGD

As shown in Table 2, 15-HPGD expression in the calcium and calcium + vitamin D groups increased 68% ($P = 0.001$) and 41% ($P = 0.03$), respectively, in whole crypts, and increased 38% and 37%, respectively, in the stroma ($P = 0.01$ for both), relative to the placebo group. For the calcium group, relative treatment effects were stronger in the lower 60% ('proliferation zone') of crypts in which there was a 90% ($P = 0.002$) increase in 15-HPGD expression relative to placebo.

COX-2/15-HPGD Ratio

As shown in Table 2, in whole crypts, the COX-2/15-HPGD expression ratio decreased 46% in the calcium group ($P = 0.002$), 47% in the vitamin D group ($P = 0.001$), and 34% in the calcium + vitamin D group ($P = 0.03$), relative to the placebo group. Similar but weaker relative treatment effects were observed in the adjacent stroma in which the COX-2/15-HPGD ratio decreased 38% ($P = 0.03$), 33% ($P = 0.05$), and an estimated 17% ($P = 0.37$) in the calcium, vitamin D, and combined groups, respectively, relative to placebo. The treatment effects for each group on the COX-2/15-HPGD ratio were slightly stronger in the upper 40% of crypts than in the lower 60% of crypts.

Subgroup Analyses

The relative treatment effects on COX-2 and 15-HPGD expression in whole crypts according to DBP2 isoform (*GC* rs4588*A) are presented in Table 3. Among individuals with the DBP2-encoding *GC* rs4588*A allele, the COX-2/15-HPGD ratio statistically significantly decreased 75%, 70%, and 60% in the calcium, vitamin D, and combined supplementation

groups, respectively, relative to the placebo group. In contrast, among those without DBP2, the COX-2/15-HPGD ratio in the crypts non-statistically significantly decreased an estimated 19%, 30%, and 13% in the calcium, vitamin D, and combined supplementation groups, respectively, relative to the placebo group.

The estimated treatment effects in whole crypts or adjacent stroma did not meaningfully differ according to BMI, pill-taking adherence, or baseline blood 25(OH)D concentration for COX-2 expression (Supplementary Table S1), 15-HPGD (Supplementary Table S2), or the COX-2/15-HPGD ratio (Supplementary Table S3). However, the estimated effects of vitamin D and calcium on COX-2 and the COX-2/15-HPGD ratio in whole crypts were slightly stronger among individuals who were not regularly taking a non-aspirin NSAID at least once a week at baseline. Relative to the placebo group, in the vitamin D, calcium, combined supplementation groups, respectively, the COX-2/15-HPGD ratio in whole crypts statistically significantly decreased 51%, 50%, and 46% among participants not regularly taking a non-aspirin NSAID, but decreased an estimated 35%, 39%, and 5% among participants who were regularly took one (Supplementary Table S3). These participant subgroup findings for the COX-2/15-HPGD ratio appeared to be driven more by subgroup differences in treatment effects on COX-2 (Supplementary Table S1) than on 15-HPGD (Supplementary Table S2).

DISCUSSION

We found that vitamin D and calcium decrease the balance of COX-2 to 15-HPGD expression in the morphologically-normal colorectal mucosa of colorectal adenoma patients. As discussed below, this would indicate that vitamin D and calcium decrease inflammation in the human colorectal mucosa, and supports an important anti-carcinogenesis mechanism for these

agents against colorectal cancer—an inflammation-related disease (35). This is the first study, to our knowledge, to test the effects of vitamin D and calcium supplementation on COX-2 and 15-HPGD expression in the morphologically-normal human colorectal mucosa.

Vitamin D and calcium may reduce CRC-promoting inflammation through multiple mechanisms, including via effects on bile acid catabolism, immunomodulation, and fatty acid metabolism. The VDR is expressed in tissues throughout the body, including normal and neoplastic colon tissue (173), and regulates over 200 vitamin D-responsive genes affecting pathways involved in bile acid and xenobiotic metabolism, inflammation, and immunomodulation, among others, such as cell cycle control and growth factor signaling (2, 27). VDR activation induces *in vivo* expression of CYP3A4, a cytochrome p450 enzyme that detoxifies the secondary bile acid lithocholic acid in the intestines, which may prevent oxidative epithelial cell damage and consequent inflammation, in addition to its mutagenic and mitogenic effects in colorectal tissue (29). Also, calcium binds to bile acids in the gut lumen, thereby sequestering them and preventing their pro-inflammatory toxicity to colonocytes (25, 26). Vitamin D also has important immunomodulatory functions, including the differentiation of VDR-expressing T cells, which affects inflammatory cytokine production (174). In mice, dietary vitamin D and calcium also suppressed TNF-α transcription in the colon and reduced the severity of inflammatory bowel disease—an autoimmune disease with increased COX-2 production and higher CRC risk (35, 175). Vitamin D and calcium supplementation may also decrease levels of pro-inflammatory polyunsaturated fatty acids, including arachidonic acid—the immediate precursor to prostaglandins—by altering fatty acid desaturase enzyme expression and increasing fatty acid fecal excretion, respectively (176-179).

Importantly, findings from experimental studies indicate that vitamin D and calcium may affect prostaglandin synthesis by inhibiting the NF-κB pathway or by affecting COX-2 and 15-HPGD expression directly. NF-κB is a potent transcription factor that upregulates PG synthesis by binding directly to the COX-2 promoter (47-49). Vitamin D may block TNF-α-induced NF-κB activation through binding IκB kinase β by activated VDR (48), while higher intracellular calcium may reduce NF-κB transcription by reducing IκB kinase β activity (47). Furthermore, in cancer cell lines expressing the VDR, $1,25(OH)_2D$ reduced COX-2 mRNA and protein expression, decreased downstream prostaglandin E_2 concentrations, and attenuated PG-promoting cell proliferation (8). Also, human trophoblast cells treated with the calcium chelator BAPTA-AM decreased 15-HPGD activity, suggesting that intracellular calcium may increase 15-HPGD expression (180).

The treatment effects we observed on the COX-2/15-HPGD expression ratio in whole crypts appeared to be highly driven in the vitamin D group by a decrease in COX-2, and in the calcium and combined treatment groups by an increase in 15-HPGD. However, a pro-inflammatory COX-2/15-HPGD ratio may be a more informative biomarker of risk for CRC since high 15-HPGD expression could compensate for high COX-2 expression. In an RCT among colorectal adenoma patients, the chemoprotective effect of the selective COX-2 inhibitor, celecoxib, on adenoma recurrence was stronger among patients with pre-treatment adenomas with low 15-HPGD expression (RR, 0.60; 95% CI, 0.52–0.69; $P < 0.0001$) than among those with intact 15-HPGD expression (RR, 0.73; 95% CI, 0.47–1.12; $P = 0.15$) (46). Our findings suggest that supplemental vitamin D and calcium, alone or in combination, may reduce tumor-promoting inflammation in morphologically-normal colorectal tissue, but *via* differentially strong effects on the expression of different prostaglandin-metabolizing enzymes.

The estimated vitamin D treatment effects on COX-2 and 15-HPGD were stronger among individuals with the functional DBP2-encoding *GC* rs4588*A polymorphism. Findings from observational studies suggest that the associations of higher circulating 25(OH)D concentrations with lower risk of colorectal adenoma (118) and CRC (172) may be stronger among individuals with DBP2 (*GC* rs4588*AC or AA) than among those without it (*GC* rs4588*CC). Additionally, in a large RCT (n=2,259), the effects of vitamin D supplementation on increasing 25(OH)D blood concentrations and reducing colorectal adenoma recurrence risk were stronger with each DBP2-encoding *GC*-rs4588*A allele inherited (100, 101). These effect-modification findings are consistent with those found in our study. Relative to the DBP1 isoforms, DBP2 is associated with an approximately 2- to 4-fold lower 25(OH)D binding affinity (135) and 2- to 3-fold higher vitamin D-pathway induction by 25(OH)D *in vitro* (139), providing biologic plausibility for these clinically relevant genotype-specific associations. Interaction between calcium intake and DBP2 isoform in relation to colorectal neoplasms has not been reported; however, DBP2 is associated with lower DBP concentrations (131), which may increase parathyroid hormone (PTH) secretion and calcium reabsorption, potentially leading to stronger supplemental calcium effects on COX-2/15-HPGD among individuals with this genotype (181).

We estimated that the relative treatment effects of vitamin D and/or calcium supplementation on COX-2 expression in whole crypts were slightly stronger among patients who were not regularly taking non-aspirin NSAIDs. In a sensitivity analysis, substituting all NSAID use (aspirin + non-aspirin) for non-aspirin NSAID use did not materially affect these findings. We hypothesize that among patients who are already taking drugs that inhibit COX-2, additional COX-2 suppression by vitamin D or calcium supplementation may be relatively small. In experimental studies, there is evidence that vitamin D and NSAIDs (both non-selective and

COX-2 selective) may act synergistically to reduce prostate cancer cell growth by affecting PG synthesis (8), but potential synergistic effects on COX-2 and 15-HPGD in morphologically-normal colorectal tissue has not been reported. Our NSAID-stratified results should be cautiously interpreted given the time-varying nature and potential misclassification of self-reported NSAID use, and the small sample sizes within each subgroup.

We found no evidence of a synergistic effect of combined vitamin D and calcium supplementation on COX-2 or 15-HPGD expression. This is consistent with findings from several of our previous calcium and vitamin D biomarker trials, including those of APC/β-catenin, p21, and bax expression in normal colorectal tissue, and combined circulating inflammation markers (C-reactive protein [CRP], TNF-α, interleukin [IL]-6, IL-1β, IL-8, and IL-10) (66). A potential explanation for these findings is that combined calcium and vitamin D supplementation may act synergistically to lower PTH concentrations, thereby reducing $1,25(OH)_2D$ synthesis in the kidneys and calcium absorption in the gut, compared to the use of either supplement alone (68).

Strengths of this study are that we quantified biomarker expression using automated immunohistochemistry and precise image analysis, and analyzed a large number of crypts per patient, thereby reducing measurement error and bias due to outcome misclassification. Our powerful image analysis tool also allowed us to quantify and compare biomarker expression in different functional zones of the colorectal crypts and in the adjacent stroma, which has not been previously reported for COX-2 and 15-HPGD. Additional strengths include the high adherence to study protocol, complete follow-up, high biomarker scorer reliability, and 2x2 factorial randomization allowing us to assess treatment effects of calcium and vitamin D alone and in combination.

The primary limitation of this study is its small size, limiting the statistical stability of the estimated treatment effects and the ability to conduct stratified analyses. While we assessed two important, validated biomarkers of risk for colorectal neoplasms involved in prostaglandin metabolism, we did not collect information on prostaglandin concentrations. We also assessed COX-2 and 15-HPGD expression only in the normal rectal mucosa; thus, treatment effects in other parts of the colon are unknown. In addition, we did not quantify biomarker expression beyond 1-year follow-up or in tumor tissue, warranting future studies to investigate long-term vitamin D and calcium effects on COX-2 and 15-HPGD as well as potential differences in effects in normal versus neoplastic tissue. Last, since most of our study participants were white, our results may not be generalizable to other races.

In conclusion, we found that supplemental vitamin D and calcium decrease the balance of COX-2 to 15-HPGD expression in the morphologically-normal colorectal mucosa of colorectal adenoma patients. These findings, taken together with previous literature, would indicate that vitamin D and calcium may decrease inflammation in the human colorectal mucosa, and support an important mechanism by which vitamin D and calcium may prevent colorectal cancer—an inflammation-related disease.

Table 1. Selected baseline characteristics of participants, by treatment group, in the adjunct biomarker study (n = 62)[a]

	Treatment group				
Characteristic	**Placebo (n = 12)**	**Calcium (n = 16)**	**Vitamin D (n = 17)**	**Calcium + vit. D (n = 17)**	***P*[b]**
Age, years	60 (7)	60 (7)	59 (8)	58 (7)	0.79
Men, %	75	81	71	82	0.83
White, %	83	75	71	94	0.49
College graduate, %	67	38	65	53	0.35
Family history of CRC[c], %	0	13	20	6	0.33
Regularly take non-aspirin NSAID[d], %	33	44	24	29	0.65
Regularly take aspirin[d], %	50	69	41	41	0.35
Multivitamin user, %	42	81	47	65	0.11
Current smoker, %	25	6	0	6	0.12
Alcohol intake, drinks/day	0.7 (0.7)	0.8 (1.0)	0.9 (0.9)	0.9 (0.9)	0.92
Physical activity, MET-mins./wk., median (IQR)[e]	80 (480)	840 (1,360)	300 (960)	480 (2,400)	0.003
Body mass index, kg/m^2	29 (5)	32 (8)	29 (6)	30 (5)	0.32
Dietary intakes					
Total energy[f], kcal/day	1,314 (381)	1,737 (556)	1,437 (527)	1,631 (550)	0.18
Total fat[f], g/day	57(22)	69 (26)	60 (27)	63 (27)	0.69
Dietary fiber[f], g/day	10 (4)	16 (5)	14 (6)	16 (6)	0.03
Total calcium[g], mg/day	715 (455)	895 (264)	671 (278)	670 (255)	0.14
Total vitamin D[h], IU/day	354 (307)	457 (189)	313 (278)	421 (296)	0.48
Serum concentrations					
Calcium, mg/dL	9.2 (0.2)	9.3 (0.3)	9.3 (0.3)	9.4 (0.3)	0.22
25-hydroxyvitamin D, ng/ml	22 (8)	24 (13)	23 (9)	22 (7)	0.93

Abbreviations: CRC, colorectal cancer; IQR, interquartile range; IU, international units; kcal, kilocalories; MET, metabolic equivalents of task; NSAID, nonsteroidal anti-inflammatory drug; vit. D, vitamin D.

[a]Data are given as mean (standard deviation) unless otherwise specified.

[b]*P* values calculated using Fisher's exact test for categorical variables and ANOVA for continuous variables.

[c]In a first-degree relative; missing values for 2 participants in the vitamin D group.

[d]At least once a week.

[e]Median (IQR) is presented because of the right-skewed distribution of the physical activity variable. The variable was log-transformed, yielding a normal distribution, prior to the ANOVA test.

[f]Missing values for 2 participants in the placebo group and 1 participant in the calcium group.

[g]From foods and supplements; missing values for 2 participants in the placebo group, 1 participant in the calcium group, and 1 participant in the vitamin D group.

[h]From foods and supplements; missing values for 3 participants in the placebo group, 2 participants in the calcium group, and 2 participants in the vitamin D group.

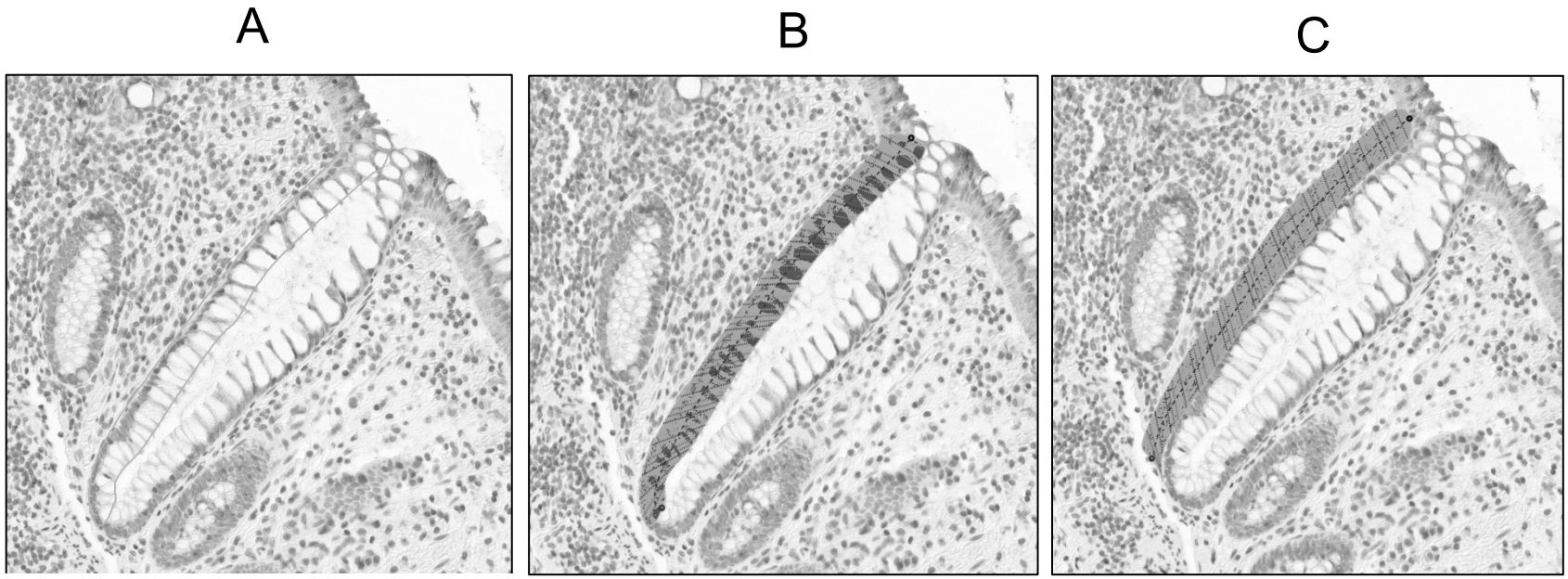


Figure 1. Measurement of COX-2 expression in crypts and stroma of normal-appearing rectal mucosa using custom-designed quantitative image analysis software. The scoring process entailed (**A**) finding and tracing a full-length hemicrypt and then (**B**) automated sectioning and quantification of biomarker labeling optical density, overall, and within each segment of the hemicrypt; (**C**) the stroma adjacent to previously scored hemicrypts was also outlined and scored.

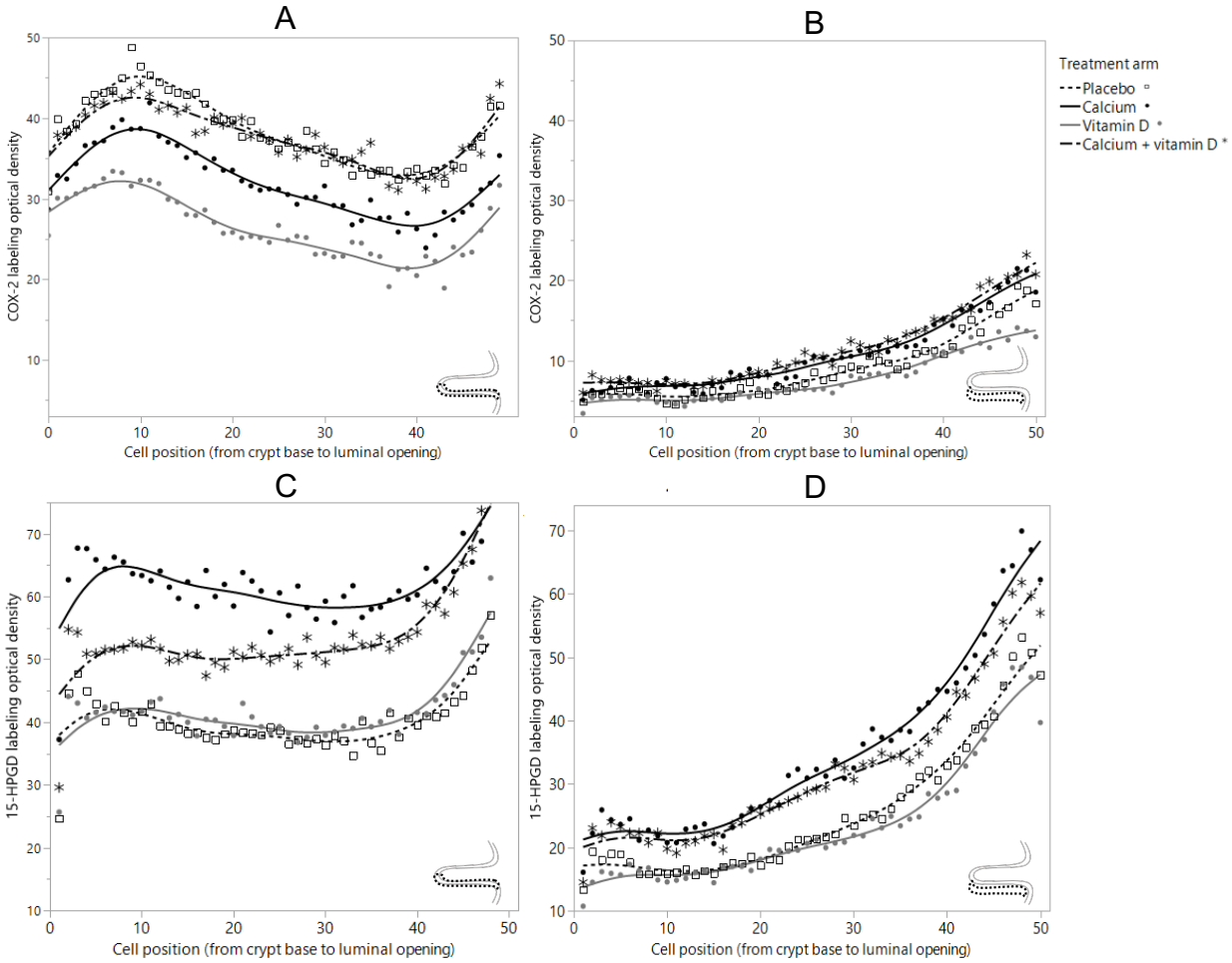


Figure 2. Distribution of COX-2 and 15-HPGD labeling optical density, by treatment arm, at 1-year follow-up among adjunct biomarker study participants (n = 62). Labeling optical densities presented for: (**A**) COX-2 in crypts, (**B**) COX-2 in stroma, (**C**) 15-HPGD in crypts, and (**D**) 15-HPGD in stroma. The dashed outlines in the bottom-right corner of each panel depict the areas (i.e., the crypt or adjacent stroma) in which biomarker expression was quantified using image analysis software.

