

Vitamin D, Inflammation, and Colorectal Cancer Progression: A Review of Mechanistic Studies and Future Directions for Epidemiological Studies

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Abstract

Survival from colorectal cancer is positively associated with vitamin D status. However, whether this association is causal remains unclear. Inflammatory processes may link vitamin D to colorectal cancer survival, and therefore investigating inflammatory markers as potential mediators may be a valuable next step. This review starts with an overview of inflammatory processes suggested to be involved in colorectal cancer progression and regulated by vitamin D. Next, we provide recommendations on how to study inflammatory markers in future epidemiologic studies on vitamin D and colorectal cancer survival. Mechanistic studies have shown that calcitriol—active form of vitamin D— influences inflammatory processes involved in cancer progres-

sion, including the enzyme cyclooxygenase 2, the NF- κ B pathway, and the expression of the cytokines TNF α , IL1 β , IL6, IL8, IL17, and TGF β 1. Based on this and taking into account methodologic issues, we recommend to include analysis of specific soluble peptides and proteins, such as cytokines, in future epidemiologic studies on this issue. Vitamin D and the markers should preferably be measured at multiple time points during disease progression or recovery and analyzed using mediation analysis. Including these markers in epidemiologic studies may help answer whether inflammation mediates a causal relationship between vitamin D and colorectal cancer survival. *Cancer Epidemiol Biomarkers Prev*; 24(12); 1820–8. ©2015 AACR.

Introduction

Colorectal cancer is the third most common cancer in men and the second most common cancer in women (1). Five years after diagnosis of colorectal cancer, approximately 65% of the patients is still alive (2). Survival of colorectal cancer is positively associated with vitamin D status (25-hydroxyvitamin D). This association is observed in prospective cohort studies and is summarized in multiple reviews, including two meta-analyses (3–8). These meta-analyses were based on five prospective cohort studies in patients with colorectal cancer in which vitamin D status was measured pre- or postdiagnosis. Both meta-analyses showed that patients with colorectal cancer with high vitamin D levels compared with patients with low vitamin D levels had a significantly reduced overall and colorectal cancer-specific mortality (4, 5). One recent additional study that focused on postoperative vitamin D levels also showed that vitamin D status was inversely associated with both overall and colorectal cancer-specific

mortality (9). Table 1 provides details of all published prospective studies on vitamin D status and colorectal cancer survival.

Effects of vitamin D on health have been studied in both observational studies and randomized trials. However, the results are not always consistent (10, 11), fueling the debate whether low vitamin D levels are the cause or the result of diseases. It has been proposed that vitamin D levels could be reduced as a consequence of poor health, for example, due to disease-related inflammatory processes, and therefore would just be a marker of inflammation (10). However, it is also possible that vitamin D directly affects health status, and that the above-mentioned associations between vitamin D and cancer survival are mediated by inflammation, because vitamin D has anti-inflammatory effects (12).

To gain more insight in the exact role of vitamin D and its active metabolites in colorectal cancer progression, we propose to study vitamin D levels and inflammatory markers during colorectal cancer in observational epidemiologic studies, before conducting relatively expensive trials in patients with cancer. However, this leads to several questions: (i) Which inflammatory markers would be relevant and suitable to measure in these epidemiologic studies? (ii) How should these markers be measured in these studies? And, (iii) how should the data be analyzed? Recently, a review was published on the application of inflammatory markers in epidemiologic studies (13). This review provided an overview on laboratory techniques and tissue requirements to measure inflammatory markers and on inflammatory markers in relation to cancer in general. In the current review, we aim to provide an overview of inflammatory processes, based on mechanistic studies, which are specifically suggested to be involved in colorectal cancer

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Table 1. Study characteristics and main results of epidemiologic studies investigating the association between vitamin D and survival in patients with colorectal cancer