Table 2. Effects of vitamin D and/or calcium supplementation on COX-2 and 15-HPGD expression[a] in the crypts and stroma of the normal-appearing colorectal mucosa among adjunct biomarker study participants (n = 62)

Biomarkers and treatment groups	N	Baseline means[b] (95% CI)	1-yr follow-up means[b] (95% CI)	Relative Rx effects[c] (95% CI)	*P*	Absolute Rx effects[d] (OD)
Whole crypts						
COX-2						
Placebo	12	1,658 (1,161-2,369)	1,708 (1,125-2,593)	1.0 (Ref.)		
Calcium	16	1,663 (1,221-2,265)	1,541 (1,073-2,211)	0.90 (0.60-1.35)	0.60	-172
Vitamin D	17	1,743 (1,280-2,374)	1,002 (698-1,438)	0.56 (0.39-0.86)	0.008	-791
Calcium + vit. D	17	1,509 (1,119-2,037)	1,441 (1,015-2,047)	0.93 (0.62-1.38)	0.71	-118
15-HPGD						
Placebo	12	2,929 (2,339-3,667)	2,110 (1,684-2,643)	1.0 (Ref.)		
Calcium	16	2,595 (2,136-3,153)	3,144 (2,586-3,820)	1.68 (1.24-2.28)	0.001	1,368
Vitamin D	17	2,707 (2,228-3,289)	2,043 (1,681-2,483)	1.05 (0.77-1.42)	0.76	155
Calcium + vit. D	17	2,717 (2,250-3,282)	2,756 (2,280-3,330)	1.41 (1.04-1.91)	0.03	858
COX-2 / 15-HPGD[f]						
Placebo	12	0.57 (0.39-0.82)	0.81 (0.57-1.15)	1.0 (Ref.)		
Calcium	16	0.64 (0.47-0.88)	0.49 (0.36-0.66)	0.54 (0.37-0.80)	0.002	-0.39
Vitamin D	17	0.64 (0.47-0.88)	0.49 (0.36-0.66)	0.53 (0.36-0.78)	0.001	-0.39
Calcium + vit. D	17	0.56 (0.41-0.75)	0.52 (0.39-0.70)	0.66 (0.45-0.96)	0.03	-0.28
Crypt upper 40% ('differentiation' zone)						
COX-2						
Placebo	12	490 (331-725)	602 (392-923)	1.0 (Ref.)		
Calcium	16	631 (445-896)	526 (359-771)	0.63 (0.40-1.00)	0.05	-217
Vitamin D	17	622 (443-874)	330 (228-478)	0.40 (0.26-0.63)	0.0002	-404
Calcium + vit. D	17	514 (370-714)	532 (371-762)	0.79 (0.5-1.23)	0.29	-94
15-HPGD						
Placebo	12	1,072 (871-1,320)	849 (684-1,056)	1.0 (Ref.)		
Calcium	16	1,027 (858-1,230)	1258 (1,043-1,519)	1.55 (1.17-2.04)	0.003	454
Vitamin D	17	1,103 (921-1,320)	850 (704-1,025)	0.97 (0.74-1.28)	0.84	-30
Calcium + vit. D	17	1,093 (918-1,302)	1,159 (966-1,391)	1.34 (1.02-1.76)	0.04	289

COX-2 / 15-HPGD[f]						
Placebo	12	0.46 (0.32-0.66)	0.71 (0.50-1.01)	1.0 (Ref.)		
Calcium	16	0.62 (0.45-0.87)	0.42 (0.31-0.58)	0.44 (0.28-0.68)	0.0004	-0.45
Vitamin D	17	0.56 (0.41-0.78)	0.39 (0.29-0.53)	0.44 (0.29-0.68)	0.0004	-0.42
Calcium + vit. D	17	0.47 (0.34-0.64)	0.46 (0.34-0.62)	0.63 (0.41-0.96)	0.03	-0.26
Crypt lower 60% ('proliferation' zone)						
COX-2						
Placebo	12	984 (713-1,356)	891 (639-1,537)	1.0 (Ref.)		
Calcium	16	915 (676-1,237)	838 (601-1,319)	0.99 (0.63-1.55)	0.95	16
Vitamin D	17	1051 (770-1,434)	615 (420-900)	0.64 (0.41-0.99)	0.05	-343
Calcium + vit. D	17	1080 (754-1,547)	991 (580-1,212)	1.00 (0.64-1.55)	0.99	4
15-HPGD						
Placebo	12	1,660 (1,225-2,251)	1,106 (850-1,441)	1.0 (Ref.)		
Calcium	16	1,343 (1,032-1,747)	1,701 (1,353-2,138)	1.90 (1.27-2.85)	0.002	912
Vitamin D	17	1,403 (1,078-1,825)	1,020 (812-1,283)	1.09 (0.73-1.63)	0.66	852
Calcium + vit. D	17	1,400 (1,084-1,808)	1,377 (1,103-1,720)	1.48 (0.99-2.2)	0.06	531
COX-2 / 15-HPGD[f]						
Placebo	12	0.65 (0.43-0.98)	0.90 (0.62-1.30)	1.0 (Ref.)		
Calcium	16	0.75 (0.52-1.07)	0.53 (0.38-0.74)	0.52 (0.32-0.83)	0.008	-0.47
Vitamin D	17	0.75 (0.53-1.07)	0.60 (0.44-0.83)	0.58 (0.37-0.94)	0.03	-0.40
Calcium + vit. D	17	0.65 (0.46-0.92)	0.61 (0.45-0.83)	0.68 (0.43-1.08)	0.09	-0.29
Stroma						
COX-2[e]						
Placebo	12	517 (394-639)	482 (367-598)	1.0 (Ref.)		
Calcium	16	656 (550-762)	567 (467-667)	0.93 (0.71-1.15)	0.52	-54
Vitamin D	17	548 (445-651)	409 (312-506)	0.80 (0.54-1.06)	0.10	-104
Calcium + vit. D	17	572 (467-678)	655 (555-756)	1.23 (0.94-1.52)	0.17	76
15-HPGD[e]						
Placebo	12	1,879 (1,537-2,220)	1,350 (1,003-1,696)	1.0 (Ref.)		
Calcium	16	1,873 (1,577-2,169)	1,855 (1,555-2,155)	1.38 (1.13-1.63)	0.01	511
Vitamin D	17	1,578 (1,291-1,865)	1,242 (951-1,533)	1.10 (0.85-1.35)	0.47	193
Calcium + vit. D[g]	16	1,711 (1,415-2,007)	1,702 (1,402-2,002)	1.37 (1.12-1.62)	0.01	520

COX-2 / 15-HPGD[f]						
Placebo	12	0.26 (0.19-0.36)	0.36 (0.26-0.49)	1.0 (Ref.)		
Calcium	16	0.38 (0.29-0.51)	0.33 (0.25-0.43)	0.62 (0.41-0.94)	0.03	-0.15
Vitamin D	17	0.34 (0.26-0.45)	0.32 (0.25-0.41)	0.67 (0.45-1.01)	0.05	-0.12
Calcium + vit. D[g]	16	0.32 (0.24-0.43)	0.37 (0.28-0.48)	0.83 (0.55-1.25)	0.37	-0.05

Abbreviations: CI, confidence interval; OD, optical density; Ref., reference; Rx, treatment; vit. D, vitamin D.

[a]Biomarker expression measured as labeling optical density using automated immunohistochemistry and image analysis. Mean biomarker labeling densities were calculated using mixed linear models. Unless otherwise noted in the table[e], biomarker labeling density distributions were normalized with natural log-transformation, and the relative effects[c], 95% CIs, and *P*-values were taken directly from the mixed linear models' output, and the absolute effects[d] were hand calculated from the geometric means.

[b]Unless otherwise noted in the table[e], data were natural log-transformed and reported values are optical density geometric mean (95% CI).

[c]Relative effect = [(treatment group follow-up) / (treatment group baseline)] / [(placebo group follow-up) / (placebo group baseline)]; interpretation is similar to that for an odds ratio (e.g., a value of 1.50 would be interpreted as a 50% increase in biomarker expression in the treatment group relative to the placebo group after 1 year).

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

[e]For the COX-2 and 15-HPGD stroma data, since the biomarker labeling densities were normally distributed, the optical density crude means are presented, the absolute effects were taken directly from the mixed linear models' output, the relative effects were hand calculated from the crude means, and the 95% CIs and *P*-values were calculated using the delta method.

[f]Ratio of mean COX-2 to mean 15-HPGD labeling optical densities in whole crypts or stroma adjacent to whole crypts.

[g]One subject excluded due to missing values for the measurement of 15-HPGD expression in the stroma.

Table 3. Effects of vitamin D and/or calcium supplementation on COX-2 and 15-HPGD expression[a] in whole crypts of the normal-appearing colorectal mucosa, according to vitamin D-binding protein isoform-2 genotype, among adjunct biomarker study participants (n = 60)

	No DBP2 (rs4588*CC)			DBP2 (rs4588*AC/AA)		
Biomarkers and treatment groups	**N[b]**	**Relative Rx effects[c] (95% CI)**	***P***	**N[b]**	**Relative Rx effects[c] (95% CI)**	***P***
COX-2						
Placebo	9	1.0 (Ref.)		3	1.0 (Ref.)	
Calcium	9	1.27 (0.81-1.98)	0.28	7	0.43 (0.20-0.89)	0.02
Vitamin D	7	0.96 (0.59-1.54)	0.84	9	0.24 (0.12-0.49)	0.001
Calcium + vit. D	8	1.34 (0.85-2.12)	0.20	8	0.47 (0.23-0.97)	0.04
15-HPGD						
Placebo	9	1.0 (Ref.)		3	1.0 (Ref.)	
Calcium	9	1.71 (1.00-2.92)	0.05	7	1.57 (1.05-2.34)	0.03
Vitamin D	7	0.82 (0.48-1.38)	0.43	9	1.36 (0.89-2.09)	0.15
Calcium + vit. D	8	1.18 (0.70-1.99)	0.52	8	1.54 (1.02-2.33)	0.04
COX-2 / 15-HPGD[d]						
Placebo	9	1.0 (Ref.)		3	1.0 (Ref.)	
Calcium	9	0.81 (0.51-1.28)	0.35	7	0.25 (0.13-0.47)	0.0002
Vitamin D	7	0.70 (0.43-1.14)	0.15	9	0.30 (0.16-0.55)	0.0006
Calcium + vit. D	8	0.87 (0.54-1.40)	0.55	8	0.40 (0.21-0.75)	0.006

Abbreviations: CI, confidence interval; DBP, vitamin D-binding protein; Ref., reference; Rx, treatment; vit. D, vitamin D.

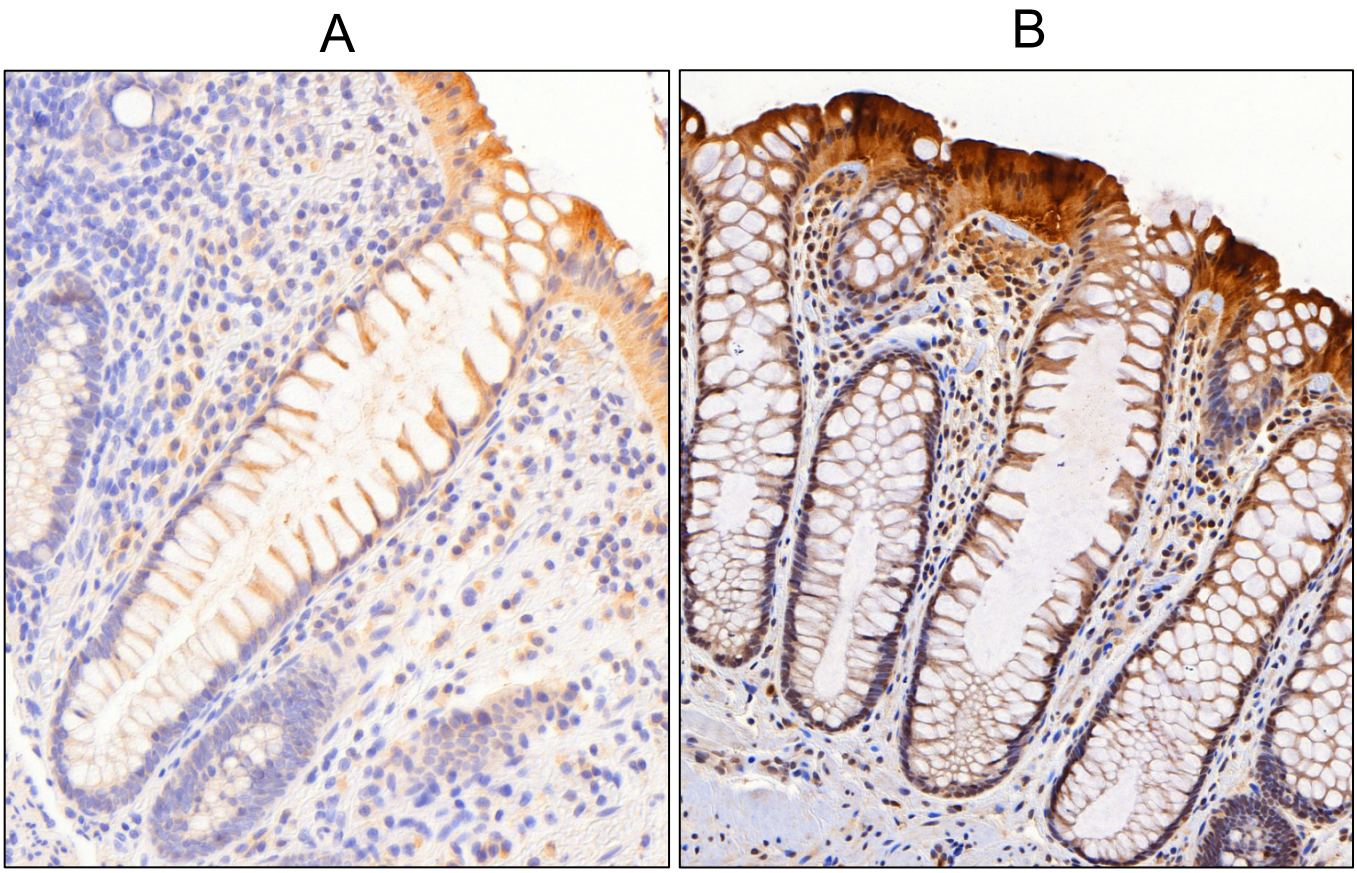
[a]Biomarker expression measured as labeling optical density using automated immunohistochemistry and image analysis. Biomarker labeling density distributions were normalized with natural log-transformation and the relative effects[c], 95% CIs, and *P*-values were taken directly from the mixed linear models' output. Geometric means and 95% CIs at baseline and 1-year follow-up for COX-2, 15-HPGD, and COX-2/15-HPGD are presented in Supplementary Tables S2, S3, and S4, respectively.

[b]One subject excluded from the vitamin D group, and one from the calcium + vitamin D group due to missing *GC* rs4588 genotyping data.

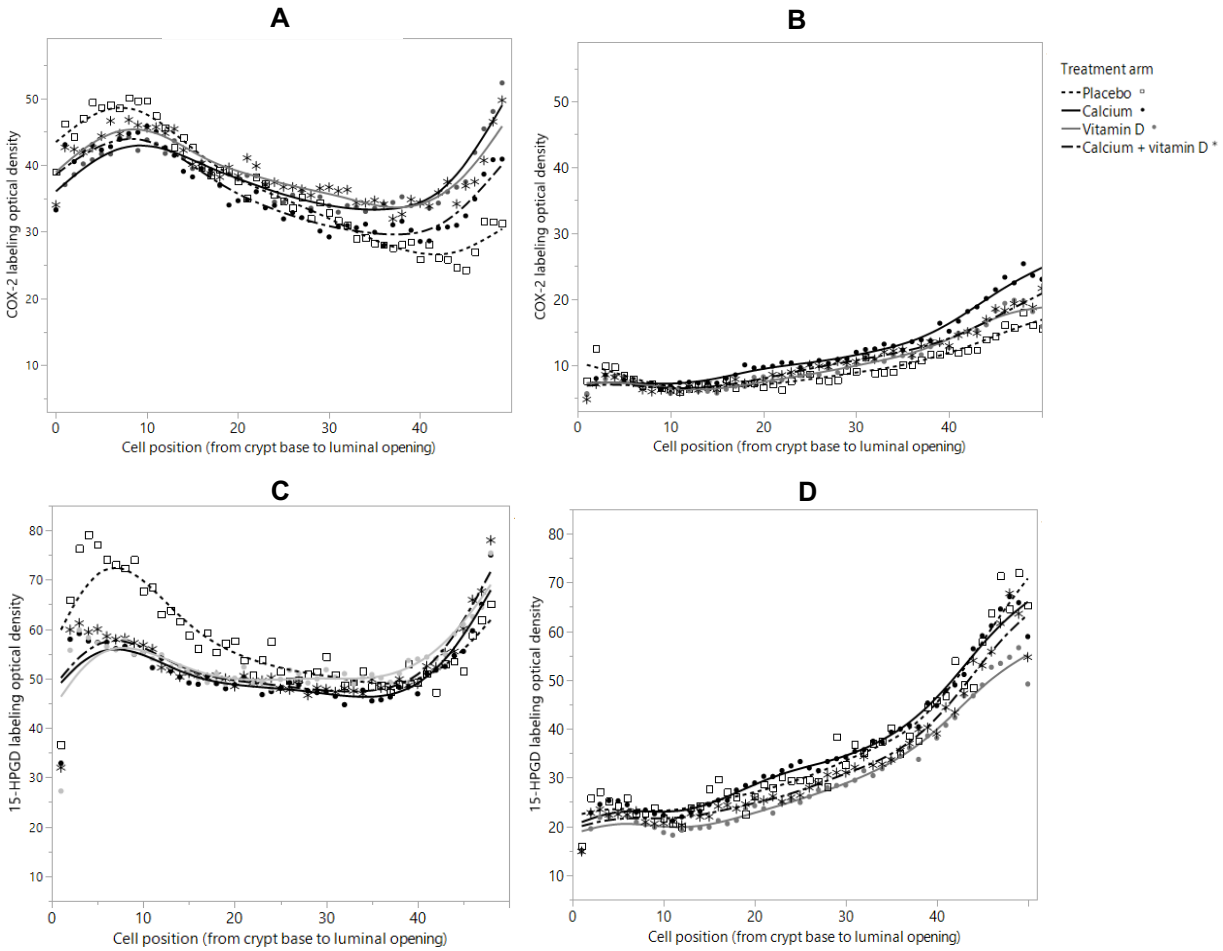
[c]Relative effect = [(treatment group follow-up) / (treatment group baseline)] / [(placebo group follow-up) / (placebo group baseline)]; interpretation is similar to that for an odds ratio (e.g., a value of 1.50 would be interpreted as a 50% increase in biomarker expression in the treatment group relative to the placebo group after 1 year).

[d]Ratio of mean COX-2 to mean 15-HPGD labeling optical densities in whole crypts.

SUPPLEMENTARY TABLES AND FIGURES



Supplementary Figure S1. Representative images of histologic sections of biopsies of normal-appearing rectal mucosa immunohistochemically processed for (**A**) COX-2 and (**B**) 15-HPGD, and counterstained with hematoxylin.



Supplementary Figure S2. Distribution of COX-2 and 15-HPGD labeling optical density, by treatment arm, in the crypts and in the stroma between crypts at baseline among adjunct biomarker study participants (n = 62). Labeling optical densities presented for: (**A**) COX-2 in crypts, (**B**) COX-2 in stroma, (**C**) 15-HPGD in crypts, and (**D**) 15-HPGD in stroma.

Supplementary Table S1. Effects of vitamin D and/or calcium supplementation on COX-2 expression in the crypts and stroma of the normal-appearing colorectal mucosa, according to selected participant characteristics, among adjunct biomarker study participants (n = 62)[a]

	Whole crypts[b]					Stroma[c]				
Strata	N	Baseline geometric means[b] (95% CI)	1-yr follow-up geometric means[b] (95% CI)	Relative Rx effects[d] (95% CI)	*P*	N	Baseline crude means[c] (95% CI)	1-yr follow-up crude means[c] (95% CI)	Absolute Rx effects[e] (95% CI)	*P*
NSAID Use										
Weekly non-aspirin NSAID use										
Placebo	4	2,130 (983-4,615)	2,195 (1,035-4,657)	1.00 (Ref)		4	554 (322-787)	536 (319-753)	0 (Ref)	
Calcium	7	1,682 (937-3,017)	1,752 (992-3,094)	1.01 (0.54 to 1.88)	0.97	7	629 (454-805)	555 (391-719)	-56 (-296 to 184)	0.63
Vitamin D	4	1,085 (444-2,651)	730 (306-1,741)	0.65 (0.31 to 1.39)	0.25	4	400 (168-633)	507 (290-724)	124 (-146 to 395)	0.34
Calcium + vit. D	5	1,248 (625-2,491)	1,418 (724-2,779)	1.10 (0.57 to 2.15)	0.76	5	562 (329-794)	611 (394-828)	67 (-2014 to 337)	0.61
No weekly non-aspirin NSAID use										
Placebo	8	1,464 (974-2,200)	1,507 (882-2,574)	1.00 (Ref)		8	498 (346-650)	455 (309-601)	0 (Ref)	
Calcium	9	1,649 (1,123-2,422)	1,394 (841-2,310)	0.82 (0.47 to 1.43)	0.48	9	677 (534-820)	576 (439-714)	-58 (-294 to 179)	0.62
Vitamin D	13	1,945 (1,413-2,678)	1,077 (708-1,640)	0.54 (0.32 to 0.90)	0.02	13	594 (475-713)	379 (265-494)	-171 (-390 to 47)	0.12
Calcium + vit. D	12	1,634 (1,171-2,279)	1,451 (937-2,248)	0.86 (0.51 to 1.46)	0.57	12	577 (453-700)	671 (551-790)	137 (-85 to 360)	0.22
BMI										
Not obese (< 30 kg/m^2)										
Placebo	9	1,939 (1,332-2,823)	2,117 (1,301-3,444)	1.00 (Ref)		9	568 (441-694)	455 (309-601)	0 (Ref)	
Calcium	8	1,977 (1,327-2,945)	1,462 (872-2,449)	0.68 (0.40 to 1.14)	0.14	8	730 (596-863)	576 (439-714)	-28 (-249 to 192)	0.79
Vitamin D	11	1,364 (955-1,948)	702 (443-1,114)	0.47 (0.29 to 0.77)	0.004	11	556 (442-671)	379 (265-494)	-71 (-275 to 133)	0.49
Calcium + vit. D	9	1,122 (770-1,633)	1,069 (657-1,740)	0.87 (0.53 to 1.45)	0.59	9	483 (349-617)	671 (551-790)	129 (-92 to 350)	0.24
Obese										
Placebo	3	1,038 (513-2,100)	897 (460-1,750)	1.00 (Ref)		3	364 (87-641)	511 (315-707)	0 (Ref)	
Calcium	8	1,400 (909-2,155)	1,624 (1,079-2,445)	1.34 (0.64 to 2.80)	0.42	8	583 (413-752)	528 (409-648)	-201 (-539 to 136)	0.23
Vitamin D	6	2,625 (1,595-4,322)	1,810 (1,129-2,903)	0.80 (0.37 to 1.72)	0.55	6	533 (337-729)	444 (305-582)	-236 (-589 to 116)	0.18
Calcium + vit. D	8	2,107 (13,69-3,245)	2,017 (1,340-3,037)	1.11 (0.53 to 2.31)	0.78	8	635 (475-794)	796 (672-919)	14 (-322 to 349)	0.93
Pill adherence										
Below median (< 99%)										
Placebo	6	1,458 (807-2,632)	1,297 (715-2,354)	1.00 (Ref)		6	567 (389-745)	455 (309-601)	0 (Ref)	
Calcium	8	1,543 (925-2,574)	1,260 (752-2,110)	0.92 (0.55 to 1.54)	0.73	8	625 (470-779)	576 (439-714)	1 (-256 to 259)	0.99
Vitamin D	10	1,523 (843-2,750)	927 (511-1,681)	0.68 (0.39 to 1.19)	0.17	10	533 (395-671)	379 (265-494)	-93 (-339 to 153)	0.44
Calcium + vit. D	6	1,568 (1,013-2426)	1,494 (962-2,319)	1.07 (0.66 to 1.74)	0.78	6	652 (457-847)	671 (551-790)	50 (-239 to 339)	0.72
At or above median										
Placebo	6	1,887 (1,194-2,982)	2,249 (1,204-4,200)	1.00 (Ref)		6	467 (283-651)	438 (274-602)	0 (Ref)	
Calcium	8	1,793 (1,206-2,665)	1,884 (1,097-3236)	0.88 (0.45 to 1.73)	0.70	8	688 (528-847)	549 (406-691)	-110 (-374 to 154)	0.40
Vitamin D	7	1,891 (1,326-2,695)	1,049 (647-1,703)	0.47 (0.25 to 0.88)	0.02	7	570 (399-740)	423 (270-474)	-118 (-390 to 154)	0.38
Calcium + vit. D	11	1,408 (891-2,225)	1,351 (723-2,522)	0.80 (0.39 to 1.65)	0.54	11	534 (400-669)	652 (529-774)	146 (-102 to 394)	0.24
Baseline 25(OH)D										
Below median (< 21.3 ng/mL)										
Placebo	7	1,443 (890-2,338)	1,476 (874-2,493)	1.00 (Ref)		7	458 (277-638)	455 (309-601)	0 (Ref)	
Calcium	8	1,374 (875-2,158)	1,457 (892-2,378)	1.04 (0.59 to 1.83)	0.90	8	581 (412-751)	576 (439-714)	-81 (-344 to 182)	0.53
Vitamin D	9	1,846 (1,175-2,900)	1,109 (679-1,810)	0.59 (0.33 to 1.04)	0.07	9	514 (354-673)	379 (265-494)	-49 (-305 to 207)	0.70