First author, year, country	Population	Follow-up	Time point blood draw	Overall mortality			CRC-specific mortality		
				25(OH)D (nmol/L)	HR (95% CI)	P for trend	25(OH)D (nmol/L)	HR (95% CI)	P for trend
Ng, 2008, United States (64)	304 CRC cases Mean age: 68 Stage: I-IV 123 deaths, 96 CRC-specific deaths	Median 6.5 years	Prediagnosis (median 6 years before diagnosis)	Quartile 1 ^a	1.0	0.02	Quartile 1 ^a	1.0	0.23
				Quartile 2	0.81 (0.49-1.35)		Quartile 2	0.76 (0.41-1.42)	
				Quartile 3	0.81 (0.48-1.37)		Quartile 3	1.04 (0.58-1.89)	
				Quartile 4	0.52 (0.29-0.94)		Quartile 4	0.61 (0.31-1.19)	
Fedirko, 2012, Western Europe (EPIC) (65)	1,201 CRC cases Mean age: 62 Stage: I-IV 541 deaths, 444 CRC-specific deaths	Mean 6.1 years	Prediagnosis (on average 3.8 years before diagnosis)	Quintile 1 (<36.3)	1.0	<0.01	Quintile 1 (<36.3)	1.0	0.04
				Quintile 2 (36.4-48.6)	0.82 (0.63-1.07)		Quintile 2 (36.4-48.6)	0.76 (0.56-1.02)	
				Quintile 3 (48.7-60.5)	0.91 (0.70-1.18)		Quintile 3 (48.7-60.5)	0.93 (0.69-1.24)	
				Quintile 4 (60.6-76.8)	0.78 (0.59-1.03)		Quintile 4 (60.6-76.8)	0.78 (0.58-1.06)	
				Quintile 5 (>76.8)	0.67 (0.50-0.88)		Quintile 5 (>76.8)	0.69 (0.50-0.93)	
Mezawa, 2010, Japan (66)	257 CRC cases Mean age: 65 Stage: I-IV 39 deaths, 30 CRC-specific deaths	Median 2.7 years	Peri-operative	Quartile 1 (7.5-17.5)	0.50 (0.16-1.54)	NA	Continuous per 2.5 nmol/L	0.98 (0.89-1.08)	NA
				Quartile 2 (20-25)	0.55 (0.18-1.65)				
				Quartile 3 (27.5-37.5)	1.0				
				Quartile 4 (40-90)	0.16 (0.04-0.63)				
Tretli, 2012, Norway (67)	52 colon cancer cases Median age: 59 Stage: I-IV 26 deaths	From 1973-2005 until December 2008	Postdiagnosis (median 30.5 days after diagnosis)	Quartile 1 (<46)	1.0	0.23	Quartile 1 (<44)	1.0	0.16
				Quartile 2 (46-61)	0.48 (0.18-1.29)		Quartile 2 (45-56)	0.46 (0.15-1.48)	
				Quartile 3 (62-81)	0.61 (0.23-1.59)		Quartile 3 (57-77)	0.73 (0.25-2.15)	
				Quartile 4 (>81)	0.40 (0.10-1.60)		Quartile 4 (>77)	0.20 (0.04-1.10)	
Ng, 2011, United States/Canada (68)	515 CRC cases Mean age: 61 Stage: IV 475 deaths	Median 5.1 years	Post diagnosis, but before chemotherapy	Quartile 1 (5.8-32.8)	1.0	0.55	NA	NA	NA
				Quartile 2 (33-49.8)	0.78 (0.60-1.02)				
				Quartile 3 (50-67.8)	1.13 (0.87-1.47)				
				Quartile 4 (68-188.5)	0.94 (0.72-1.23)				
Zgaga, 2014, Scotland (9)	1,598 CRC cases Mean age: 62 Stage: I-III 531 deaths, 363 CRC-specific deaths	Median 8.9 years	Postoperatively (median 105 days after treatment)	Tertile 1 (<18.1)	1.0	0.003	Tertile 1 (<18.1)	1.0	0.009
				Tertile 2 (18.2-33.1)	0.81 (0.65-1.01)		Tertile 2 (18.2-33.1)	0.86 (0.66-1.13)	
				Tertile 3 (>33.2)	0.70 (0.55-0.89)		Tertile 3 (>33.2)	0.68 (0.50-0.90)	

NOTE: Vitamin D levels reported in ng/mL were converted into nmol/L using a conversion factor (1 ng/mL = 2.5 nmol/L). Abbreviations: CRC, colorectal cancer; BMI, body mass index; NA, not available; EPIC, European Prospective Investigation into Cancer and Nutrition. ^aQuartile ranges differ per laboratory run (3 runs). Quartile 1: 22.8-47.0, 15.0-48.5, 25.8-56.5; Quartile 2: 47.3-59.3, 51.3-64.0, 57.0-67.8; Quartile 3: 60.0-71.3, 64.3-83.5, 68.0-82.8; Quartile 4: 72.5-175.0, 84.3-142.0, 83.5-138.5.

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progression and at the same time regulated by vitamin D. Furthermore, we will briefly describe findings on markers of these inflammatory processes in patients with colorectal cancer. We will conclude with recommendations on how to study these inflammatory markers in future epidemiologic studies on vitamin D and colorectal cancer progression.