Calcium + vit. D	7	1,368 (844-2,217)	1,293 (765-2,183)	0.92 (0.51 to 1.66)	0.78	7	542 (369-715)	671 (551-790)	61 (-208 to 311)	0.64
At or above median										
Placebo	5	2,015 (1,135-3,579)	2,095 (1,007-4,355)	1.00 (Ref)		5	599 (426-773)	492 (293-690)	0 (Ref)	
Calcium	8	2,014 (1,279-3,171)	1,629 (913-2,905)	0.78 (0.41 to 1.48)	0.43	8	731 (594-868)	616 (460-773)	-6 (-244 to 232)	0.96
Vitamin D	8	1,646 (1,046-2,592)	905 (507-1,614)	0.53 (0.28 to 1.01)	0.05	8	587 (450-724)	327 (169-484)	-152 (-390 to 86)	0.20
Calcium + vit. D	10	1,617 (1,077-2,426)	1,556 (927-2,611)	0.93 (0.50 to 1.72)	0.80	10	586 (457-715)	677 (529-825)	199 (-34 to 432)	0.09
DBP Isoform										
DBP1-1										
Placebo	9	1,675 (1,125-2,492)	1,444 (935-2,231)	1.00 (Ref)		9	532 (376-688)	496 (375-617)	0 (Ref)	
Calcium	9	1,290 (867-1,920)	1,410 (912-2,178)	1.27 (0.81 to 1.98)	0.28	9	631 (476-787)	593 (471-714)	-3 (-207 to 200)	0.97
Vitamin D[f]	7	1,693 (1,079-2,656)	1,394 (851-2,283)	0.96 (0.59 to 1.54)	0.84	7	502 (326-679)	411 (273-549)	-56 (-273 to 162)	0.60
Calcium + vit. D[f]	8	1,746 (1,146-2,662)	2,023 (1,275-3,209)	1.34 (0.85 to 2.12)	0.20	8	604 (438-769)	779 (651-908)	211 (2 to 421)	0.05
DBP1-2/DBP2-2										
Placebo	3	1,611 (728-3,567)	2,826 (1,119-7,138)	1.00 (Ref)		3	471 (232-711)	440 (185-694)	0 (Ref)	
Calcium	7	2,306 (1,371-3,880)	1,727 (942-3,167)	0.43 (0.20 to 0.89)	0.02	7	688 (531-844)	534 (368-701)	-122 (-495 to 252)	0.51
Vitamin D[f]	9	1,914 (1,177-3,114)	809 (459-1,427)	0.24 (0.12 to 0.49)	0.001	9	593 (455-731)	424 (277-571)	-137 (-498 to 224)	0.44
Calcium + vit. D[f]	8	1,380 (848-2,246)	1,141 (647-2,012)	0.47 (0.23 to 0.97)	0.04	8	590 (432-748)	572 (407-737)	13 (-360 to 387)	0.94

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; CI, confidence interval; DBP, vitamin D-binding protein; NSAID, non-steroidal anti-inflammatory drug; Ref., reference; Rx, treatment; vit. D, vitamin D.

[a]Biomarker expression measured as labeling optical density using automated immunohistochemistry and image analysis.

[b]Crypt COX-2 biomarker labeling densities were natural log-transformed due to right-skewness; thus, we provide the geometric means and relative effects[d] estimated by the mixed linear model.

[c]Stromal COX-2 biomarker labeling densities were normally distributed; thus, we provide the crude means and absolute effects[e] estimated by the mixed linear model.

[d]Relative effect = [(treatment group follow-up) / (treatment group baseline)] / [(placebo group follow-up) / (placebo group baseline)]; interpretation is similar to that for an odds ratio (e.g., a value of 1.50 would be interpreted as a 50% increase in biomarker expression in the treatment group relative to the placebo group after 1 year).

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

[f]One subject excluded from the vitamin D group, and one from the calcium + vitamin D group due to missing DBP isoform information.

Supplementary Table S2. Effects of vitamin D and/or calcium supplementation on 15-HPGD expression in the crypts and stroma of the normal-appearing colorectal mucosa, according to potential effect-modifiers, among adjunct biomarker study participants (n = 62)[a]

	Whole crypts[b]					Stroma[c]				
Strata	**N**	**Baseline geometric means[b] (95% CI)**	**1-yr follow-up geometric means[b] (95% CI)**	**Relative Rx effects[d] (95% CI)[d]**	***P***	**N[a]**	**Baseline crude means[c] (95% CI)**	**1-yr follow-up crude means[c] (95% CI)**	**Absolute Rx effects[e] (95% CI)**	***P***
NSAID Use										
Weekly non-aspirin NSAID use										
Placebo	4	2,890 (1,809-4,617)	2,373 (1,853-3,038)	1.00 (Ref)		4	1,644 (947-2,342)	1,293 (705-1,881)	0 (Ref)	
Calcium	7	2,369 (1,663-3,376)	3,227 (2,677-3,890)	1.66 (1.09 to 2.53)	0.02	7	2,014 (1,486-2,541)	1,832 (1,388-2,277)	169 (-428 to 767)	0.55
Vitamin D	4	3,064 (1,784-5,263)	2,538 (1,908-3,377)	1.01 (0.60 to 1.68)	0.97	4	1,394 (696-2,091)	1,166 (579-1,754)	124 (-550 to 798)	0.70
Calcium + vit. D	5	2,635 (1,733-4,007)	2,501 (2,005-3,119)	1.16 (0.74 to 1.81)	0.50	4	1,676 (978-2,373)	1,477 (889-2,065)	153 (-521 to 826)	0.64
No weekly non-aspirin NSAID use										
Placebo	8	2,948 (2,242-3,877)	1,989 (1,447-2,735)	1.00 (Ref)		8	1,996 (1,581-2,411)	1,378 (921-1,835)	0 (Ref)	
Calcium	9	2,786 (2,152-3,606)	3,080 (2,281-4,158)	1.64 (1.06 to 2.52)	0.03	9	1,763 (1,372-2,155)	1,872 (1,442-2,303)	727 (1.82 to 1,273)	0.01
Vitamin D	13	2,631 (2,122-3,261)	1,943 (1,514-2,494)	1.09 (0.73 to 1.63)	0.65	13	1,635 (1,309-1,961)	1,266 (907-1,624)	249 (-256 to 754)	0.32
Calcium + vit. D	12	2,752 (2,201-3,442)	2,870 (2,213-3,721)	1.54 (1.03 to 2.32)	0.04	12	1,711 (1,370-2,052)	1,761 (1,391-2,130)	668 (156 to 1,180)	0.01
BMI										
Not obese (< 30 kg/m^2)										
Placebo	9	2,702 (2,178-3,352)	2,054 (1,554-2,715)	1.00 (Ref)		9	1,663 (1,329-1,998)	1,208 (894-1,523)	0 (Ref)	
Calcium	8	2,818 (2,242-3,542)	3,075 (2,287-4,135)	1.44 (0.97 to 2.11)	0.07	8	1,801 (1,446-2,156)	1,799 (1,465-2,132)	453 (-12 to 917)	0.06
Vitamin D	11	2,523 (2,056-3,095)	1,643 (1,261-2,141)	0.86 (0.59 to 1.23)	0.39	11	1,462 (1,160-1,765)	1,015 (731-1,300)	8 (-422 to 437)	0.97
Calcium + vit. D	9	2,753 (2,219-3,415)	2,513 (1,901-3,322)	1.20 (0.82 to 1.75)	0.33	8	1,628 (1,273-1,983)	1,582 (1,248-1,915)	409 (-56 to 873)	0.08
Obese										
Placebo	3	3,730 (2,085-6,671)	2,285 (1,610-3,244)	1.00 (Ref)		3	2,525 (1,695-3,355)	1,774 (913-2,636)	0 (Ref)	
Calcium	8	2,390 (1,674-3,412)	3,213 (2,593-3,981)	2.19 (1.27 to 3.80)	0.01	8	1,944 (1,436-2,453)	1,911 (1,383-2,438)	718 (-142 to 1,577)	0.10
Vitamin D	6	3,045 (2,018-4,593)	2,937 (2,293-3,763)	1.57 (0.89 to 2.80)	0.12	6	1,790 (1,203-2,377)	1,659 (1,049-2,268)	620 (-278 to 1,518)	0.17
Calcium + vit. D	8	2,678 (1,876-3,823)	3,057 (2,467-3,788)	1.86 (1.07 to 3.23)	0.03	8	1,789 (1,278-2,299)	1,814 (1,289-2,339)	777 (-83 to 1,636)	0.07
Compliance										
Below median (< 99%)										
Placebo	6	2,980 (2,267-3,917)	2,178 (1,509-3,144)	1.00 (Ref)		6	1,723 (1,238-2,208)	1,261 (903-1,618)	0 (Ref)	
Calcium	8	2,684 (2,118-3,401)	3,169 (2,307-4,355)	1.51 (1.03 to 2.22)	0.04	8	2,066 (1,646-2,486)	1,834 (1,524-2,143)	230 (-350 to 810)	0.42
Vitamin D	10	2,426 (1,963-2,998)	1,910 (1,438-2,538)	1.03 (0.70 to 1.51)	0.89	10	1,614 (1,238-1,990)	1,306 (1,029-1,583)	154 (-400 to 709)	0.57
Calcium + vit. D	6	3,088 (2,349-4,059)	2,499 (1,732-3,607)	1.60 (1.07 to 2.38)	0.02	5	1,818 (1,287-2,350)	1,374 (982-1,766)	18 (-632 to 669)	0.95
At or above median										
Placebo	6	2,878 (1,981-4,182)	2,043 (1,513-2,758)	1.00 (Ref)		6	2,035 (1,518-2,551)	1,439 (828-2,049)	0 (Ref)	
Calcium	8	2,509 (1,816-3,468)	3,118 (2,404-4,043)	1.97 (1.21 to 3.19)	0.01	8	1,680 (1,233-2,126)	1,876 (1,347-2,404)	792 (260 to 1,325)	0.01
Vitamin D	7	3,251 (2,237-4,723)	2,284 (1,692-3,083)	1.12 (0.69 to 1.82)	0.63	7	1,527 (1,049-2,004)	1,152 (587-1,716)	221 (-328 to 769)	0.42
Calcium + vit. D	11	2,534 (1,923-3,340)	2,906 (2,329-3,628)	1.40 (0.88 to 2.23)	0.15	11	1,610 (1,227-1,994)	1,785 (1,345-2,225)	771 (272 to 1,169)	0.004
Baseline 25(OH)D										
Below median (< 21.3 ng/mL)										
Placebo	7	3,028 (2,278-4,027)	2,462 (1,808-3,353)	1.00 (Ref)		7	2,067 (1,659-2,474)	1,626 (1,256-1,997)	0 (Ref)	
Calcium	8	2,923 (2,239-3,817)	3,589 (2,689-4,791)	1.51 (1.03 to 2.22)	0.04	8	2,101 (1,720-2,482)	2,146 (1,800-2,493)	486 (-53 to 1,025)	0.08
Vitamin D	9	2,317 (1,775-3,024)	1,933 (1,448-2,580)	1.03 (0.70 to 1.51)	0.89	9	1,421 (1,062-1,780)	1,207 (880-1,534)	227 (-298 to 752)	0.38
Calcium + vit. D	7	2,373 (1,784-3,155)	3,084 (2,265-4,199)	1.60 (1.07 to 2.38)	0.02	7	1,648 (1,247-2,049)	1,880 (1,505-2,255)	673 (117 to 1,229)	0.02

At or above median										
Placebo	5	2,794 (1,940-4,026)	1,699 (1,211-2,383)	1.00 (Ref)		5	1,615 (1,028-2,202)	962 (359-1,566)	0 (Ref)	
Calcium	8	2,304 (1,726-3,075)	2,753 (2,107-3,597)	1.97 (1.21 to 3.19)	0.01	8	1,644 (1,180-2,109)	1,563 (1,086-2,041)	572 (-50 to 1,193)	0.07
Vitamin D	8	3,163 (2,370-4,221)	2,159 (1,652-2,821)	1.12 (0.69 to 1.82)	0.63	8	1,755 (1,291-2,219)	1,282 (805-1,759)	180 (-441 to 802)	0.56
Calcium + vit. D	10	2,988 (2,308-3,868)	2,547 (2,005-3,235)	1.40 (0.88 to 2.23)	0.15	9	1,784 (1,346-2,221)	1,588 (1,138-2,038)	457 (-151 to 1,065)	0.13
DBP Isoform										
DBP1-1										
Placebo	9	2,878 (2,117-3,913)	1,991 (1,608-2,464)	1.00 (Ref)		9	1,845 (1,379-2,310)	1,356 (962-1,750)	0 (Ref)	
Calcium	9	2,659 (1,956-3,614)	2,891 (2,335-3,579)	1.71 (1.00 to 2.92)	0.05	9	2,041 (1,576-2,507)	1,818 (1,423-2,212)	265 (-238 to 768)	0.29
Vitamin D[f]	7	2,560 (1,808-3,627)	2,415 (1,895-3,076)	0.82 (0.48 to 1.38)	0.43	7	1,704 (1,176-2,232)	1,426 (979-1,873)	211 (-327 to 749)	0.43
Calcium + vit. D[f]	8	2,626 (1,896-3,638)	2,803 (2,235-3,515)	1.18 (0.70 to 1.99)	0.52	8	1,647 (1,153-2,141)	1,607 (1,189-2,025)	449 (-70 to 967)	0.09
DBP1-2/DBP2-2										
Placebo	3	3,084 (2,065-4,605)	2,512 (1,404-4,493)	1.00 (Ref)		3	1,981 (1,377-2,584)	1,330 (529-2,131)	0 (Ref)	
Calcium	7	2,516 (1,935-3,271)	3,501 (2,393-5,124)	1.57 (1.05 to 2.34)	0.03	7	1,656 (1,262-2,051)	1,903 (1,378-2,427)	897 (132 to 1,662)	0.02
Vitamin D[f]	9	2,687 (2,102-3,435)	1,787 (1,251-2,551)	1.36 (0.89 to 2.09)	0.15	9	1,490 (1,142-1,838)	1,170 (707-1,632)	330 (-409 to 1,069)	0.36
Calcium + vit. D[f]	8	2,837 (2,219-3,626)	2,722 (1,906-3,887)	1.54 (1.02 to 2.33)	0.04	7	1,706 (1,304-2,109)	1,709 (1,217-2,201)	653 (-104 to 1,411)	0.09

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; BMI, body mass index; CI, confidence interval; DBP, vitamin D-binding protein; NSAID, non-steroidal anti-inflammatory drug; Ref., reference; Rx, treatment; vit. D, vitamin D.

[a]Biomarker expression measured as labeling optical density using automated immunohistochemistry and image analysis.

[b]Crypt 15-HPGD biomarker labeling densities were natural log-transformed due to right-skewness; thus, we provide the geometric means and relative effects[d] estimated by the mixed linear model.

[c]Stromal 15-HPGD biomarker labeling densities were normally distributed; thus, we provide the crude means and absolute effects[e] estimated by the mixed linear model; one subject excluded from stroma estimates due to missing 15-HPGD expression values.

[d]Relative effect = [(treatment group follow-up) / (treatment group baseline)] / [(placebo group follow-up) / (placebo group baseline)]; interpretation is similar to that for an odds ratio (e.g., a value of 1.50 would be interpreted as a 50% increase in biomarker expression in the treatment group relative to the placebo group after 1 year).

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

[f]One subject excluded from the vitamin D group, and one from the calcium + vitamin D group due to missing DBP isoform information.

Supplementary Table S3. Effects of vitamin D and/or calcium supplementation on the ratio of COX-2 to 15-HPGD expression in the crypts and stroma of the normal-appearing colorectal mucosa, according to potential effect-modifiers, among adjunct biomarker study participants (n = 62)[a]

	Whole crypts[b]					Stroma[b,c]				
Strata	**N**	**Baseline geometric means[b] (95% CI)**	**1-yr follow-up geometric means[b] (95% CI)**	**Relative Rx effects[d] (95% CI)**	***P***	**N**	**Baseline geometric means[b] (95% CI)**	**1-yr follow-up geometric means[b] (95% CI)**	**Relative Rx effects[d] (95% CI)**	***P***
NSAID Use										
Weekly non-aspirin NSAID use										
Placebo	4	0.74 (0.32-1.68)	0.93 (0.45-1.89)	1.00 (Ref)		4	0.35 (0.17-0.71)	0.42 (0.21-0.85)	1.00 (Ref)	
Calcium	7	0.71 (0.38-1.32)	0.54 (0.32-0.93)	0.61 (0.28 to 1.33)	0.20	7	0.35 (0.21-0.60)	0.34 (0.20-0.58)	0.80 (0.43 to 1.51)	0.47
Vitamin D	4	0.35 (0.14-0.92)	0.29 (0.13-0.65)	0.65 (0.25 to 1.67)	0.34	4	0.29 (0.14-0.59)	0.42 (0.21-0.85)	1.19 (0.59 to 2.43)	0.60
Calcium + vit. D	5	0.47 (0.23-0.99)	0.57 (0.30-1.07)	0.95 (0.41 to 2.20)	0.91	4	0.30 (0.15-0.60)	0.37 (0.18-0.75)	1.03 (0.50 to 2.10)	0.93
No weekly non-aspirin NSAID use										
Placebo	8	0.50 (0.33-0.74)	0.76 (0.50-1.15)	1.00 (Ref)		8	0.23 (0.16-0.33)	0.33 (0.23-0.48)	1.00 (Ref)	
Calcium	9	0.59 (0.41-0.86)	0.45 (0.31-0.67)	0.50 (0.31 to 0.80)	0.005	9	0.41 (0.29-0.58)	0.32 (0.23-0.45)	0.53 (0.31 to 0.92)	0.03
Vitamin D	13	0.74 (0.54-1.01)	0.55 (0.40-0.77)	0.49 (0.32 to 0.76)	0.002	13	0.36 (0.27-0.48)	0.29 (0.22-0.39)	0.55 (0.33 to 0.92)	0.02
Calcium + vit. D	12	0.59 (0.43-0.82)	0.51 (0.36-0.71)	0.56 (0.36 to 0.87)	0.01	12	0.34 (0.25-0.46)	0.38 (0.28-0.51)	0.75 (0.45 to 1.26)	0.27
BMI										
Not obese (< 30 kg/m^2)										
Placebo	9	0.72 (0.50-1.03)	1.03 (0.67-1.57)	1.00 (Ref)		9	0.32 (0.24-0.44)	0.39 (0.28-0.55)	1.00 (Ref)	
Calcium	8	0.70 (0.48-1.03)	0.48 (0.30-0.74)	0.47 (0.29 to 0.77)	0.004	8	0.44 (0.31-0.61)	0.36 (0.25-0.51)	0.67 (0.41 to 1.08)	0.10
Vitamin D	11	0.54 (0.38-0.76)	0.43 (0.29-0.64)	0.55 (0.35 to 0.88)	0.01	11	0.37 (0.28-0.50)	0.36 (0.27-0.49)	0.80 (0.51 to 1.24)	0.31
Calcium + vit. D	9	0.41 (0.28-0.58)	0.43 (0.28-0.65)	0.73 (0.45 to 1.17)	0.18	8	0.29 (0.21-0.40)	0.29 (0.20-0.42)	0.84 (0.52 to 1.35)	0.45
Obese										
Placebo	3	0.28 (0.13-0.61)	0.39 (0.22-0.71)	1.00 (Ref)		3	0.14 (0.06-0.30)	0.28 (0.14-0.55)	1.00 (Ref)	
Calcium	8	0.59 (0.36-0.95)	0.51 (0.35-0.72)	0.61 (0.29 to 1.29)	0.18	8	0.33 (0.21-0.54)	0.30 (0.20-0.46)	0.45 (0.19 to 1.07)	0.07
Vitamin D	6	0.86 (0.50-1.5)	0.62 (0.41-0.93)	0.51 (0.23 to 1.10)	0.08	6	0.29 (0.17-0.51)	0.25 (0.16-0.41)	0.43 (0.17 to 1.06)	0.06
Calcium + vit. D	8	0.79 (0.49-1.27)	0.66 (0.46-0.95)	0.59 (0.28 to 1.25)	0.16	8	0.36 (0.22-0.57)	0.47 (0.31-0.71)	0.65 (0.27 to 1.54)	0.31
Compliance										
Below median (< 99%)										
Placebo	6	0.51 (0.28-0.91)	0.63 (0.37-1.09)	1.00 (Ref)		6	0.32 (0.20-0.51)	0.41 (0.28-0.60)	1.00 (Ref)	
Calcium	8	0.61 (0.37-1.03)	0.40 (0.25-0.65)	0.52 (0.31 to 0.88)	0.02	8	0.31 (0.20-0.46)	0.32 (0.23-0.44)	0.81 (0.44 to 1.47)	0.47
Vitamin D	10	0.47 (0.26-0.85)	0.41 (0.24-0.70)	0.69 (0.39 to 1.21)	0.19	10	0.32 (0.23-0.46)	0.29 (0.22-0.39)	0.71 (0.40 to 1.26)	0.23
Calcium + vit. D	6	0.62 (0.40-0.96)	0.51 (0.34-0.77)	0.66 (0.41 to 1.08)	0.10	5	0.36 (0.21-0.59)	0.41 (0.27-0.62)	0.89 (0.46 to 1.75)	0.73
At or above median										
Placebo	6	0.63 (0.40-1.00)	1.03 (0.65-1.64)	1.00 (Ref)		6	0.21 (0.13-0.34)	0.31 (0.19-0.52)	1.00 (Ref)	
Calcium	8	0.67 (0.45-0.99)	0.59 (0.40-0.89)	0.55 (0.31 to 0.97)	0.04	8	0.48 (0.32-0.73)	0.34 (0.22-0.53)	0.48 (0.26 to 0.89)	0.02
Vitamin D	7	0.78 (0.55-1.11)	0.55 (0.38-0.79)	0.43 (0.25 to 0.75)	0.004	7	0.37 (0.24-0.59)	0.36 (0.22-0.57)	0.65 (0.35 to 1.21)	0.17
Calcium + vit. D	11	0.46 (0.29-0.72)	0.54 (0.34-0.86)	0.73 (0.39 to 1.35)	0.30	11	0.32 (0.22-0.46)	0.36 (0.25-0.53)	0.77 (0.43 to 1.37)	0.36
Baseline 25(OH)D										
Below median (< 21.3 ng/mL)										
Placebo	7	0.48 (0.30-0.76)	0.6 (0.39-0.92)	1.00 (Ref)		7	0.20 (0.13-0.30)	0.28 (0.21-0.38)	1.00 (Ref)	
Calcium	8	0.47 (0.30-0.73)	0.41 (0.27-0.60)	0.69 (0.39 to 1.20)	0.18	8	0.27 (0.18-0.39)	0.24 (0.18-0.32)	0.64 (0.34 to 1.19)	0.15
Vitamin D	9	0.80 (0.51-1.23)	0.57 (0.39-0.85)	0.57 (0.33 to 1.00)	0.05	9	0.36 (0.25-0.51)	0.38 (0.29-0.50)	0.77 (0.42 to 1.41)	0.38
Calcium + vit. D	7	0.58 (0.36-0.92)	0.42 (0.27-0.64)	0.58 (0.32 to 1.03)	0.06	7	0.32 (0.21-0.47)	0.34 (0.24-0.46)	0.76 (0.40 to 1.44)	0.39
At or above median										
Placebo	5	0.72 (0.41-1.28)	1.23 (0.70-2.16)	1.00 (Ref)		5	0.37 (0.23-0.62)	0.51 (0.30-0.85)	1.00 (Ref)	