Vitamin D: Metabolism and Mechanism of Action

Vitamin D is synthesized in the skin from its precursor 7-dehydrocholesterol under the influence of UV radiation (14). Then vitamin D undergoes two hydroxylation steps to yield the biologically active 1,25-dihydroxyvitamin D (1,25(OH)₂D), also known as calcitriol. The first hydroxylation step occurs in the liver by the enzyme 25-hydroxylase, which yields 25-hydroxyvitamin D (25(OH)D). The second hydroxylation is performed by another enzyme of the cytochrome P450 (CYP) system, CYP27B1, which is present in the kidney but also in human colon tissue (15–17). Calcitriol is inactivated by different enzymes including CYP24A1, which is overexpressed in colon cancer cells (18, 19). The concentration of 25(OH)D in the circulation is the generally accepted marker to determine vitamin D status. 25(OH)D has a relative long half-life and is a reflection of both nutrition-derived vitamin D as well as vitamin D that is synthesized under the influence of UV radiation.

The biologic effects of calcitriol are mediated by the vitamin D receptor (VDR), which serves as a nuclear transcription factor and binds to DNA sequences known as vitamin D response elements (14). VDR is expressed in many tissues, including human colon tissue (16, 17). The presence of CYP27B1, CYP24A1, and VDR suggest that vitamin D might interact with colon tissue, as will be explained in the next section.

Vitamin D, Inflammation, and Cancer Progression: Proposed Pathways

Vitamin D can affect multiple inflammatory processes involved in colorectal cancer progression, including the cyclooxygenase and NF- κ B pathways, and several cytokines (Fig. 1). Here, we discuss, based on mechanistic studies, how these inflammatory processes are related to cancer progression and how vitamin D affects these processes.

Prostaglandin-endoperoxide synthase 2

Prostaglandin-endoperoxide synthase 2 (PTGS2), also known as COX2, synthesizes prostaglandins (PG) from arachidonic acid and other fatty acids (Fig. 1). These PGs, such as PGE₂, are critical mediators of inflammatory processes and regulate cell migration and activation (12, 20). In addition, PGs stimulate tumor proliferation in colon cancer cells (21, 22). These effects have contributed to the view that COX2 can be regarded as an oncogene, and that inhibition of COX2 may have benefits for patients with colorectal cancer (12).

Calcitriol inhibits COX2 and decreases levels of PGE₂ and two PG receptors (EP2 and FP) in prostate cancer cells, thereby reducing the protumorigenic effect of PGE₂ (23). Effects of calcitriol on COX2 in colon cancer cells have not been reported, but are likely because the PG receptor EP2 is also present in colon cells (24).

In addition to effects on synthesis, calcitriol also stimulates breakdown of PGs. Usually, PGs are hydrolyzed by the enzyme 15-hydroxyprostaglandin dehydrogenase (HPGD), but this enzyme is reduced or even absent in tumor tissue (25). Moreno and colleagues observed an increase in the expression of HPGD after administration of calcitriol to prostate cancer cells (23).

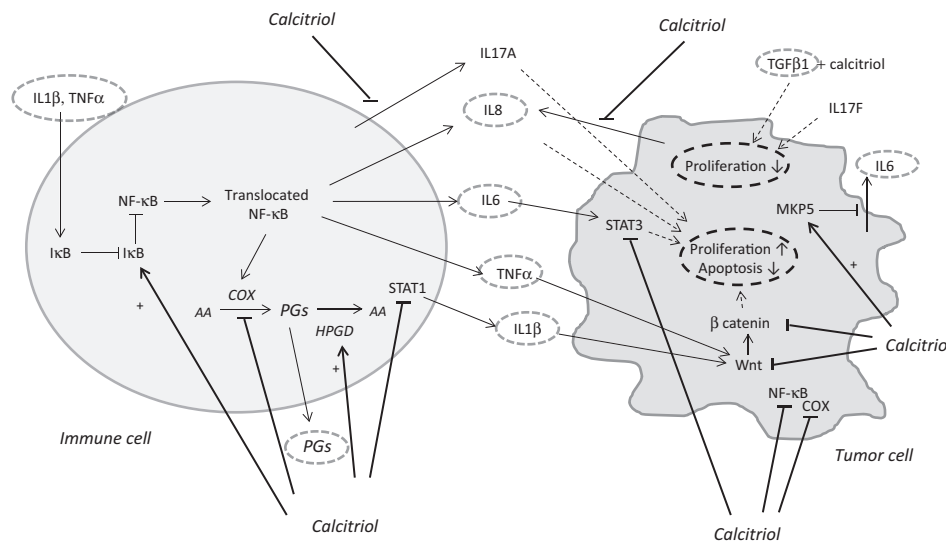


Figure 1.