Calcium	8	0.87 (0.56-1.37)	0.59 (0.38-0.92)	0.40 (0.23 to 0.68)	0.002	8	0.55 (0.37-0.81)	0.46 (0.30-0.69)	0.61 (0.34 to 1.11)	0.10
Vitamin D	8	0.52 (0.33-0.82)	0.42 (0.27-0.65)	0.47 (0.27 to 0.81)	0.008	8	0.33 (0.22-0.49)	0.26 (0.17-0.39)	0.58 (0.32 to 1.05)	0.07
Calcium + vit. D	10	0.54 (0.36-0.81)	0.61 (0.41-0.91)	0.66 (0.39 to 1.11)	0.11	9	0.32 (0.22-0.46)	0.39 (0.27-0.58)	0.91 (0.51 to 1.62)	0.74
DBP Isoform										
DBP1-1										
Placebo	9	0.58 (0.37-0.92)	0.73 (0.47-1.11)	1.00 (Ref)		9	0.28 (0.18-0.44)	0.37 (0.27-0.51)		
Calcium	9	0.49 (0.31-0.77)	0.49 (0.32-0.75)	0.81 (0.51 to 1.28)	0.35	9	0.34 (0.22-0.54)	0.36 (0.26-0.50)	0.80 (0.48 to 1.32)	0.37
Vitamin D[e]	7	0.66 (0.39-1.11)	0.58 (0.36-0.94)	0.70 (0.43 to 1.14)	0.15	7	0.28 (0.17-0.47)	0.28 (0.19-0.40)	0.76 (0.44 to 1.29)	0.29
Calcium + vit. D[e]	8	0.66 (0.41-1.08)	0.72 (0.46-1.14)	0.87 (0.54 to 1.40)	0.55	8	0.38 (0.23-0.62)	0.49 (0.35-0.69)	0.98 (0.58 to 1.64)	0.92
DBP1-2/DBP2-2										
Placebo	3	0.52 (0.28-0.99)	1.13 (0.58-2.18)	1.00 (Ref)		3	0.22 (0.13-0.36)	0.34 (0.17-0.68)		
Calcium	7	0.92 (0.60-1.39)	0.49 (0.32-0.76)	0.25 (0.13 to 0.47)	0.0002	7	0.44 (0.32-0.61)	0.29 (0.18-0.46)	0.43 (0.19 to 0.95)	0.04
Vitamin D[e]	9	0.71 (0.48-1.05)	0.45 (0.30-0.68)	0.30 (0.16 to 0.55)	0.0006	9	0.41 (0.31-0.54)	0.34 (0.23-0.51)	0.55 (0.25 to 1.19)	0.12
Calcium + vit. D[e]	8	0.49 (0.33-0.72)	0.42 (0.28-0.63)	0.40 (0.21 to 0.75)	0.0062	7	0.34 (0.24-0.47)	0.34 (0.22-0.52)	0.67 (0.30 to 1.48)	0.30

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; BMI, body mass index; CI, confidence interval; DBP, vitamin D-binding protein; NSAID, non-steroidal anti-inflammatory drug; Ref., reference; Rx, treatment; vit. D, vitamin D.

[a]Biomarker expression measured as labeling optical density using automated immunohistochemistry and image analysis.

[b]Both crypt and stromal biomarker labeling densities for the ratio of COX-2 to 15-HPGD were natural log-transformed due to right skewness; thus, the geometric means and relative effects[c] estimated by the mixed linear model are provided.

[c]One subject excluded from stroma estimates due to missing values for the measurement of 15-HPGD expression in the stroma.

[d]Relative effect = [(treatment group follow-up) / (treatment group baseline)] / [(placebo group follow-up) / (placebo group baseline)]; interpretation is similar to that for an odds ratio (e.g., a value of 1.50 would be interpreted as a 50% increase in biomarker expression in the treatment group relative to the placebo group after 1 year).

[e]One subject excluded from the vitamin D group, and one from the calcium + vitamin D group due to missing DBP isoform information.

Chapter IV

Association of circulating vitamin D with colorectal cancer depends on vitamin D-binding protein isoforms: A pooled nested case-control study

Manuscript Information

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ABSTRACT

Higher circulating 25-hydroxyvitamin-D [25(OH)D] concentrations are consistently inversely associated with colorectal cancer (CRC) risk in observational studies. However, it is unknown whether this association depends on the functional *GC*-rs4588*A (Thr436Lys) variant encoding the vitamin D-binding protein-2 (DBP2) isoform, which may affect vitamin D status and bioavailability. We analyzed data from 1,710 incident CRC cases and 1,649 incidence-density matched controls nested within three prospective cohorts of mostly Caucasians. Study-specific incidence rate ratios (RRs) for associations of pre-diagnostic, season-standardized 25(OH)D concentrations according to DBP2 isoform with CRC were estimated using multivariable unconditional logistic regression and pooled using fixed effects models. All statistical significance tests were two-sided. The odds of having 25(OH)D concentrations below 50 nmol/L (considered insufficient by the Institute of Medicine) were 43% higher for each DBP2-encoding variant (rs4588*A) inherited (per DBP2 OR = 1.43, 95% CI: 1.27 to 1.62, P_{trend} = 1.2 x10^{-8}). The association of 25(OH)D concentrations with CRC risk differed by DBP2: 25(OH)D concentrations considered sufficient (≥50 nmol/L), relative to deficient (<30 nmol/L), were associated with a 53% lower CRC risk among individuals with the DBP2 isoform (RR = 0.47, 95% CI: 0.33 to 0.67), but a non-statistically significant 12% lower risk among individuals without it (RR = 0.88, 95% CI: 0.61 to 1.27) ($P_{heterogeneity}$ = 0.01). Our results suggest that the 25(OH)D-CRC association may differ by DBP isoform, and those with a DBP2-encoding genotype—linked to vitamin D insufficiency—may particularly benefit from adequate 25(OH)D for CRC prevention.

INTRODUCTION

Colorectal cancer (CRC) is the second leading cause of cancer-related death and the third most common cause of cancer among men and women globally (9). Strong experimental evidence supports that vitamin D may prevent colorectal carcinogenesis via several mechanisms, including increasing bile acid catabolism, decreasing inflammation and angiogenesis, and direct effects on cellular proliferation, differentiation, apoptosis (28, 182). While higher circulating 25-hydroxyvitamin D [25(OH)D] concentrations—used clinically to assess vitamin D status—are inversely associated with CRC risk in observational studies (24), randomized clinical trials of the efficacy of vitamin D supplementation in preventing colorectal neoplasms were largely null (30-32). Various limitations of these trials—including sample size, dosing, trial duration, timing of supplementation in the natural history of the disease, and compliance—that may have contributed to these null findings have been described (30-32). Additionally, the effects of vitamin D supplementation and circulating 25(OH)D concentrations on vitamin D metabolism may differ by functional genetic variants, such as those in the vitamin D-binding protein (DBP) gene, formerly known as group component (*GC*) (100, 101). However, whether the 25(OH)D-CRC risk association differs by functional *GC* variants is unknown. Addressing this is relevant to the National Institute of Health's 'Precision Medicine Initiative' aimed at tailoring health care recommendations based on individual characteristics such as genotypes (183).

Nearly 90% of circulating 25(OH)D is bound to the DBP, which maintains stable serum vitamin D stores and regulates free 25(OH)D available to target tissues (141). DBP may also play a role in fatty acid binding, actin scavenging, and complement-mediated immune cell chemotaxis (109). Two *GC* missense variants (rs7041 and rs4588) determine three common DBP protein 'isoforms' (DBP1s, DBP1f, and DBP2, also known as Gc1s, Gc1f and Gc2), which

are associated with differences in vitamin D status and vitamin D pathway induction (184, 185). Moreover, the association of 25(OH)D with, and the effects of vitamin D supplementation on, colorectal adenoma risk were reported to be stronger among those with the DBP2-encoding variant than among those without it, but whether there is a similar pattern of effect-modification in relation to CRC risk is unknown (100, 118).

Accordingly, we hypothesized that higher 25(OH)D concentrations would be more strongly inversely associated with CRC risk among individuals with the DBP2 isoform than among those without it. We investigated this hypothesis in three prospective case-control studies nested within cohort studies conducted in the US and Europe.

METHODS

Study population

We conducted an individual participant pooled analysis of data from three prospective cohort studies: (1) the European Prospective Investigation into Cancer and Nutrition (EPIC), which recruited men and women from the general population in 10 Western European countries (1992-1998) (186); (2) the Cancer Prevention Study-II Nutrition Cohort (CPS-II), which recruited men and women from 21 US states (1992-1993) (187); and (3) the Nurses' Health Study (NHS), which recruited female nurses in the US (1976) (188).

The European Prospective Investigation into Cancer and Nutrition (EPIC) study recruited over 520,000 participants from the general population in 10 Western European countries between 1992 and 1998, and blood samples were collected from most participants at recruitment prior to cancer onset or diagnosis (158, 159). After exclusions (56 cases for missing matching information, 31 cases for missing 25(OH)D data for the case-control set), 1,248 first incident

CRC cases were identified, and 1,248 cancer-free controls were matched using incidence density sampling with respect to age, sex, study center, date of blood draw, time of day and fasting status at blood draw, and, among women, menopausal status, phase of menstrual cycle, and hormone replacement therapy at blood draw (189).

The Cancer Prevention Study-II (CPS-II) recruited 184,194 men and women from 21 US states between 1992 and 1993, and blood samples were collected prior to cancer diagnosis among 39,380 participants between 1998 and 2001(156). After exclusions (10 for missing matching information, 10 non-whites), 288 incident CRC cases were identified, and 288 cancer-free controls were matched using incidence density sampling with respect to age, sex, and date of blood draw (24).

The Nurses' Health Study (NHS) enrolled 121,701 US female nurses in 1976. Blood specimens were collected from 32,826 women between 1989 and 1990 (188). Among those with available blood specimens, 378 incident CRC cases were identified and matched to 713 controls using incidence density sampling with respect to age and month/year of blood draw (148). This NHS sample includes 15 non-white participants (8 cases, 7 controls) who were included in our primary analyses in order to be consistent with prior NHS studies that investigated the 25(OH)D-CRC association among participants in the same matched set (148). In sensitivity analyses, excluding these non-white participants did not materially affect the results. Additional details concerning the study population, case ascertainment, and data collection for EPIC (158, 159), CPS-II (156, 157), and NHS (148, 188) were previously published.

Of the combined 1,914 CRC cases and 2,249 matched controls from the above-described case-control studies nested within EPIC, CPS-II, and NHS (24, 148, 189), 1,710 cases and 1,649 controls had relevant genotyping information at the *GC* locus and were included in this analysis

Each participating cohort was approved by its respective institutional review board, and written informed consent was obtained from each subject.

25(OH)D Assays

Total 25(OH)D (D_2 and D_3) was measured using the FDA-approved DiaSorin LIAISON chemiluminescence immunoassay (CLIA) in CPS-II (Heartland Assays, Ames, IA), the OCTEIA enzyme immunoassay (Immuno Diagnostic Systems, Boldon, UK) in EPIC (189), and a radioimmunoassay at the laboratory of Dr. B.W. Hollis (The Medical University of South Carolina, Charleston, SC) and the Heartland Laboratory (Heartland Assays, Ames, IA) in NHS (148). The intra-assay coefficient of variance was 4.5% for EPIC, 5.2% for CPS-II, and 13.5% for NHS.

For each study that previously measured 25(OH)D concentrations, individual 25(OH)D concentrations were first calibrated to the same assay used for the newly measured studies (direct, competitive chemiluminescence immunoassay at Heartland Assays, LLC) according to the equation:

$$Y_{calibrated} = \hat{a} + \hat{b}Y_{original}$$

where the estimates $\hat{a}$ and $\hat{b}$ were obtained by regressing Heartland Assays 25(OH)D on the original 25(OH)D values for 29 calibration samples, described previously (190). In each study, approximately three control participants were selected within each decile of the study-specific 25(OH)D distribution to serve as calibration samples and were re-assayed at Heartland Assays. Variances for the continuous 25(OH)D measurements were increased to account for laboratory error in the calibration process; variances for the categorical analyses did not need to be similarly adjusted. In CPS-II, in which the 25(OH)D concentrations were newly measured for this project

by Heartland Assays, no calibration was required.

Calibrated 25(OH)D measurements were seasonally-adjusted using the method described by Gail *et al.* (190). To remove variation in circulating 25(OH)D due to season of blood collection, individual residuals were calculated from the following study-specific sin/cosin function fitted to controls:

$$\gamma_0 + \gamma_1 \sin(2\pi t/52) + \gamma_2 \cos(2\pi t/52) + \gamma_3 \sin(4\pi t/52) + \gamma_4 \cos(4\pi t/52)$$

where t = week of blood draw (1, 2, … 52). Residuals from the model were added to the study-specific mean 25(OH)D concentration among controls averaged over the entire year. The adjusted value may be interpreted as a participant's predicted 25(OH)D concentration averaged over the entire year, accounting for study-specific seasonal-variation in 25(OH)D concentrations.

Genotyping

Genotyping was performed using a custom GoldenGate Universal-plex assay kit (Illumina, CA, USA) in EPIC (191); a custom Affymetrix genome-wide platform, the Axiom Correct Set (Affymetrix, CA, USA), in CPS-II (192); and the OmniExpress platform in NHS (Illumina, CA, USA) (192). Genotyping quality control for CPS-II and NHS samples was described previously (192). In EPIC, all *GC* genotyping was conducted using standard quality control: the lowest reproducibility frequency across 62 replicate samples was 0.98; call rates were >95% for all samples and SNPs.

Individuals with the *GC*-rs4588*A allele (CA or AA) were classified as having the DBP2 isoform, while those without the A allele (CC) were classified as having only DBP1 isoforms (131, 184). The two DBP1 (1f and 1s) isoforms, distinguished by *GC*-rs7041, were combined in this analysis based on previous studies' effect-modification findings and our hypothesis (100,

118, 120). These genotypes perfectly predict the expected amino acid changes of the circulating protein isoforms as determined in previous proteomic analyses (131). *GC* rs3755967 (G>A) was used as a proxy rs4588 in EPIC; these SNPs are in complete linkage disequilibrium ($r^2 = 1.0$) in the HapMap Spanish and British populations (1000 Genomes Project Phase 3, LD link, National Cancer Institute, Washington, DC). *GC* rs3755967 and rs4588 were in Hardy-Weinberg equilibrium ($P > 0.05$) in each study.

Statistical Analyses

Calibrated 25(OH)D measurements were season-standardized using a cos/sin function described previously (190) and in the Supplementary Methods. The season-standardized value may be interpreted as a participant's predicted 25(OH)D concentration averaged over the entire year, accounting for study-specific seasonal-variation in 25(OH)D (190).

We estimated the association of DBP2 inheritance (*GC*-rs4588 genotype) with 25(OH)D concentrations <50 nmol/L using unconditional logistic regression; 50 nmol/L is considered the cut-point for vitamin D sufficiency by the Institute of Medicine (IOM, now the National Academy of Medicine). A two-stage approach was used to estimate summary odds ratios (ORs): study-specific ORs were calculated in separate unconditional logistic regression models, and then combined using fixed effects models (in sensitivity analyses, the use of mixed effects models did not materially affect the results). All study-specific ORs were adjusted for age, sex, and case-control status; EPIC models were further adjusted for study center. Study-specific mean 25(OH)D concentrations among DBP1-1, DBP1-2, and DBP2-2 participants were calculated using general linear regression models adjusted for the same covariates.

We estimated the association of 25(OH)D concentrations, categorized using IOM-

recommended cut-points, with CRC risk using unconditional logistic regression models stratified by DBP2 isoform inheritance (i.e., *GC*-rs4588 using a dominant inheritance model). We report associations as incidence rate ratios (RRs), which are estimated by odds ratios in nested case-control studies in which controls are selected using incidence density sampling (189). Conditional logistic regression necessitated excluding participants in matched pairs who were discordant on DPB2-encoding genotypes; however, in sensitivity analyses, the results from conditional and unconditional logistic regression did not materially differ, so unconditional logistic regression was chosen to maximize our sample size and statistical power. A dominant inheritance model was chosen based on previous findings of effect-modification by DBP2 for the association of 25(OH)D with colorectal adenoma risk [15] and to maximize statistical efficiency given the rarity of the DBP2-2 genotype, especially in the smaller CPS-II and NHS studies. A two-stage approach was used to estimate summary RRs: study-specific RRs were calculated in separate logistic regression models, and then combined using fixed effects models (in sensitivity analyses, the use of mixed effects models did not materially affect the results). Study-specific RRs were adjusted for study-specific matching factors (Supplementary Methods), body mass index (continuous, kg/m^2), and physical activity (combined recreational and household activity MET-hrs/week, quartiles). Potential covariates, chosen based on biological plausibility and previous literature, included education, smoking, and total dietary intakes of energy, calcium (from food and supplements), fruits and vegetables, red and processed meats, and alcohol; of these, only those that affected the RRs by ≥10% were included in the final models (see Tables' footnotes). Between-study heterogeneity was evaluated using the I^2 statistic. Effect modification evaluating interaction between 25(OH)D and DBP2 on the multiplicative scale was evaluated using meta-regression (193).

Since the cut-points for vitamin D status are debated, in separate analyses we included an additional upper category (≥75 nmol/L) and collapsed the lower IOM categories (<50 nmol/L), as other professional societies use these values to define vitamin D sufficiency and deficiency, respectively (56). In all models, the lowest 25(OH)D category was used as the reference. To assess the significance of trend in CRC risk across the three- and four-level 25(OH)D categories, participants were assigned the study-specific median value of their respective 25(OH)D category, and the study-specific coefficients were pooled using fixed-effects models (193).

To assess interaction on the additive scale, we estimated the associations of a joint variable combining 25(OH)D concentrations and the DBP2 isoform with CRC risk to calculate the relative excess risk due to interaction (RERI) (194): $RERI = RR_{11} - RR_{10} - RR_{01} + 1$, where RR_{ij} is the relative risk associated with the joint variable that combines 25(OH)D (*i*, coded 0 for sufficient [≥ 50 nmol/L], and 1 for deficient [<30 nmol/L]) and DBP isoform (*j,* coded 0 for no DBP2 isoform, and 1 for the DBP2 isoform).

All statistical tests were two-sided; a *P*-value < 0.05 or a 95% confidence interval (CI) that excluded 1.0 was considered statistically significant. Analyses were performed using SAS version 9.3 (Cary, NC), except for the meta-analyses performed in STATA version 12.1 (College Station, TX).

RESULTS

Selected characteristics of the study participants, by cohort and case-control status, are summarized in **Table 1**; tumor characteristics (site and stage) of CRC cases are presented in Supplementary Table 1. In EPIC, CPS-II, and NHS, the median ages at blood draw were 59, 75,

and 59 years; the median times from blood draw to CRC diagnosis were 3.6, 3.2, and 9.6 years; and the frequencies of the DBP2-encoding allele were 0.29, 0.26, and 0.28, respectively.

Individuals with the DBP2 isoform were more likely to have 25(OH)D concentrations <50 nmol/L than were those with DBP1 isoforms (per DBP2 OR = 1.43, 95% CI: 1.27 to 1.62, P_{trend} = 1.2 x10^{-8}) (**Table 2**). Mean 25(OH)D concentrations were lower in EPIC (DBP1-1: 43.1, DBP2-2: 40.8, DBP2-2: 37.5 nmol/L) than in NHS (DBP1-1: 69.2, DBP1-2: 55.5, DBP2-2: 63.6 nmol/L) or CPS-II (DBP1-1: 62.3, DBP1-2: 61.5, DBP2-2: 64.3 nmol/L) (Supplementary Table 2).

Higher 25(OH)D concentrations were more strongly associated with lower CRC risk among individuals with the DBP2 isoform, than among those with only DBP1 isoforms (**Table 3**). Among those with DBP2, 25(OH)D concentrations of 30-<50, 50-<75, and ≥75 nmol/L, relative to <30 nmol/L, were associated with statistically significant 31%, 56%, and 60% lower risk of CRC, respectively (P_{trend} = 5.8 x 10^{-5}). Among those with only DBP1 isoforms, the corresponding RRs for CRC risk were 20% higher, 8% lower, and 34% lower (for concentrations of 30-<50, 50-75, and ≥75, relative to <30 nmol/L [P_{trend} = 0.01; $P_{heterogeneity\ for\ DBP2}$ = 0.02, 0.02, and 0.21], respectively). Concentrations ≥50 nmol/L relative to <30 nmol/L, were associated with statistically significant 53% lower CRC risk among those with DBP2 (P_{trend} = 0.0001), and non-statistically significant 12% lower risk among individuals with only DBP1 isoforms (P_{trend} = 0.09; $P_{heterogeneity\ by\ DBP2}$ = 0.01).

The pattern of effect-modification by DBP2 was most pronounced in the larger EPIC study (Supplementary Table 3), but there was no evidence of significant study heterogeneity in the meta-analyses (I^2 = 0.0 – 20.1%, $P_{heterogeneity\ by\ study}$ > 0.28 for all meta-estimates [Supplementary Table 4]).

In the joint/combined analysis, relative to those with only DBP1 isoforms and vitamin D sufficiency, those with DBP2 and vitamin D deficiency had a statistically significant 68% higher CRC risk. This association was 72% higher than expected (RERI = 0.72) indicating a greater than additive interaction (RERI > 0) (**Table 4).**

DISCUSSION

Our findings suggest that associations of 25(OH)D concentrations with CRC risk differ by common, inherited vitamin-D binding protein isoforms, and that individuals with DBP2—who may be predisposed to vitamin D insufficiency relative to individuals with DBP1 isoforms—may particularly benefit from maintaining sufficient vitamin D concentrations for CRC prevention. To our knowledge, this is the first study to report that the 25(OH)D-CRC association differs by DBP isoform.

DBP2 is encoded by the functional *GC*-rs4588 polymorphism (C>A) resulting in a Thr (DBP1)→ Lys (DBP2) amino acid substitution at residue 436 (105, 133). Although the physiologic consequences of the isoforms have not been fully elucidated, consistent with previous studies (124-126), the DBP2-encoding variant was strongly associated with lower circulating 25(OH)D concentrations and higher odds of vitamin D insufficiency in our study population. This association may be due to differences in circulating DBP concentrations (20-30% lower among DBP2 homozygotes relative to DBP1 homozygotes were reported in studies that did not use the isoform-biased monoclonal R&D assay (127-131)), as DBP mediates the renal reabsorption of 25(OH)D and prolongs its circulating half-life (105, 120, 195). Some studies suggest that the DBP2 isoform also has the lowest binding affinity to 25(OH)D, which, in addition to lower DBP concentrations, could lead to higher levels of free 25(OH)D (131, 135,

141, 196). This may underlie the higher induction of vitamin D target genes by 25(OH)D in cultured monocytes and colon cancer cell lines with DBP2 relative to cells cultured with DBP1 isoforms (119, 139). Normal and neoplastic colon tissues express the vitamin D-receptor (VDR) and are able to locally convert 25(OH)D to the VDR-activating $1,25(OH)_2D$ form, which may play an important role in colorectal carcinogenesis via modulating cell growth, inflammation, angiogenesis, and apoptosis (2, 173). Taken together, we hypothesize that individuals with the DBP2 isoform may particularly benefit from higher 25(OH)D concentrations as these concentrations may lead to higher vitamin D-pathway activation and may be needed to compensate for DBP2 individuals' reduced capacity to otherwise maintain adequate 25(OH)D concentrations.

Supporting this hypothesis are findings from other observational studies and randomized controlled trials (RCT) that reported similar patterns of effect-modification by DBP2. In a US case-control study of individuals of European ancestry, 25(OH)D concentrations ≥50 relative to <50 nmol/L were associated with lower risk of incident, sporadic colorectal adenoma among those with DBP2 (OR = 0.51, 95% CI: 0.33 to 0.81), but not among those without DBP2 (OR = 1.11, 95% CI: 0.68 to 1.92) ($P_{interaction}$ = 0.05) (118). Findings from two other observational studies (including an NHS study that used the same matched case-control set used in our analysis) suggest that the 25(OH)D-CRC risk association is stronger among those with DBP concentrations below the median, which provides indirect support of our findings given the strong association of DBP2 with lower DBP concentrations (148, 197). Additionally, although the reported effects of vitamin D supplementation on colorectal neoplasm prevention in randomized controlled trials have largely been null (32, 97), it is possible that the effects of vitamin D supplementation on 25(OH)D concentrations and colorectal neoplasm prevention may

also depend on the functional DBP2 isoform (100, 101). In two trials, vitamin D supplementation increased 25(OH)D concentrations more among those with the DBP2-encoding relative to DBP1-encoding genotypes (101, 152). Moreover, in a large RCT (n = 2,259) (100), the 'interaction relative risk'—ratio of the vitamin D supplementation RR per DBP2-encoding minor allele divided by that for the DBP1-encoding major allele—was 0.82 (95% CI: 0.69 to 0.98), indicating that the effect of vitamin D supplementation on reducing adenoma recurrence was significantly stronger with each DBP2-encoding variant inherited ($P_{interaction}$ = 0.03).

Our findings may help explain certain inconsistencies in the literature regarding vitamin D concentrations, *GC* genotypes, and CRC risk. In a recent international pooling project of 17 cohorts, the study-specific RRs for CRC with each 25 nmol/L increase in 25(OH)D were mostly inverse, but varied from 1.17 to 0.63, with only five being statistically significant (24). In addition to differences in sample size that may affect the precision of the estimates, this heterogeneity may, in part, be due to differences in DBP2 frequency in different study populations, as DBP2 frequency varies by geographic area and ethnicity (from 0.01 to 0.41 internationally, and from 0.21 to 0.41 in European and white American populations) (116). Additionally, while the DBP2-encoding *GC*-rs4588 variant is associated with lower 25(OH)D concentrations, it was not associated with CRC risk in genome-wide association or Mendelian randomization studies (198-200). The potential interaction between 25(OH)D concentrations and DBP2 in relation to CRC risk could contribute to these null findings.

Epidemiologists have argued that measures of interaction on the additive scale, such as the RERI, are important when considering public health implications since they can indicate whether an intervention is more likely to have a greater absolute effect on a health outcome in a certain sub-population (201, 202). Our finding of a RERI > 0 suggests that the association of

inheriting the DBP2 isoform *and* being vitamin D deficient with CRC risk is greater than that expected given the risks associated with the DBP2 isoform and 25(OH)D deficiency alone. Given the high prevalence of DBP2 (40 – 50% with European ancestry (116)) and vitamin D concentrations <50 nmol/L in the US and Europe (26 – 76% (56, 203, 204)), these findings, if confirmed, could inform future clinical recommendations related to vitamin D and CRC prevention and have significant public health impact.

Strengths of our study include the use of data from three prospective cohorts in the US and Europe, with participants from geographically diverse areas. We also used season-adjusted 25(OH)D concentrations, thereby reducing misclassification of vitamin D status, which may vary throughout the year and in study populations living at different latitudes. Given that 25(OH)D measurements may vary by assay type, harmonization of 25(OH)D levels to a standard assay is another strength of this study, providing more reliable meta-estimates and the ability to assess 25(OH)D using absolute clinical cut-points—a limitation in most prior meta-analyses.

Our study also has several limitations. IOM cut-points for vitamin D status are based on skeletal health research, as their guidelines currently cite insufficient evidence to inform recommendations for non-skeletal health outcomes (56, 67). Larger studies are needed to investigate more precise categories of 25(OH)D that may be relevant to CRC risk. Data for certain potential confounding factors (e.g., aspirin or multivitamin use) were not available in EPIC; however, adjusting for these covariates in the CPS-II and NHS models did not materially affect the results. Additionally, pre-diagnostic 25(OH)D was measured only once, although it may still have been a relatively good indicator of long-term vitamin D status given that previous studies estimated within-person correlations between 0.53 and 0.81 for repeated 25(OH)D measures taken 1 to 11 years apart (205, 206). Although our meta-estimates were largely driven

by the estimates from EPIC due to its much larger sample size—especially for those with lower 25(OH)D concentrations—between-study heterogeneity was minimal. Due to logistic issues related to primary data transfers, we were unable to pool the individual datasets; therefore, we conducted the analyses separately within each cohort and then summarized the estimates using a meta-analytic approach. We would expect this to yield estimates similar to those from a pooled analysis, and to be more conservative. Last, because the frequency and effects of DBP isoforms may differ by race/ethnicity (105, 116), our findings may not be generalizable to other populations.

In conclusion, our findings suggest that the association of circulating vitamin D concentration with CRC risk may differ by common, inherited genotypes encoding vitamin D-binding protein isoforms. Individuals with the DBP2 isoform—linked to vitamin D insufficiency—may particularly benefit from maintaining adequate vitamin D concentrations for CRC prevention.

Table 1. Selected characteristics of participants in three case-control studies nested within the EPIC, CPS-II, and NHS cohorts[a]

	EPIC		CPS-II		NHS	
Variable	**Cases (n=1,106)**	**Controls (n=719)**	**Cases (n=246)**	**Controls (n=217)**	**Cases (n=358)**	**Controls (n=713)**
Circulating 25(OH)D, nmol/L, mean (SD)[b]	40.7 (16.0)	42.9 (14.6)	59.4 (20.5)	63.5 (21.6)	62.8 (27.3)	67.3 (27.0)
Vitamin D binding protein (DBP) isoforms (rs4588 genotype)						
DBP1-1 (CC), %	52	50	54	58	56	52
DBP1-2 (CA), %	39	41	40	34	37	38
DBP2-2 (AA), %	9	9	6	8	7	10
Age, years, mean (SD)	58.6 (7.1)	58.7 (8.0)	74.6 (5.7)	75.0 (5.7)	58.7(6.7)	58.7(6.7)
Female,%	50	52	53	52	100	100
Educational level						
None/primary, %	38	46	4	4	0	0
Secondary (high school), %	15	12	25	23	0	0
Technical/professional, %	26	22	8	5	70[d]	66[d]
University or higher, %	18	17	63	69	27	30
Missing, %	3	3	1	0	4	4
Body mass index, kg/m^2, mean (SD)	26.7 (4.2)	26.3 (3.7)	26.4 (5.0)	25.8 (4.2)	25.3(4.4)	24.7(4.3)
Smoking status						
Never smokers, %	41	45	45	47	42	44
Former smokers, %	33	32	46	45	44	43
Current smokers, %	25	21	4	2	14	12
Missing, %	1	1	5	6	1	0
Physical activity, MET-hrs/wk[c]						
Median (IQR)	73.5 (44.5-120.6)	88.0 (48.8-126.0)	13.5 (6.8-23.0)	13.4 (7.0-22.0)	10.8 (4.2-19.4)	10.4 (4.2-20.7)
Quartile 1, %	24	20	24	24	24	25
Quartile 2, %	24	17	25	24	25	25
Quartile 3, %	21	23	25	23	26	25
Quartile 4, %	26	32	24	26	24	25
Missing	6	8	2	2	1	1
Menopausal status[e]						
Pre-menopausal, %l	9	12	0	0	13	12
Post-menopausal, %	12	10	100	100	87	88
Peri-menopausal/unknown, %	80	79	0	0	0	0

Hormone replacement therapy at time of blood draw[e]						
No, %	83	83	61	45	65	57
Yes, %	14	12	35	48	31	40
Unknown, %	3	4	4	7	4	3
Dietary intakes						
Total energy, kcal/day, mean (SD)	2,149 (681)	2,065 (621)	1,729 (463)	1,774 (606)	1,711 (461)	1708.6 (442.4)
Total fruits, g/day, median (IQR)[f]	178 (93-288)	207 (117-328)	160 (105-238)	161 (97-247)	2.2 (1.5-2.9)	2.2 (1.5-2.9)
Total vegetables, g/day, median (IQR)[f]	153 (97-227)	161 (102-255)	175 (124-235)	186 (113-252)	2.8 (2.2-3.5)	2.9 (2.1-3.7)
Total red and processed meats, g/day, median (IQR)[f]	48 (25-79)	38 (20-64)	45 (31-67)	41 (30-60)	0.9 (0.6-1.3)	0.8 (0.6-1.2)
Total alcohol, g/day, median (IQR)	9.1 (1.4-24.2)	6.1 (0.9-16.3)	1.6 (0-8.0)	1.6 (0-10.7)	2.2 (0.4-8.3)	2.3 (0.4-9.0)
Total vitamin D, IU/day, median (IQR)	137 (93-199)	128 (84-188)	361 (177-565)	454 (174-575)	270 (178-413)	299 (199-457)
Total calcium, mg/day, median (IQR)	930 (716-1,219)	948 (724-1,206)	1011 (665-1,419)	1,067 (732-1,559)	853 (662-1,079)	896(717-1165)

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; CPS-II, Cancer Prevention Study-II; DBP, vitamin D-binding protein; EPIC, European Prospective Investigation into Cancer and Nutrition; IQR, interquartile range; MET, metabolic equivalents of task; NHS, Nurses' Health Study

[a] Percentages given for categorical variables; may not sum to 100 due to rounding. Mean and SD given for normally distributed continuous variables; median and IQR given for non-normally distributed continuous variables.

[b] 25(OH)D blood concentrations in EPIC and NHS were calibrated to the assay used for the CPS-II cohort; all 25(OH)D blood concentrations were seasonally adjusted.

[c] MET-hours/week calculated from self-reported combined recreational and household activity in the EPIC study, and leisure time recreational physical activity in the CPS-II and NHS studies.

[d] Includes nurses who checked RN as highest completed degree.

[e] Among women.

[f] Presented in servings per day for the NHS Cohort.

Table 2. Study-specific and summary associations of vitamin D-binding protein isoforms with vitamin D non-sufficiency[a] in the EPIC, CPS-II, and NHS cohorts

Study DBP isoform (rs4588 genotype)	<50 nmol/L (Non-sufficient)	≥50 nmol/L (Sufficient)	<50 vs. ≥50 nmol/L OR (95% CI)[b]	P_{trend}
EPIC				
DBP1-1 (CC)	674	254	1.00 (Ref)	
DBP1-2 (CA)	560	173	1.41 (1.11 to 1.78)	
DBP2-2 (AA)	138	26	2.59 (1.63 to 4.42)	
Per DBP2 isoform (per A allele)	1,372	453	1.52 (1.26 to 1.82)	8.7×10^{-6}
CPS-II				
DBP1-1 (CC)	78	180	1.00 (Ref)	
DBP1-2 (CA)	53	120	1.03 (0.67 to 1.57)	
DBP2-2 (AA)	12	20	1.47 (0.68 to 3.18)	
Per DBP2 isoform (per A allele)	143	320	1.13 (0.82 to 1.55)	0.42
NHS				
DBP1-1 (CC)	149	421	1.00 (Ref)	
DBP1-2 (CA)	120	282	1.20 (0.91 to 1.60)	
DBP2-2 (AA)	47	52	2.55 (1.65 to 3.95)	
Per DBP2 isoform (per A allele)	316	755	1.46 (1.20 to 1.77)	0.0002
All studies[c]				
DBP1-1 (CC)	901	855	1.00 (Ref)	
DBP1-2 (CA)	733	575	1.27 (1.08 to 1.50)	
DBP2-2 (AA)	197	98	2.36 (1.74 to 3.19)	
Per DBP2 isoform (per A allele)	912	2,447	1.43 (1.27 to 1.62)	1.2×10^{-8}

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; CI, confidence interval; CPS-II, Cancer Prevention Study-II; DBP, vitamin D-binding protein; EPIC, European Prospective Investigation into Cancer and Nutrition; NHS, Nurses' Health Study; OR, odds ratio

[a] According to 2011 Institute of Medicine recommendations based on circulating 25(OH)D concentrations. 25(OH)D blood concentrations calibrated to the same assay and seasonally-adjusted using the method described by Gail *et al.* (PMCID: PMC4853926).

[b] OR and 95% CI estimated in logistic regression models adjusted for age (continuous), sex, study center (for EPIC models), and case-control status.

[c] ORs and 95% CIs estimated in fixed effects meta-analyses (I^2 = 0.0 to 22.1; $P_{heterogeneity\ by\ study} > 0.25$ for all summary estimates).

Table 3. Summary incidence rate ratios (RR) of colorectal cancer according to vitamin D status and functional vitamin D-binding protein (DBP) isoforms in the EPIC, CPS-II, and NHS cohorts

	DBP1-1 (rs4588 CC)[b]				DBP1-2 or DBP2-2 (rs4588 CA or AA)[c]				
25(OH)D concentration (IOM-defined vitamin D status)[a]	**No. cases**	**No. controls**	**RR (95% CI)[d]**	**I^2**	**No. cases**	**No. controls**	**RR (95% CI)[d]**	**I^2**	***$P_{heterogeneity\ by\ DBP2}$***
<30 nmol/L (Deficient)*	144	104	1.00 (ref)		218	107	1.00 (ref)		
30-<50 nmol/L (Insufficient)	386	267	1.20 (0.86 to 1.67)	0.0	320	285	0.69 (0.51 to 0.95)	0.0	0.02
50-<75 nmol/L (Sufficient)	266	288	0.92 (0.63 to 1.34)	0.0	191	266	0.44 (0.27 to 0.73)	0.0	0.02
≥ 75 nmol/L (Beyond sufficient)	105	196	0.66 (0.37 to 1.16)	20.1	80	136	0.40 (0.23 to 0.68)	0.0	0.21
P_{trend}			0.01				5.8×10^{-5}		
<30 nmol/L (Deficient)	144	104	1.00 (ref)		218	107	1.00 (ref)		
30-<50 nmol/L (Insufficient)	386	267	1.19 (0.85 to 1.66)	0.0	320	285	0.69 (0.50 to 0.94)	0.0	0.02
≥ 50 nmol/L (Sufficient)	371	484	0.88 (0.61 to 1.27)	0.0	271	402	0.47 (0.33 to 0.67)	0.0	0.01
P_{trend}			0.09				0.0001		
< 50 nmol/L (Non-sufficient)	530	371	1.00 (ref)		538	392	1.00 (ref)		
≥ 50 nmol/L (Sufficient)	371	484	0.79 (0.63 to 1.00)	0.0	271	402	0.60 (0.47 to 0.76)	0.0	0.10

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; CI, confidence interval; CPS-II, Cancer Prevention Study-II; DBP, vitamin D-binding protein; EPIC, European Prospective Investigation into Cancer and Nutrition; IOM, Institute of Medicine; NHS, Nurses' Health Study; RR, incidence rate ratio

[a] 25(OH)D blood concentrations calibrated to the same assay and seasonally-adjusted using the method described by Gail *et al.* (PMCID: PMC4853926).

[b] Participants with no minor allele at *GC*-rs4588 (rs4588*CC genotype) were defined as not having the DBP2 isoform (or only DBP1 isoforms).

[c] Participants with minor allele at *GC*-rs4588 (rs4588*CA or rs4588*AA genotypes) were defined as having the DBP2 isoform.

Table 4. Meta-analytic associations of the joint vitamin D status and DBP isoform variable with colorectal cancer risk in case-control studies nested in the EPIC, CPS-II, and NHS cohorts.

Joint Variable	No. cases	No. controls	RR (95% CI)[a]	RERI[b]
≥50 nmol/L (Sufficient) + no DBP2	371	484	1.00 (Ref)	
≥50 nmol/L (Sufficient) + DBP2	271	402	0.95 (0.71 to 1.29)	
<30 nmol/L (Deficient) + no DBP2	530	371	1.01 (0.69 to 1.51)	
<30 nmol/L (Deficient) + DBP2	538	392	1.68 (1.15 to 2.45)	0.72

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; CI, confidence interval; CPS-II, Cancer Prevention Study-II; DBP, vitamin D-binding protein; EPIC, European Prospective Investigation into Cancer and Nutrition; NHS, Nurses Health Study; RR, relative risk

[a] Meta-RRs and 95% CIs estimated in fixed-effect meta-analyses combining study-specific RRs and 95% CIs that were estimated in unconditional logistic regression models adjusted for study-specific matching factors, BMI (continuous, kg/m^2), and physical activity (combined recreational and household activity MET-hours/week, quartiles).

[b] Relative excess risk due to interaction (RERI) calculated as: $1.68 – 1.01 – 0.95 + 1$ ($RR_{11} – RR_{10} – RR_{01} + RR_{00}$).

SUPPLEMENTARY TABLES AND FIGURES

Supplementary Table 1. Distribution of tumor characteristics for colorectal cancer cases in the EPIC, CPS-II and NHS cohort studies

	Study		
Tumor characteristic	**EPIC (n=1,106)**	**CPS-II (n=246)**	**NHS (n=358)**
Site[a]			
Distal colon	336 (30)	63 (26)	106 (27)
Proximal colon	289 (26)	146 (59)	191 (49)
Rectum	410 (37)	26 (11)	78 (20)
Missing/not specified	71 (6)	11 (4)	16 (4)
Stage			
I	274 (25)	105 (43)	85 (22)
II	216 (20)	45 (18)	113 (29)
III	334 (30)	54 (22)	85 (22)
IV	109 (10)	27 (11)	57 (14)
Missing/not specified	173 (15)	15 (6)	51 (13)

[a]Distal colon includes malignant neoplasms of the cecum, appendix, ascending colon, hepatic flexure and transverse colon (ICD-10 codes 18.0-18.4); proximal colon cancer includes malignant neoplasms of the splenic flexure, descending colon and sigmoid colon; missing/not-specific includes participants with ICD-10 codes indicating unspecific or overlapping colorectal neoplasm locations (18.8-18.9) or that were missing ICD-10 code information.

Supplementary Table 2. Associations of vitamin D binding protein (DBP) isoforms with circulating seasonally-adjusted 25(OH)D concentrations (nmol/L) in the EPIC, CPS-II, and NHS cohorts

	EPIC					CPS-II					NHS				
DBP isoforms (rs4588 genotype)_	**No.**	**Estimated mean[a]**	**95% CI**		***P***	**No.**	**Estimated mean[a]**	**95% CI**		***P***	**No.**	**Estimated mean[a]**	**95% CI**		***P***
DBP1-1 (CC)	928	43.1	41.9	44.2	Ref	258	62.9	60.1	65.7	Ref	570	69.2	67.0	71.4	Ref
DBP1-2 (CA)	733	40.8	39.5	42.0	0.002	173	61.5	58.1	65.0	0.54	402	55.5	50.2	60.7	<0.001
DBP2-2 (AA)	164	37.5	35.1	39.9	<0.001	32	64.3	56.5	72.2	0.75	99	63.6	61.0	66.2	0.001

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; CI, confidence interval; CPS-II, Cancer Prevention Study-II; DBP, vitamin D-binding protein; EPIC, European Prospective Investigation into Cancer and Nutrition; NHS, Nurses' Health Study

[a] Least squares means estimated in linear regression models adjusted for age (continuous), sex, study center (for EPIC models) and case-control status.

Supplementary Table 3. Study-specific incidence rate ratio (RR) for colorectal cancer according to vitamin D status and functional vitamin D-binding protein (DBP) isoforms in the EPIC, CPS-II, and NHS cohorts

Study	DBP1-1 (rs4588 CC)[b]					DBP1-2 or DBP2-2 (rs4588 CA or AA)[c]				
25(OH)D concentration (IOM-defined vitamin D status)	No. cases	No. controls	RR[d]	95% CI		No. cases	No. controls	RR[d]	95% CI	
EPIC										
<30 nmol/L (Deficient)	126	84	1.00 (ref)			193	86	1.00 (ref)		
30-<50 nmol/L (Insufficient)	295	169	1.28	0.88	1.85	247	172	0.75	0.53	1.08
50-<75 (Sufficient)	125	95	0.99	0.64	1.53	81	83	0.49	0.31	0.77
≥ 75 nmol/L (Beyond sufficient)	23	11	1.13	0.47	2.75	16	19	0.42	0.19	0.93
<30 nmol/L (Deficient)	126	84	1.00 (ref)			193	86	1.00 (ref)		
30-<50 nmol/L (Insufficient)	295	169	1.28	0.88	1.85	247	172	0.75	0.53	1.08
≥ 50 nmol/L (Sufficient)	148	106	1.01	0.66	1.54	97	102	0.48	0.31	0.73
< 50 nmol/L (Non-sufficient)	421	253	1.00 (ref)			440	258	1.00 (ref)		
≥ 50 nmol/L (Sufficient)	148	106	0.89	0.63	1.20	97	102	0.54	0.40	0.77
CPS-II										
<30 nmol/L (Deficient)	7	4	1.00 (ref)			10	4	1.00 (ref)		
30-<50 nmol/L (Insufficient)	38	29	0.80	0.20	3.24	26	25	0.39	0.10	1.44
50-<75 (Sufficient)	59	58	0.59	0.15	2.24	53	34	0.58	0.16	2.05
≥ 75 nmol/L (Beyond sufficient)	28	35	0.49	0.12	1.97	25	28	0.31	0.08	1.18
<30 nmol/L (Deficient)	7	4	1.00 (ref)			10	4	1.00 (ref)		
30-<50 nmol/L (Insufficient)	38	29	0.80	0.20	3.24	26	25	0.40	0.11	1.47
≥ 50 nmol/L (Sufficient)	87	93	0.55	0.15	2.08	78	62	0.47	0.14	1.63
< 50 nmol/L (Non-sufficient)	45	33	1.00 (ref)			36	29	1.00 (ref)		
≥ 50 nmol/L (Sufficient)	87	93	0.78	0.41	1.34	78	62	0.59	0.30	1.14
NHS										
<30 nmol/L (Deficient)	11	16	1.00 (ref)			15	17	1.00 (ref)		

30-<50 nmol/L (Insufficient)	53	69	0.95	0.39	2.33	47	88	0.57	0.25	1.30
50-<75 (Sufficient)	82	135	0.83	0.35	1.99	57	149	0.43	0.19	0.97
≥ 75 nmol/L (Beyond sufficient)	54	150	0.43	0.18	1.05	39	89	0.42	0.18	1.00
<30 nmol/L (Deficient)	11	16	1.00 (ref)			15	17	1.00 (ref)		
30-<50 nmol/L (Insufficient)	53	69	0.93	0.38	2.28	47	88	0.53	0.23	1.22
≥ 50 nmol/L (Sufficient)	136	285	0.62	0.27	1.44	96	238	0.42	0.19	0.93
< 50 nmol/L (Non-sufficient)	64	85	1.00 (ref)			62	105	1.00 (ref)		
≥ 50 nmol/L (Sufficient)	136	285	0.66	0.44	0.99	96	238	0.71	0.47	1.06

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; CI, confidence interval; CPS-II, Cancer Prevention Study-II; DBP, vitamin D-binding protein; EPIC, European Prospective Investigation into Cancer and Nutrition; IOM, Institute of Medicine; NHS, Nurses' Health Study; RR, incidence rate ratio

[a] 25(OH)D blood concentrations seasonally-adjusted using the method described by Gail *et al.* (PMCID: PMC4853926).

[b] Participants with no minor allele at *GC*-rs4588 (rs4588*CC genotype) were defined as not having the DBP2 isoform (or only DBP1 isoforms)

[c] Participants with minor allele at *GC*-rs4588 (rs4588*CA or rs4588*AA genotypes) were defined as having the DBP2 isoform.

[d] Study-specific RRs and 95% CIs estimated in unconditional logistic regression models adjusted for study-specific matching factors, BMI (continuous, kg/m2), and physical activity (combined recreational and household activity MET-hours/week, quartiles).

Supplementary Table 4. Study heterogeneity for the meta-analytic incidence rate ratios (RR) of colorectal cancer according to vitamin D status and functional vitamin D-binding protein (DBP) isoforms in the EPIC, CPS-II, and NHS cohorts

25(OH)D concentration (IOM-defined vitamin D status)[a]	DBP1-1 (rs4588 CC)[b]					DBP1-2 or DBP2-2 (rs4588 CA or AA)[c]				
	No. cases	No. controls	RR (95% CI)[d]	I^2	$P_{heterogeneity\ by\ study}$	No. cases	No. controls	RR (95% CI)[d]	I^2	$P_{heterogeneity\ by\ study}$
<30 nmol/L (Deficient)	144	104	1.00 (ref)			218	107	1.00 (ref)		
30-<50 nmol/L (Insufficient)	386	267	1.20 (0.86 to 1.67)	0.0	0.70	320	285	0.69 (0.51 to 0.95)	0.0	0.57
50-<75 nmol/L (Sufficient)	266	288	0.92 (0.63 to 1.34)	0.0	0.75	191	266	0.44 (0.27 to 0.73)	0.0	0.91
≥75 nmol/L (Beyond sufficient)	105	196	0.66 (0.37 to 1.16)	20.1	0.29	80	136	0.40 (0.23 to 0.68)	0.0	0.92
<30 nmol/L (Deficient)	144	104	1.00 (ref)			218	107	1.00 (ref)		
30-<50 nmol/L (Insufficient)	386	267	1.19 (0.85 to 1.66)	0.0	0.69	320	285	0.69 (0.50 to 0.94)	0.0	0.53
≥50 nmol/L (Sufficient)	371	484	0.88 (0.61 to 1.27)	0.0	0.45	271	402	0.47 (0.33 to 0.67)	0.0	0.96
<50 nmol/L (Non-sufficient)	530	371	1.00 (ref)			538	392	1.00 (ref)		
≥50 nmol/L (Sufficient)	371	484	0.79 (0.63 to 1.00)	0.0	0.53	271	402	0.60 (0.47 to 0.76)	0.0	0.59

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; CI, confidence interval; CPS-II, Cancer Prevention Study-II; DBP, vitamin D-binding protein; EPIC, European Prospective Investigation into Cancer and Nutrition; IOM, Institute of Medicine; NHS, Nurses' Health Study; RR, incidence rate ratio

[a] 25(OH)D blood concentrations calibrated to the same assay and seasonally-adjusted using the method described by Gail *et al.* (PMCID: PMC4853926).

[b] Participants with no minor allele at *GC*-rs4588 (rs4588*CC genotype) were defined as not having the DBP2 isoform (or only DBP1 isoforms).

[c] Participants with minor allele at *GC*-rs4588 (rs4588*CA or rs4588*AA genotypes) were defined as having the DBP2 isoform.

[d] Meta-RRs and 95% CIs estimated in fixed-effect meta-analyses combining study-specific RRs and 95% CIs that were estimated in unconditional logistic regression models adjusted for study-specific matching factors, BMI (continuous, kg/m^2), and physical activity (combined recreational and household activity MET-hours/week, quartiles).

CHAPTER V

Association of pre-diagnostic vitamin D status with mortality among colorectal cancer patients differs by common, inherited vitamin D-binding protein isoforms

Manuscript Information

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ABSTRACT

Lower pre-diagnostic circulating 25-hydroxyvitamin D [25(OH)D] is associated with higher mortality risk among colorectal cancer (CRC) patients. However, it is unknown whether this association differs by the vitamin D-binding protein (DBP)-2 isoform (encoded by *GC* rs4588*A, Thr→Lys), which may substantially affect vitamin D metabolism and modify associations of 25(OH)D with colorectal neoplasm risk. Associations of pre-diagnostic 25(OH)D with mortality according to DBP2 isoform were estimated using multivariable Cox proportional hazards regression among 1,281 CRC cases (635 deaths, 483 from CRC) from two large prospective cohorts conducted in the US and Europe. 25(OH)D measurements were calibrated to a single assay, season standardized, and categorized using Institute of Medicine recommendations [deficient (<30), insufficient (30 – <50), sufficient (≥50 nmol/L)]. In the pooled analysis, multivariable-adjusted hazard ratios (HRs) for CRC-specific mortality associated with deficient relative to sufficient 25(OH)D concentrations were 2.24 (95% CI, 1.44 to 3.49) among cases with the DBP2 isoform, and 0.94 (95% CI, 0.68 to 1.22) among cases without DBP2 ($P_{interaction}$ = 0.0002). The corresponding HRs for all-cause mortality were 1.80 (95% CI, 1.24 to 2.60) among those with DBP2, and 1.12 (95% CI, 0.84 to 1.51) among those without DBP2 ($P_{interaction}$ = 0.004). Our findings suggest that pre-diagnostic vitamin D deficiency relative to sufficiency may be associated with higher mortality risk among CRC patients, but only among those with the common DBP2-encoding *GC*-rs4588*A functional polymorphism. The potential clinical utility of 25(OH)D concentration as a prognostic factor for CRC patients may depend on DBP isoform.

INTRODUCTION

Colorectal cancer (CRC) is the second leading cause of cancer death among men and women globally (9). Vitamin D regulates several important signaling pathways relevant to cancer progression and prognosis, including proliferation, differentiation, angiogenesis, apoptosis, inflammation, and metastasis (2). Circulating 25-hydroxyvitamin D concentrations [collective term for D_2 and D_3, 25(OH)D]—used clinically to assess vitamin D status—is associated with mortality risk among CRC patients in observational studies (89-91, 207); however, vitamin D status and bioavailability may be impacted by functional variants in the vitamin D-binding protein (DBP) gene, *GC* (126).

Nearly 90% of circulating 25(OH)D is bound to the DBP, which delivers vitamin D to target tissues and helps maintain stable 25(OH)D stores (105, 141, 195, 208). Two missense variants—*GC*-rs4588 and rs7041—encode for three common DBP isoforms: DBP1s, DBP1f, and DBP2, also known as Gc1s, Gc1f, and Gc2 (120, 127, 184). We recently reported that associations of 25(OH)D concentrations with incident, sporadic colorectal adenoma (118) and CRC (172) were stronger among individuals with the DBP2 isoform. Relative to the DBP1 isoforms, DBP2 is associated with an approximately 2- to 4-fold lower 25(OH)D binding affinity (135) and 2- to 3-fold higher vitamin D-pathway induction by 25(OH)D *in vitro* (139), providing biologic plausibility for these clinically relevant genotype-specific associations.

Accordingly, we hypothesized that the association of pre-diagnostic 25(OH)D concentrations with mortality risk among CRC patients would be stronger among individuals with the DBP2 isoform than among those without it. We investigated whether associations of 25(OH)D with CRC-specific and all-cause mortality differed by DBP2 isoform among 1,281 CRC patients in two large prospective cohort studies in the United States and Europe.

METHODS

Study population

We analyzed individual patient data from the European Prospective Investigation into Cancer and Nutrition (EPIC) and the Cancer Prevention Study-II Nutrition (CPS-II) prospective cohort studies. Details of the study populations and data collection were published previously for EPIC (158) and CPS-II (156). Briefly, EPIC recruited over 520,000 men and women from the general population in 10 western European countries from 1992 to 1998 (189), and CPS-II recruited 184,194 men and women across 21 US states from 1992 to 1993 (156). Blood samples were collected prior to cancer diagnosis from EPIC participants between 1992 and 1998, and from CPS-II participants between 1998 and 2001. Pre-diagnostic circulating 25(OH)D concentrations were measured for 1,248 and 298 incident CRC cases for previous case-control studies with 1:1 matching nested in EPIC (189) and CPS-II (24), respectively. Detailed descriptions of case selection and exclusions for these studies are described elsewhere (24, 89, 189). Of these 1,546 CRC cases, we further excluded 142 EPIC cases and 44 CPS-II cases with missing *GC* genotyping information, 7 non-white CPS-II cases, 25 EPIC cases with missing cause of death information, and 38 EPIC cases and 9 CPS-II cases with missing follow-up or vital status information, leaving 1,281 CRC cases for these analyses. The EPIC and CPS-II studies were approved by their respective institutional review boards, and written informed consent was obtained from each subject.

Follow-up

Follow-up for CRC incidence occurred during 1993–2004 in EPIC (89, 189) and 1999–2007 in CPS-II (209). In EPIC, vital status and cancer incidence information was collected via linkage to

regional and/or national mortality registries in all countries except France, Germany, and Greece, where participants were followed using a combination of cancer/pathology registries, health insurance records, and active follow-up, as described previously (89). Censoring dates for complete follow-up in EPIC occurred in 2012 (Netherlands, Greece), 2013 (France, Italy, Spain, UK, Denmark), and 2014 (Germany, Sweden). In CPS-II, CRC cases were followed through 2014, and vital status and cause of death information were collected via linkage to the National Death Index (209). CRC-attributable deaths were determined using the International Classification of Diseases for Oncology (ICD-O) 10th revision codes C18.0-18.8 and C19 for colon cancer (including C18.1 for appendix cancer), C20 for rectal cancer, and C18.8-18.9 for overlapping/unspecified colorectal origin.

25(OH)D Measurements

Total serum 25(OH)D (D_2 and D_3) was measured using the FDA-approved DiaSorin Liaison chemiluminescence immunoassay (CLIA) in CPS-II (24) (Heartland Assays, Ames, IA), and the OCTEIA enzyme immunoassay (Immuno Diagnostic Systems, Boldon, UK) in EPIC (189). Inter-assay coefficients of variation were 5.2% in CPS-II and 5.7% in EPIC. EPIC 25(OH)D measurements were calibrated to the same assay used in CPS-II using a robust linear regression calculated by re-measuring a subset of 40 EPIC samples within each 25(OH)D decile using the DiaSorin CLIA, described previously (190). Each assay batch included National Institute of Standards and Technology (NIST) standard reference materials, for which the coefficients of variation were 16%, 9%, and 9% at 17.7, 32.3, and 49.8 nmol/L, respectively.

Genotyping

Genotyping was performed using a custom GoldenGate Universal-plex assay kit (Illumina, CA, USA) in EPIC, and a custom Affymetrix genome-wide platform, the Axiom Correct Set (Affymetrix, CA, USA), in CPS-II. Quality control measures for CPS-II (192) and EPIC (210) were reported previously. Individuals with the *GC*-rs4588 CC, CA, and AA genotypes were classified as having DBP1-1, DBP1-2, and DBP2-2 isoform combinations, respectively (184). These genotypes perfectly predict the expected amino acid changes of the circulating protein isoforms as determined in previous proteomic analyses (131). In EPIC, *GC*-rs3755967 was used as a proxy for rs4588 since these SNPs are in complete linkage disequilibrium (r^2=1.0) in the HapMap Spanish and British Western European populations similar to EPIC's (LDproxy, 1000 Genomes Project Phase 3). *GC*-rs3755967 and rs4588 were in Hardy-Weinberg equilibrium in both studies.

Statistical Analyses

To seasonally-adjust 25(OH)D measurements, calibrated (EPIC) or newly measured (CPS-II) 25(OH)D values were regressed on week of blood draw using a cos/sin function, and residuals from the model were added to the study- and sex-specific mean among cases (details in references: (24, 190)). The adjusted value may be interpreted as the predicted 25(OH)D concentration for a participant averaged over the entire year, accounting for study- and sex-specific 25(OH)D seasonal variation.

CRC-specific mortality was the primary endpoint, and all-cause mortality the secondary endpoint. Our primary exposure was circulating 25(OH)D categorized *a priori* according to Institute of Medicine (IOM, now the National Academy of Medicine) vitamin D status clinical guidelines for skeletal health: <30 nmol/L (deficient), 30 – <50 nmol/L (insufficient), and ≥50

nmol/L (sufficient). For our primary analysis, effect modification by DBP2 was evaluated using a dominant inheritance model, given the low frequency of DBP2-2 homozygotes. As a secondary analysis, we coded DBP2 using a co-dominant inheritance model as we would expect the 25(OH)D-CRC survival association to be stronger with an increasing number of DBP2-encoding alleles; here, 25(OH)D was dichotomized at 50 nmol/L (IOM cut-point for sufficiency) to maximize statistical power.

Cox proportional hazards models, stratified by country of cancer diagnosis, were used to calculate hazard ratios (HRs) and 95% confidence intervals (CIs) for CRC-specific and all-cause mortality according to 25(OH)D concentrations and DBP2 isoform. Age between diagnosis and censorship or death was used as the time-scale, which may better control for age and reduce bias (211). Covariates included year of diagnosis (continuous), sex, tumor site (colon, rectum, missing/not specified), BMI (continuous), physical activity (quartiles 1 – 4, missing), smoking status (never, former, current, missing), and stage (I – IV, missing/not specified). Potential covariates were selected based on biological plausibility, causal structure, and previous literature; of those selected, education, dietary calcium, and alcohol consumption were not included in final models because they did not materially affect the estimated HRs. The proportional hazards assumption was evaluated by including a time-dependent covariate in the Cox model and by assessing the correlation between the Schoenfeld residuals and survival time (212). Estimates were calculated in each study separately and in a pooled analysis using aggregated data. Results presented hereafter are based on the pooled analysis unless otherwise stated. Multiplicative interaction between 25(OH)D and the DBP2 isoform was evaluated by comparing the pooled, adjusted Cox models with and without an interaction term using a likelihood ratio test.

To assess whether competing causes of death may have influenced the observed associations, adjusted cumulative incidence curves for CRC-specific mortality risk were estimated according to 25(OH)D and DBP2 isoform using Fine and Gray's competing-risks regression (213).

To assess interaction on the additive scale, we estimated the associations of a joint variable combining 25(OH)D concentrations and the DBP2 isoform (i.e., cross-classification analysis) with CRC-specific mortality to calculate the relative excess risk due to interaction (RERI) (194) calculated as: $RERI = HR_{11} - HR_{10} - HR_{01} + 1$, where HR_{ij} is the hazard ratio associated with the joint variable that combines 25(OH)D (*i*, coded 0 for sufficient, and 1 for deficient) and DBP isoform (*j,* coded 0 for no DBP2 isoform, and 1 for the DBP2 isoform).

All statistical tests were two-sided; a *P*-value <0.05 or a 95% confidence interval (CI) that excluded 1.0 was considered statistically significant. Analyses were performed in SAS version 9.4 (Cary, NC).

RESULTS

Study Population and Follow-Up

During follow-up, of the 1,281 CRC cases, 635 died, including 483 from CRC. Mean follow-up duration was 8.3 years in EPIC and 7.3 years in CPS-II. Characteristics of CRC cases according to IOM-defined vitamin D status categories are summarized in **Table 1**.

25(OH)D and Mortality According to DBP2

Associations of 25(OH)D concentrations with mortality among all participants and according to DBP2 isoform, assuming a dominant inheritance model, are summarized in **Table 2**. Relative to

those with 25(OH)D concentrations considered sufficient by the IOM (≥50 nmol/L), CRC-specific mortality risk for those with concentrations considered deficient (<30 nmol/L) was statistically significantly 33% higher among all cases, 124% higher among cases with DBP2, and non-statistically significantly 6% lower among cases without DBP2 ($P_{interaction}$ = 0.0002). There was a dose-response association trend between lower (poorer) vitamin D status and higher mortality risk among those with DBP2 (P_{trend} = <0.0001and 0.0002 for CRC-specific and overall mortality, respectively), but not among those without DBP2 (P_{trend} = 0.69 and 0.49 for CRC-specific and overall mortality, respectively). This pattern of effect modification by DBP2 was similar in both EPIC and CPS-II (Supplementary Table 1).

Associations of 25(OH)D concentrations with CRC-specific and all-cause mortality among all participants and according to DBP2 isoform, assuming a co-dominant inheritance model, are summarized in **Table 3.** Relative to those with 25(OH)D concentrations considered sufficient, CRC-specific mortality risk for those with non-sufficient concentrations (<50 nmol/L) was close to the null among DBP1-1 cases, statistically significantly 54% higher among DBP1-2 cases, and non-statistically significantly 150% higher among DBP2-2 cases ($P_{interaction}$ = 0.003). Estimated all-cause mortality risk for those with non-sufficient relative to sufficient 25(OH)D concentrations varied from 6% to 33% higher among DBP1-1, DBP1-2, and DBP2-2 cases, but did not statistically significantly differ by DBP2 ($P_{interaction}$ = 0.09). The pattern of effect modification by number of DBP2-encoding alleles for CRC-specific mortality was similar in EPIC and CPS-II (Supplementary Table 2).

Competing Risks Regression and Cumulative Incidence Curves

Using multivariable-adjusted competing-risks regression, we observed a dose-response association of lower 25(OH)D concentrations with higher CRC-specific mortality among those with the DBP2 isoform, but not among those without DBP2 (**Figure 1**). Among individuals with DBP2, the estimated risk dying from CRC within 5 years of diagnosis was approximately 15% if vitamin D sufficient, 20% if vitamin D insufficient, and 30% if vitamin D deficient prior to diagnosis, controlling for all other covariates and competing causes of death.

Additive Interaction

In joint/combined analysis (**Table 4**), among those who were vitamin D sufficient, the DBP2 isoform, relative to only DBP1 isoforms, was associated with a statistically significant 43% lower risk of CRC-specific mortality. Additionally, among those with only DBP1 isoforms, vitamin D deficiency relative to sufficiency was associated with a non-statistically significant 13% lower risk of CRC-specific mortality. However, those with the DBP2 isoform *and* who were vitamin D deficient (relative to those with only DBP1 isoforms and who were vitamin D sufficient) had a non-statistically significant 11% higher risk of CRC-specific mortality, which was 71% higher than expected (RERI = 0.71) indicating a greater than additive interaction (RERI > 0).

Subgroup and Sensitivity Analyses

The association of 25(OH)D concentrations <50 relative to ≥50 nmol/L with CRC-specific mortality among individuals with and without the DBP2 isoform did not statistically significantly differ according to sex, stage, tumor site, or calcium intake; however, the observed effect-modification pattern by DBP2 was slightly more pronounced among rectal cancer cases, stage I-

II cases, and individuals with above mean dietary calcium intakes (Supplementary Table 3). In sensitivity analyses, our effect-modification findings were slightly stronger when we excluded metastatic CRC cases (Supplementary Table 4) or cases diagnosed within 1 or 3 years of their pre-diagnostic blood draw (Supplementary Table 5). There was also a similar pattern of effect modification by DBP2 when we categorized 25(OH)D using study-specific 25(OH)D tertile cut-points (Supplementary Table 6), further supporting the robustness of our findings.

DISCUSSION

Our findings suggest that pre-diagnostic vitamin D deficiency relative to sufficiency, based on IOM recommendations, may be associated with higher mortality risk among CRC patients, particularly those with the functional, inherited DBP2 isoform. This association was stronger for CRC-specific mortality, which may have been due to non-vitamin D-related deaths in the all-cause mortality group. To our knowledge, this is the first study to investigate 25(OH)D-mortality associations among CRC patients, by DBP isoform.

Strong evidence from observational studies supports an association of circulating 25(OH)D concentrations—including those measured before (89, 90) and after diagnosis (91, 92)—with CRC-specific mortality. Findings from some studies also suggest that 25(OH)D may be a clinically useful prognostic factor and add value to predictive survival models for CRC patients (91, 93); however, our findings suggest that this utility may depend on DBP isoform. If our findings are confirmed, they would support DBP genotyping, which could be easily and affordably obtained in clinical settings, for guiding vitamin D-related therapy and survival stratification.

To our knowledge, only one randomized controlled trial (RCT) of vitamin D

supplementation among CRC patients has been reported, but the results were promising. In a phase-II, multi-US-center RCT with 139 patients with advanced or metastatic CRC, those randomized to high-dose (4,000 IU/day) relative to low-dose (400 IU/day) vitamin D supplementation had longer progression-free survival (HR=0.64; 1-sided 95% CI: 0 to 0.90; *P*=0.02), which was the primary outcome (99). Importantly, findings from a larger RCT (n=2,259) suggest that the effects of vitamin D supplementation on increasing 25(OH)D concentrations (101) and reducing colorectal adenoma risk (100) may be stronger among individuals with the DBP2-encoding variant. The effect of vitamin D supplementation on adenoma risk was statistically significantly 18% lower with each DBP2-encoding-rs4588 variant inherited (interaction relative risk = 0.82; 95% CI: 0.69 to 0.98; $P_{interaction}$ = 0.03) (100). Those findings are consistent with ours, and collectively suggest that future trials should consider potential differences in supplementation effects depending on DBP2 isoform.

The DBP2 isoform is determined by the missense *GC*-rs4588 polymorphism encoding a Threonine (Thr) to Lysine (Lys) amino acid substitution at position 436 (105, 133). The DBP2-encoding variant is strongly associated with lower 25(OH)D concentrations and vitamin D insufficiency (124-126). This may be due to differences in DBP concentration [20 – 30% lower among DBP2-2 relative to DBP1-1 (127, 128, 130, 131)] since DBP mediates renal reabsorption of 25(OH)D (109). Putative isoform differences in DBP concentration and/or 25(OH)D binding affinity (135) may also increase relative free 25(OH)D concentrations and explain higher induction of vitamin D-target genes by 25(OH)D in monocytes and colon cancer cell lines cultured with DBP2 relative to DBP1 isoforms (119, 139). Activation of the vitamin D receptor, ubiquitously expressed in colonic epithelia, regulates over 200 genes, including those involved in cell proliferation, differentiation, apoptosis, angiogenesis, and metastasis (2, 214). Taken

together, we hypothesize that 25(OH)D may be more strongly associated with CRC mortality among patients with DBP2, as they may have increased vitamin D-pathway activation as 25(OH)D increases, and a reduced capacity for maintaining stable 25(OH)D stores.

Supporting this hypothesis are other studies that reported a similar pattern of effect modification for colorectal adenoma and CRC risk. In a pooled US case-control study of individuals of European ancestry, 25(OH)D concentrations ≥50 relative to <50 nmol/L were associated with a statistically significantly lower risk of incident, sporadic colorectal adenoma, but only among those with the DBP2-isoform genotype (OR among DBP2 = 0.51, 95% CI: 0.33 to 0.81; OR among non-DBP2 = 1.11, 95% CI: 0.68 to 1.82) ($P_{interaction}$ = 0.05) (118). Additionally, in a large pooled nested case-control study using EPIC, CPS-II, and Nurses' Health Study data (n=2), 25(OH)D concentrations ≥50 relative to <30 nmol/L were associated with a significantly lower risk of colorectal cancer, but only among those with DBP2-isoform genotype (RR among DBP2 = 0.47, 95%: 0.33 to 0.67; RR among non-DBP2 = 0.88, 95% CI: 0.61 to 1.27) ($P_{interaction}$ = 0.01) (172).

Our study strengths include its prospective design, long follow-up, and use of data from two independently conducted cohort studies. Additional strengths include using seasonally-adjusted 25(OH)D concentrations (limiting exposure misclassification) and calibrating 25(OH)D measurements to the same widely-used assay allowing us to estimate hazards on the same absolute scale.

Our study has several limitations. The CPS-II sample size was small; however, the direction of the HRs within strata were consistent across studies, supporting the validity and reproducibility of our findings. Larger studies are needed to yield more precise estimates among individuals with the rare DBP2-2 genotype. There may have been some misclassification of

vitamin D status related to using the DiaSorin immunoassay; however, this assay is among the most commonly used clinically, and yields results highly concordant (r^2>0.95) with those from liquid chromatography-mass spectrometry (57). Thus, we would expect this misclassification to be small and comparable to that found in real-world clinical practice. Additionally, while 25(OH)D was measured only once prior to diagnosis, estimated within-person correlations for repeated 25(OH)D measures taken 1 to 11 years apart were 0.53 – 0.81 in other studies, suggesting that single 25(OH)D measurements may be a relatively valid marker of long-term vitamin D status (205, 206). Furthermore, using 25(OH)D measurements prior to diagnosis limits the concern for reverse causality (e.g., patients with aggressive tumors may be sicker and thus develop lower 25(OH)D concentrations near diagnosis), and our results were similar when we excluded patients diagnosed within 3 years of 25(OH)D measurement. We lacked data on CRC treatment, but adjusted for year of cancer diagnosis and stratified by country to account for potential temporal or country-specific treatment differences. 25(OH)D may be a marker of an overall healthier lifestyle that could influence survival; however, we adjusted for BMI, smoking, and physical activity, and further adjusting for factors, such as alcohol intake and education, did not materially affect our results. Adjusting for these potential shared risk factors for CRC risk and survival also reduces the possibility of a spurious association due to collider-stratification bias (215). Last, our findings among Europeans and US whites with European ancestry may not be generalizable to other races or populations.

In conclusion, our findings, together with previous literature, suggest that the association of pre-diagnostic 25(OH)D with mortality risk among CRC patients may differ by common, inherited genotypes encoding DBP isoforms, such that individuals with the functional DBP2 isoform may benefit most from a sufficient vitamin D status.

Table 1. Selected Characteristics of CRC Cases According to Pre-diagnostic Vitamin D Status* in the EPIC and CPS-II Cohorts (n = 1,281)

	EPIC (n = 1,043)			CPS-II (n = 238)			Pooled cohort (n = 1,281)			
	25(OH)D, nmol/L			25(OH)D, nmol/L			25(OH)D, nmol/L			
Characteristic	**< 30 (deficient) n = 331**	**30 – < 50 (insufficient) n = 520**	**≥ 50 (sufficient) n = 192**	**< 30 (deficient) n = 35**	**30 – < 50 (insufficient) n = 73**	**≥ 50 (sufficient) n = 130**	**< 30 (deficient) n = 366**	**30 – < 50 (insufficient) n = 593**	**≥ 50 (sufficient) n = 322**	***P*†**
Age at diagnosis, mean (SD), yrs.	62.5 (7.4)	62.0 (7.5)	62.0 (6.8)	74.2 (5.9)	75.2 (5.7)	74.5 (5.7)	63.7 (8.0)	63.6 (8.5)	67.2 (8.9)	<0.0001
Women, %	59	49	41	69	55	57	60	50	43	<0.0001
Stage, %										
I	23	29	20	40	45	45	25	31	30	
II	24	17	22	20	18	20	24	17	21	
III	31	32	32	31	25	19	31	31	27	
IV	10	10	11	9	8	14	9	10	12	0.11
Tumor location, %										
Left colon	36	35	27	40	25	24	36	34	27	
Right colon	35	31	30	46	63	65	36	35	44	
Rectum	24	28	33	14	10	11	23	26	24	0.08
Body-mass index, mean (SD), kg/m^2	27.0 (4.9)	26.8 (4.1)	26.0 (3.5)	29.0 (7.2)	26.8 (5.1)	25.5 (4.1)	27.2 (5.2)	26.8 (4.2)	25.8 (3.7)	<0.0001
Smoking status, %										
Never	44	42	35	40	42	49	44	41	41	
Former	23	37	43	57	45	42	27	38	43	
Current	31	22	22	3	4	3	28	20	15	<0.0001
Physical activity quartiles‡, %										
1	28	22	22	37	30	17	29	23	20	
2	20	25	24	26	26	25	21	25	24	
3	23	22	19	17	23	28	22	22	23	
4	26	25	28	20	19	29	25	24	28	0.05

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; CPS-II, Cancer Prevention Study-II; EPIC, European Prospective Investigation into Cancer and Nutrition; MET, metabolic equivalent; SD, standard deviation; yrs, years.

* According to Institute of Medicine 2011 recommendations based on 25(OH)D blood concentrations. Column percentages (i.e., within each vitamin D status category) are presented for categorical variables; percentages may not sum to 100 due to rounding and missing values.

† *P* value calculated among the pooled sample using one-way analysis of variance for continuous variables and the χ^2 test for categorical variables.

‡ Study-specific quartiles based on recreational metabolic-equivalent hours (MET-hours) per week.

Table 2. Multivariable-adjusted Associations of Pre-diagnostic Vitamin D Status* with CRC-specific and All-cause Mortality Among All CRC Cases and According to Vitamin D-binding Protein (DBP) Isoform, Assuming a Dominant Inheritance Model, in the EPIC and CPS-II Cohorts Combined (n = 1,281)

	Circulating 25(OH)D concentrations										
	≥ 50 nmol/L (sufficient)			30 – <50 nmol/L (insufficient)			< 30 nmol/L (deficient)				
Outcome and DBP strata	**No. total**	**No. died**	**HR (95% CI)†**	**No. total**	**No. died**	**HR (95% CI)†**	**No. total**	**No. died**	**HR (95% CI)†**	***P*$_{trend}$‡**	***P*$_{interaction}$§**
CRC-specific mortality											
All CRC cases	322	106	1.00 (Ref)	593	241	1.09 (0.83 to 1.43)	366	136	1.33 (1.03 to 1.72)	0.02	
No DBP2 (*GC* rs4588*CC)	187	72	1.00 (Ref)	309	114	1.11 (0.78 to 1.57)	164	70	0.94 (0.68 to 1.22)	0.69	
DBP2 (*GC* rs4588*CA or AA)	135	34	1.00 (Ref)	284	127	1.29 (0.81 to 2.06)	202	66	2.24 (1.44 to 3.49)	<0.0001	0.0002
All-cause mortality											
All CRC cases	322	146	1.00 (Ref)	593	301	1.13 (0.90 to 1.43)	366	188	1.36 (1.09 to 1.70)	0.005	
No DBP2 (*GC* rs4588*CC)	187	93	1.00 (Ref)	309	148	1.26 (0.93 to 1.72)	164	93	1.12 (0.84 to 1.51)	0.49	
DBP2 (*GC* rs4588*CA or AA)	135	53	1.00 (Ref)	284	153	1.09 (0.75 to 1.61)	202	95	1.80 (1.24 to 2.60)	0.0002	0.004

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; CI, confidence interval; CPS-II, Cancer Prevention Study-II; CRC, colorectal cancer; DBP, vitamin D-binding protein; EPIC, European Prospective Investigation into Cancer and Nutrition; HR, hazard ratio

* According to Institute of Medicine 2011 recommendations.

† From multivariable Cox proportional hazards models, adjusted for year of diagnosis (continuous), sex, tumor site (colon, rectum, missing/not specified), BMI (continuous), physical activity (quartiles 1 – 4, missing), smoking status (never, former, current, missing), and stage (I – IV, missing/not specified), and stratified by country.

‡ P_{trend} calculated by using vitamin D status as a continuous variable in the model.

§ $P_{interaction}$ between vitamin D status and DBP isoform calculated using a likelihood ratio test.

Table 3. Multivariable-adjusted Associations of Pre-diagnostic Vitamin D Status* with CRC-specific and All-cause Mortality Among All CRC Cases and According to Vitamin D-binding Protein (DBP) Isoform, Assuming a Co-dominant Inheritance Model, in the EPIC and CPS-II Cohorts Combined (n = 1,281)

	Circulating 25(OH)D concentrations						
	≥ 50 nmol/L (sufficient)			< 50 nmol/L (non-sufficient)			
Outcome and DBP strata	**No. total**	**No. died**	**HR (95% CI)†**	**No. total**	**No. died**	**HR (95% CI)†**	***P*$_{interaction}$‡**
CRC-specific mortality							
All CRC cases	322	106	1.00 (Ref)	959	377	1.22 (0.97 to 1.52)	
DBP1-1 (*GC* rs4588*CC)	187	72	1.00 (Ref)	473	184	0.96 (0.72 to 1.29)	
DBP1-2 (*GC* rs4588*CA)	120	32	1.00 (Ref)	390	149	1.54 (1.02 to 2.32)	0.003
DBP2-2 (*GC* rs4588*AA)	15	2	1.00 (Ref)	96	44	2.50 (0.56 to 11.1)	
All-cause mortality							
All CRC cases	322	146	1.00 (Ref)	959	489	1.21 (1.00 to 1.47)	
DBP1-1 (*GC* rs4588*CC)	187	93	1.00 (Ref)	473	241	1.06 (0.83 to 1.37)	
DBP1-2 (*GC* rs4588*CA)	120	48	1.00 (Ref)	390	194	1.33 (0.94 to 1.86)	0.09
DBP2-2 (*GC* rs4588*AA)	15	5	1.00 (Ref)	96	54	1.13 (0.41 to 3.05)	

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; CI, confidence interval; CPS-II, Cancer Prevention Study-II; CRC, colorectal cancer; DBP, vitamin D-binding protein; EPIC, European Prospective Investigation into Cancer and Nutrition; HR, hazard ratio

* According to Institute of Medicine 2011 recommendations.

† From multivariable Cox proportional hazards models adjusted for age at diagnosis, year of diagnosis, sex, tumor site (colon, rectum, missing/not specified), BMI (continuous), physical activity (quartiles 1 – 4, missing), smoking status (never, former, current, missing), and stage (I – IV, missing/not specified) and stratified by country.

‡ $P_{interaction}$ between vitamin D status and DBP isoform calculated using a likelihood ratio test.

Table 4. Multivariable-adjusted Joint Associations of Pre-diagnostic Vitamin D Status* and DBP2 isoform with CRC-specific Mortality Among All CRC in the EPIC and CPS-II Cohorts Combined

Joint Variable	No. total	No. died	HR (95% CI)†	RERI‡
≥50 nmol/L (Sufficient) + no DBP2	187	72	1.00 (Ref)	
≥50 nmol/L (Sufficient) + DBP2	135	34	0.53 (0.35 to 0.83)	
<30 nmol/L (Deficient) + no DBP2	164	70	0.87 (0.64 to 1.18)	
<30 nmol/L (Deficient) + DBP2	202	66	1.11 (0.82 to 1.48)	0.71

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; CI, confidence interval; CPS-II, Cancer Prevention Study-II; CRC, colorectal cancer; DBP, vitamin D-binding protein; EPIC, European Prospective Investigation into Cancer and Nutrition; HR, hazard ratio

* According to Institute of Medicine 2011 recommendations.

† From multivariable Cox proportional hazards models adjusted for age at diagnosis, year of diagnosis, sex, tumor site (colon, rectum, missing/not specified), BMI (continuous), physical activity (quartiles 1 – 4, missing), smoking status (never, former, current, missing), and stage (I – IV, missing/not specified) and stratified by country.

‡ Relative excess risk due to interaction (RERI) calculated as: $1.11 – 0.87 – 0.53 + 1$ ($HR_{11} – HR_{10} – HR_{01} + HR_{00}$).

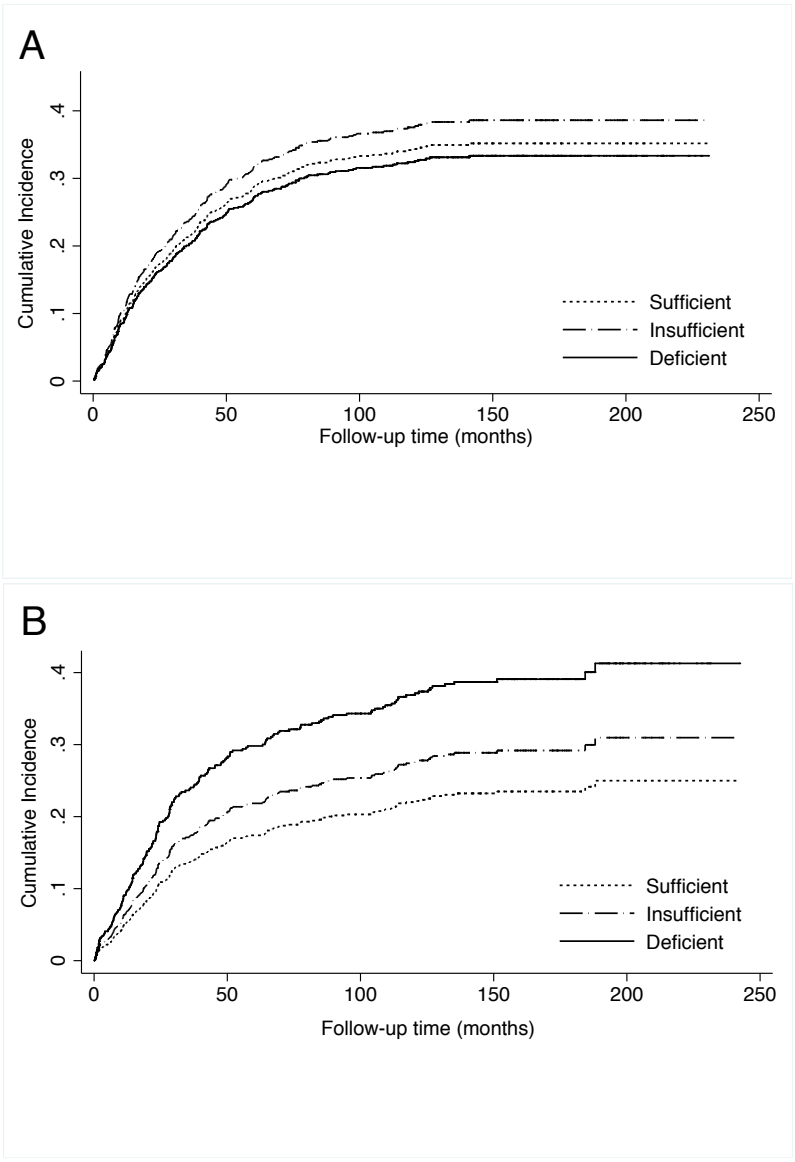

Figure 1. Adjusted cumulative incidence of CRC-specific death accounting for competing causes of death according to vitamin D status—using Institute of Medicine recommended 25-hydroxyvitamin D cut-points—in the combined EPIC and CPS-II cohort (n = 1,281) among (**A**) patients without DBP2 (*GC* rs4588*CC) and (**B**) patients with DBP2 (*GC* rs4588*CA or AA). Cumulative incidence curves were estimated using Fine and Gray's competing-risks regression models adjusted for age at diagnosis (continuous), year of diagnosis (continuous), sex, tumor site (colon, rectum, missing/not specified), BMI (continuous), physical activity (quartiles 1 – 4, missing), smoking status (never, former, current, missing), stage (I – IV, missing/not specified), and country. 25(OH)D concentrations <30, 30 – <50, and ≥50 nmol/L categorized as deficient, insufficient, and sufficient, respectively, based on Institute of Medicine guidelines.

SUPPLEMENTARY TABLES AND FIGURES

Supplementary Table 1. Study-specific Multivariable-adjusted Associations of Pre-diagnostic Vitamin D Status* with CRC-specific and All-cause Mortality According to Vitamin D-binding Protein (DBP) Isoform, Assuming a Dominant Inheritance Model, in the EPIC and CPS-II Cohorts

	Circulating 25(OH)D concentrations								
	≥ 50 nmol/L (sufficient)			30 – <50 nmol/L (insufficient)			<30 nmol/L (Deficient)		
Outcome and strata	No. total	No. died	HR (95% CI)†	No. total	No. Died	HR (95% CI)†	No. total	No. Died	HR (95% CI)†
CRC-specific mortality									
EPIC									
DBP1-1	121	55	1.00 (Ref)	267	99	1.00 (0.68 to 1.48)	145	63	0.86 (0.60 to 1.22)
DBP1-2 or DBP2-2	71	20	1.00 (Ref)	253	116	1.18 (0.69 to 2.02)	186	63	2.25 (1.36 to 3.71)
CPS-II									
DBP1-1	66	17	1.00 (Ref)	42	15	2.27 (0.94 to 5.48)	19	7	0.80 (0.24 to 2.64)
DBP1-2 or DBP2-2	64	14	1.00 (Ref)	31	11	2.97 (1.03 to 8.51)	16	3	1.86 (0.40 to 8.71)
All-cause mortality									
EPIC									
DBP1-1	121	59	1.00 (Ref)	267	126	1.23 (0.86 to 1.78)	145	80	1.02 (0.73 to 1.42)
DBP1-2 or DBP2-2	71	26	1.00 (Ref)	253	137	1.12 (0.70 to 1.78)	186	85	1.85 (1.19 to 2.88)
CPS-II									
DBP1-1	66	34	1.00 (Ref)	42	22	1.31 (0.69 to 2.49)	19	13	1.47 (0.65 to 3.34)
DBP1-2 or DBP2-2	64	27	1.00 (Ref)	31	16	0.80 (0.36 to 1.75)	16	10	1.58 (0.64 to 3.87)

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; CI, confidence interval; CPS-II, Cancer Prevention Study-II; CRC, colorectal cancer; DBP, vitamin D-binding protein; EPIC, European Prospective Investigation into Cancer and Nutrition; HR, hazard ratio

* According to Institute of Medicine 2011 recommendations.

† Adjusted for age at diagnosis, year of diagnosis, sex, tumor site (colon, rectum, missing/not specified), BMI (continuous), physical activity (quartiles 1-4, missing), smoking status (never, former, current, missing), and stage (I-IV, missing/not specified) and stratified by country.

Supplementary Table 2. Study-specific Multivariable-adjusted Associations of Pre-diagnostic Vitamin D Status* with CRC-specific and All-cause Mortality Among All CRC Cases and According to Vitamin D-binding Protein (DBP) Isoform, Assuming a Co-dominant Inheritance Model, in the EPIC and CPS-II Cohorts

	Circulating 25(OH)D concentrations					
	≥ 50 nmol/L (sufficient)			< 50 nmol/L (non-sufficient)		
Outcome and strata	**No. total**	**No. died**	**HR (95% CI)†**	**No. total**	**No. died**	**HR (95% CI)†**
CRC-specific mortality						
EPIC						
DBP1-1	121	55	1.00 (Ref)	412	162	0.90 (0.65 to 1.26)
DBP1-2	64	18	1.00 (Ref)	349	137	1.63 (0.97 to 2.74)
DBP2-2	7	2	1.00 (Ref)	90	42	1.30 (0.16 to 10.8)
CPS-II						
DBP1-1	66	17	1.00 (Ref)	61	22	1.59 (0.72 to 3.55)
DBP1-2	56	14	1.00 (Ref)	41	12	1.92 (0.66 to 5.45)
DBP2-2	8	0	1.00 (Ref)	6	2	Not estimable
All-cause mortality						
EPIC						
DBP1-1	121	59	1.00 (Ref)	412	206	1.09 (0.80 to 1.49)
DBP1-2	64	23	1.00 (Ref)	349	171	1.45 (0.91 to 2.30)
DBP2-2	7	3	1.00 (Ref)	90	51	0.82 (0.16 to 4.33)
CPS-II						
DBP1-1	66	34	1.00 (Ref)	61	35	1.04 (0.51 to 2.15)
DBP1-2	56	25	1.00 (Ref)	41	23	1.36 (0.77 to 2.41)
DBP2-2	8	2	1.00 (Ref)	6	3	Not estimable

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; CI, confidence interval; CPS-II, Cancer Prevention Study-II; CRC, colorectal cancer; DBP, vitamin D-binding protein; EPIC, European Prospective Investigation into Cancer and Nutrition; HR, hazard ratio

* According to Institute of Medicine 2011 recommendations

† Adjusted for age at diagnosis, year of diagnosis, sex, tumor site (colon, rectum, missing/not specified), BMI (continuous), physical activity (quartiles 1 – 4, missing), smoking status (never, former, current, missing), and stage (I – IV, missing/not specified) and stratified by country.

Supplementary Table 3. Multivariable-adjusted Associations of Pre-diagnostic Vitamin D Status* with CRC-specific Mortality Among CRC Cases According to Vitamin D-binding Protein (DBP) Isoform and Other Effect Modifiers in the EPIC and CPS-II Cohorts Combined

	Circulating 25(OH)D concentrations							
	≥ 50 nmol/L (sufficient)			< 50 nmol/L (non-sufficient)				
Strata	**No. total**	**No. died**	**HR (95% CI)†**	**No. total**	**No. died**	**HR (95% CI)†**	$P_{interaction\ by\ DBP2}$‡	$P_{interaction\ by\ site,\ etc.}$§
Site								
Colon								
DBP1-1	131	48	1.00 (Ref)	337	129	0.98 (0.68 to 1.41)		
DBP1-2 or DBP2-2	100	27	1.00 (Ref)	337	132	1.57 (0.94 to 2.51)	0.05	
Rectum								
DBP1-1	45	18	1.00 (Ref)	110	40	0.76 (0.36 to 1.56)		
DBP1-2 or DBP2-2	33	7	1.00 (Ref)	127	51	2.68 (1.02 to 7.07)	0.07	0.71, 0.61
Stage								
I and II								
DBP1-1	87	16	1.00 (Ref)	225	45	0.86 (0.45 to 1.62)		
DBP1-2 or DBP2-2	79	8	1.00 (Ref)	234	56	2.53 (1.02 to 6.25)	0.11	
III and IV								
DBP1-1	83	50	1.00 (Ref)	201	121	1.14 (0.81 to 1.63)		
DBP1-2 or DBP2-2	44	24	1.00 (Ref)	192	118	1.41 (0.86 to 2.32)	0.15	0.37, 0.17
Calcium intake ‖								
Below median								
DBP1-1	82	30	1.00 (Ref)	237	88	0.81 (0.48 to 1.36)		
DBP1-2 or DBP2-2	64	21	1.00 (Ref)	261	101	1.25 (0.70 to 2.22)	0.27	
At or above median								
DBP1-1	105	42	1.00 (Ref)	236	96	1.16 (0.77 to 1.74)		
DBP1-2 or DBP2-2	71	13	1.00 (Ref)	225	92	2.20 (1.13 to 4.30)	0.01	0.40, 0.10
Sex								
Male								
DBP1-1	72	20	1.00 (Ref)	244	103	0.86 (0.55 to 1.36)		
DBP1-2 or DBP2-2	77	32	1.00 (Ref)	272	103	1.61 (0.90 to 2.89)	0.13	
Female								
DBP1-1	108	44	1.00 (Ref)	244	94	1.05 (0.68 to 1.64)		
DBP1-2 or DBP2-2	63	14	1.00 (Ref)	242	90	1.75 (0.89 to 3.41)	0.11	0.58, 0.77

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; CI, confidence interval; CPS-II, DBP, vitamin D-binding protein; EPIC, European Prospective Investigation into Cancer and Nutrition; HR, hazard ratio

* According to Institute of Medicine recommendations.

† Adjusted for age at diagnosis, year of diagnosis, sex (except for in the sex-stratified models), tumor site (colon, rectum) (except for in the site-stratified models), BMI (continuous), physical activity (quartiles 1 – 4, missing), smoking status (never, former, current, missing), and stage (I – IV, unknown) (except for in the stage-stratified models), and stratified by country.

‡ $P_{interaction}$ between vitamin D status and DBP2 calculated using a likelihood ratio test.

§ $P_{interaction}$ between vitamin D status and second effect-modifier (site, stage, calcium intake or sex) calculated using a likelihood ratio test. Two *P* values are provided which correspond to the $P_{interaction}$ values calculated among DBP1-1 cases and among DBP1-2/DBP2-2 cases, respectively.

‖ Based on study-specific median for total (dietary plus supplemental) calcium intake (988.5 mg and 924.3 mg in CPS-II and EPIC, respectively).

Supplementary Table 4. Multivariable-adjusted Associations of Pre-diagnostic Vitamin D Status* with CRC-specific and All-cause Mortality Among Non-metastatic CRC Cases According to Vitamin D-binding Protein (DBP) Isoform, Assuming a Dominant Inheritance Model, in the EPIC and CPS-II Cohorts Combined (n = 1,146)

	Circulating 25(OH)D concentrations										
	≥ 50 nmol/L (sufficient)			30 – <50 nmol/L (insufficient)			< 30 nmol/L (deficient)				
Outcome and DBP strata	**No. total**	**No. died**	**HR (95% CI)†**	**No. total**	**No. died**	**HR (95% CI)†**	**No. total**	**No. died**	**HR (95% CI)†**	**P_{trend}‡**	**$P_{interaction}$§**
CRC-specific mortality											
All non-metastatic CRC cases	282	69	1.00 (Ref)	533	188	1.21 (0.87 to 1.66)	331	102	1.43 (1.06 to 1.94)	0.02	
No DBP2 (*GC* rs4588*CC)	161	48	1.00 (Ref)	277	86	1.17 (0.77 to 1.78)	143	49	0.95 (0.64 to 1.42)	0.73	
DBP2 (*GC* rs4588*CA or AA)	121	21	1.00 (Ref)	256	102	1.61 (0.91 to 2.83)	188	53	2.74 (1.60 to 4.67)	0.0001	0.003
All-cause mortality											
All non-metastatic CRC cases	282	109	1.00 (Ref)	533	244	1.18 (0.91 to 1.54)	331	153	1.41 (1.09 to 1.81)	0.007	
No DBP2 (*GC* rs4588*CC)	161	69	1.00 (Ref)	277	117	1.34 (0.94 to 1.91)	143	72	1.14 (0.82 to 1.61)	0.52	
DBP2 (*GC* rs4588*CA or AA)	121	40	1.00 (Ref)	256	127	1.17 (0.76 to 2.87)	188	81	1.90 (1.26 to 2.87)	0.0002	0.03

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; CI, confidence interval; CPS-II, Cancer Prevention Study-II; CRC, colorectal cancer; DBP, vitamin D-binding protein; EPIC, European Prospective Investigation into Cancer and Nutrition; HR, hazard ratio

* According to Institute of Medicine 2011 recommendations.

† From multivariable Cox proportional hazards models, adjusted for year of diagnosis (continuous), sex, tumor site (colon, rectum, missing/not specified), BMI (continuous), physical activity (quartiles 1 – 4, missing), smoking status (never, former, current, missing), and stage (I – III, missing/not specified), and stratified by country.

‡ P_{trend} calculated by using vitamin D status as a continuous variable in the model.

§ $P_{interaction}$ between vitamin D status and DBP isoform calculated using a likelihood ratio test.

Supplementary Table 5. Multivariable-adjusted Associations of Pre-diagnostic Vitamin D Status* with CRC-specific and All-cause Mortality Among CRC Cases According to Vitamin D-binding Protein (DBP) Isoform and Time between 25(OH)D Assay and CRC Diagnosis in the EPIC and CPS-II Cohorts Combined

	Circulating 25(OH)D concentrations										
	≥ 50 nmol/L (sufficient)			30 – <50 nmol/L (insufficient)			< 30 nmol/L (deficient)				
Outcome and strata	**No. total**	**No. died**	**HR (95% CI)†**	**No. total**	**No. died**	**HR (95% CI)†**	**No. total**	**No. died**	**HR (95% CI)†**	P_{trend}‡	$P_{interaction}$§
CRC-specific mortality											
> 1 year between 25(OH)D and diagnosis											
DBP1-1	180	68	1.00 (Ref)	288	104	1.08 (0.75 to 1.55)	154	66	0.92 (0.66 to 1.30)	0.96	
DBP1-2 or DBP2-2	126	34	1.00 (Ref)	266	118	1.12 (0.78 to 1.99)	189	62	1.92 (1.23 to 3.00)	0.004	0.0005
> 3 years between 25(OH)D and diagnosis											
DBP1-1	119	47	1.00 (Ref)	202	68	0.96 (0.62 to 1.49)	114	47	0.76 (0.50 to 1.16)	0.20	
DBP1-2 or DBP2-2	89	20	1.00 (Ref)	192	79	1.20 (0.64 to 2.26)	120	37	2.25 (1.24 to 4.07)	0.008	<0.0001
All-cause mortality											
> 1 year between 25(OH)D and diagnosis											
DBP1-1	180	89	1.00 (Ref)	288	133	1.21 (0.88 to 1.66)	154	87	1.06 (0.78 to 1.44)	0.75	
DBP1-2 or DBP2-2	126	52	1.00 (Ref)	266	143	1.07 (0.68 to 1.49)	189	91	1.59 (1.09 to 2.30)	0.002	0.005
> 3 year between 25(OH)D and diagnosis											
DBP1-1	119	59	1.00 (Ref)	202	91	1.08 (0.72 to 1.60)	114	59	0.89 (0.61 to 1.31)	0.89	
DBP1-2 or DBP2-2	89	32	1.00 (Ref)	192	99	1.01 (0.61 to 1.69)	120	52	1.84 (1.13 to 2.98)	0.01	0.0008

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; CI, confidence interval; CPS-II, Cancer Prevention Study-II; CRC, colorectal cancer; DBP, vitamin D-binding protein; EPIC, European Prospective Investigation into Cancer and Nutrition; HR, hazard ratio

* According to Institute of Medicine 2011 recommendations.

† Adjusted for age at diagnosis, year of diagnosis, sex, tumor site (colon, rectum, missing/not specified), BMI (continuous), physical activity (quartiles 1-4, missing), smoking status (never, former, current, missing), and stage (I-IV, missing/not specified) and stratified by country.

‡ P_{trend} calculated by using vitamin D status as a continuous variable in the model.

§ $P_{interaction}$ between vitamin D status and DBP isoform calculated using a likelihood ratio test.

Supplementary Table 6. Multivariable-adjusted Associations of Pre-diagnostic Study-Specific 25(OH)D Tertiles with CRC-specific and Overall Mortality According to Vitamin D-binding Protein (DBP) Isoform, Assuming a Dominant Inheritance Model, in the EPIC and CPS-II Cohorts

	Circulating 25(OH)D concentrations									
	Tertile 3			Tertile 2			Tertile 1			
Strata	No. total	No. died	HR (95% CI)*	No. total	No. Died	HR (95% CI)*	No. total	No. Died	HR (95% CI)*	$P_{interaction}$‡
CRC-specific mortality										
EPIC (study-specific tertiles)†										
DBP1-1	214	92	1.00 (Ref)	154	66	1.08 (0.75 to 1.55)	165	59	0.78 (0.55 to 1.11)	
DBP1-2 or DBP2-2	143	50	1.00 (Ref)	196	68	1.15 (0.76 to 1.75)	171	81	1.66 (1.11 to 2.66)	
CPS-II (study-specific tertiles)†										
DBP1-1	39	11	1.00 (Ref)	45	13	1.20 (0.41 to 3.48)	43	15	2.05 (0.53 to 8.04)	
DBP1-2 or DBP2-2	40	9	1.00 (Ref)	35	7	1.05 (0.25 to 4.36)	36	12	4.35 (1.10 to 17.3)	
Pooled (study-specific tertiles)†										
DBP1-1	253	103	1.00 (Ref)	199	79	1.09 (0.78 to 1.52)	208	74	0.88 (0.63 to 1.21)	0.005
DBP1-2 or DBP2-2	183	59	1.00 (Ref)	231	75	1.15 (0.78 to 1.70)	207	93	1.77 (1.22 to 2.57)	
All-cause mortality										
EPIC (study-specific tertiles)†										
DBP1-1	214	104	1.00 (Ref)	154	84	1.19 (0.87 to 1.64)	165	77	0.95 (0.70 to 1.30)	
DBP1-2 or DBP2-2	143	65	1.00 (Ref)	196	90	0.89 (0.63 to 1.26)	171	93	1.48 (1.05 to 2.08)	
CPS-II (study-specific tertiles)†										
DBP1-1	49	17	1.00 (Ref)	45	27	1.57 (0.80 to 3.08)	43	25	1.81 (0.86 to 3.85)	
DBP1-2 or DBP2-2	40	18	1.00 (Ref)	35	21	0.72 (0.29 to 1.79)	36	14	1.11 (0.52 to 2.40)	
Pooled (study-specific tertiles)†										
DBP1-1	253	121	1.00 (Ref)	199	111	1.23 (0.93 to 1.64)	208	102	1.07 (0.81 to 1.42)	0.06
DBP1-2 or DBP2-2	183	83	1.00 (Ref)	231	111	0.90 (0.65 to 1.22)	207	107	1.48 (1.09 to 2.01)	

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; CI, confidence interval; CPS-II, Cancer Prevention Study-II; CRC, colorectal cancer; DBP, vitamin D-binding protein; EPIC, European Prospective Investigation into Cancer and Nutrition; HR, hazard ratio

*Adjusted for age at diagnosis, year of diagnosis, sex, tumor site (colon, rectum, missing/not specified), BMI (continuous), physical activity (quartiles 1 – 4, missing), smoking status (never, former, current, missing), and stage (I – IV, missing/not specified) and stratified by country.

†EPIC tertile cut-points = 33 and 47 nmol/L; CPS-II tertile cut-points = 51 and 67 nmol/L.

‡ $P_{interaction}$ between vitamin D status and DBP isoform calculated using a likelihood ratio test.

CHAPTER VI

Conclusions, Public Health Implications, and Future Research

Conclusions and Public Health Implications

The overarching goals of this dissertation were: 1) to assess the effects of supplemental vitamin D and/or calcium on inflammation-related tissue biomarkers of risk for CRC, 2) to investigate whether the association of vitamin D status with CRC risk differs by inherited genotypes that may influence vitamin D status/metabolism, and 3) to investigate whether the association of pre-diagnostic vitamin D status with mortality among CRC patients differs by inherited genotypes that may influence vitamin D status/metabolism.

For my first dissertation project, I estimated the effects of supplemental vitamin D, alone and in combination with calcium, on two inflammation-related biomarkers of risk for CRC (COX-2 and 15-HPGD) in the normal-appearing colorectal mucosa among colorectal adenoma patients in a randomized chemoprevention trial (see Chapter III). For my second dissertation project, I investigated whether the association of circulating vitamin D concentrations with CRC risk differed by functional DBP-isoform-encoding genotypes in a pooled, nested case-control study (see Chapter IV). For my third dissertation project, I investigated whether the association of pre-diagnostic vitamin D status with mortality risk among CRC patients differed by DBP-isoform-encoding genotypes in a pooled prospective cohort study (see Chapter V).

Overall, we found that vitamin D supplementation modified the balance of COX-2 and 15-HPGD expression among colorectal adenoma patients, particularly among those with the DBP2-encoding genotype (GC-rs4588*C>A; Thr→Lys substitution). Additionally, our results suggest that the association of circulating vitamin D concentrations with CRC risk and survival among CRC patients were stronger among those with the DBP2-encoding genotype. Collectively, these findings suggest that individuals with the DBP2 isoform may particularly benefit from higher vitamin D exposure for: i) lowering CRC-promoting inflammation in the

gut, ii) CRC prevention, and iii) improved survival following a CRC diagnosis.

These findings may inform future randomized clinical trials of vitamin D supplementation for CRC risk and survival among CRC patients, and could impact clinical practice. If our findings are confirmed, they would support DBP genotyping, which could be easily and affordably obtained in clinical settings, for guiding vitamin D-related therapy for CRC prevention and for survival stratification. This, in turn, could have a substantial impact on public health given the high prevalence of DBP2 (40 – 50% among those with European ancestry (116)) and vitamin D concentrations <50 nmol/L considered insufficient in the US and Europe (26 – 76%, depending on age and country (56, 203, 204)).

Future Research

There are several important avenues for future research. Firstly, all three of these projects were conducted among Europeans or US individuals with European ancestry, thus future research is needed to confirm whether these findings are generalizable to individuals of other races and ethnicities. Secondly, larger observational studies are needed to estimate the associations of 25(OH)D with CRC risk/survival among those with the rare DBP2-2 homozygous genotype (i.e., who only inherited DBP2 isoforms). Thirdly, well-conducted randomized clinical trials are needed to assess whether the effects of vitamin D supplementation on: i) biomarkers of risk for CRC, ii) colorectal neoplasm occurrence, and iii) survival among CRC patients may be stronger among those with the DBP2-encoding genotype.

Related to the first dissertation project specifically, a logical next-step will be to investigate whether the estimated changes in COX-2/15-HPGD expression by vitamin D and/or calcium supplementation are associated with a lower risk of colorectal neoplasms, and whether

this potential risk reduction is mediated by prostaglandin concentrations in gut tissue. Future studies are also needed to investigate long-term (post 1-year) effects of vitamin D and/or calcium supplementation on COX-2/15-HPGD and to investigate treatment effects on COX-2/15-HPGD expression in different parts of the normal colon and in neoplastic colorectal tissue.

Related to the second and third dissertation projects, randomized clinical trials should be conducted to test whether there is a causal association of vitamin D with CRC risk and survival only among those with the DBP2 isoform. Larger studies are also needed to investigate the association of 25(OH)D concentrations with CRC risk/survival among those with each DBP-isoform combination (i.e., diplotype). Lastly, future research is needed to investigate why DBP isoforms may modify the association of 25(OH)D with the occurrence and progression of colorectal neoplasms. This may require experimental studies (e.g., testing the effects of different 25(OH)D concentrations on VDR activation in normal and neoplastic colon cell lines cultured with different DBP isoforms) in addition to observational studies. With respect to the latter, I propose measuring blood concentrations of DBP, in addition to directly measuring free and bioavailable 25(OH)D concentrations, using available blood samples collected from participants in the EPIC, CPS-II and NHS cohort studies. Using this data, one could investigate whether our observed pattern of effect modification by DBP2 isoform may be explained by putative differences in DBP concentrations by isoform, which in turn may affect relative amounts of free and/or bioavailable vitamin D among individuals with different isoforms at a given 25(OH)D concentration.

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