Overview of mechanisms through which vitamin D inhibits inflammation and may inhibit colorectal cancer progression. Several inflammatory markers, such as PGs and cytokines, such as IL6, IL8, and IL1 β , have tumor-promoting effects in a variety of models, including models of colorectal cancer. Calcitriol, the biologically active metabolite of vitamin D, inhibits inflammatory processes by interacting at various levels. Calcitriol inhibits NF- κ B and STAT1 activation, which results in lower cyclooxygenase and cytokine expression. In addition, calcitriol may also interfere in intracellular signaling cascades, thereby reducing the sensitivity of tumor cells to protumorigenic effects of cytokines. It is good to note that some of these effects are only reported for specific cancer models. Please refer to the text for a more detailed description. AA, arachidonic acid; STAT, signal transducer and activator of transcription.

NF- κ B

NF- κ B is a transcription factor, which is present in all cells, including immune cells. NF- κ B is a key regulator of immune responses and inflammation and can bind to so-called inhibitors of kappa B proteins (I κ B); NF- κ B is inactive in this bound form (12). Upon stimulation by proinflammatory cytokines, such as TNF α (26), IL1 β (27), and IL8 (28), the I κ B-NF- κ B complex is cleaved by I κ B kinase, which results in unbound NF- κ B. This unbound NF- κ B subsequently translocates to the nucleus, where it stimulates the transcription of various genes involved in proinflammatory processes (Fig. 1; ref. 12). Several products of these genes, such as IL6 (26), IL8 (29), TNF α (30), and COX2 (26, 27), play crucial regulatory roles in inflammatory processes and have protumorigenic and anti-apoptotic properties (12, 31).

Calcitriol inhibits the activity of NF- κ B by acting on various members in the NF- κ B pathway, including NF- κ B itself (32). It suppresses activation of the p65 subunit of the NF- κ B complex in colon cancer cells, thereby preventing binding of NF- κ B to DNA (33, 34). Calcitriol also increases the expression of the inhibitor of NF- κ B, I κ B (32, 33, 35). These effects of calcitriol on NF- κ B have also been shown in a mouse model of colon cancer where an increase in dietary vitamin D resulted in a 4.5-fold decrease in NF- κ B activation in colonic epithelial cells (36). Furthermore, colon cancer cells treated with a vitamin D-receptor antagonist showed increased NF- κ B activity and reduced I κ B expression (37). Thus, calcitriol inhibits NF- κ B signaling and thereby the release of proinflammatory mediators, which may inhibit colorectal cancer progression.

TNF α

Immune cells present at the tumor site secrete several cytokines, including TNF α . Activation of NF- κ B also results in increased expression of TNF α (30). TNF α promotes tumor growth in various ways, for example, by inducing DNA damage via the production of reactive oxygen species. Furthermore, TNF α activates the NF- κ B pathway (26) and it promotes Wnt signaling (38) in colon cancer cells (Fig. 1). Because calcitriol inhibits the NF- κ B pathway (32), it could potentially also decrease the release of TNF α , but a direct effect of calcitriol on TNF α remains to be demonstrated.

IL1 β

IL1 β is an important cytokine involved in tumor progression. Colon cancer cells stimulate macrophages to secrete IL1 β through a STAT1-mediated process (Fig. 1). IL1 β stimulates the expression of other tumor growth stimulators, such as TNF α , IL6, IL8, IL17, COX2, and PGE₂, and it is an important inducer of the NF- κ B pathway and the Wnt pathway in colon cancer cells (31). Interestingly, an *in vitro* study in tumor-associated macrophages showed that calcitriol inhibits STAT1 activation, thereby reducing the production of IL1 β (38).

IL6

IL6 is a key cytokine regulating inflammatory processes (39). In addition to its well-known role in inflammation, IL6 also promotes tumor growth and reduces apoptosis through activation of the oncogene STAT3 (Fig. 1; refs. 31, 39). For example, IL6 stimulated cell proliferation in colon cancer cells (40). Furthermore, after stimulation of colon cancer cells with

IL6, the expression of two matrix metalloproteinases (MMP), MMP9 and MMP2, increased (41, 42). MMPs are enzymes that degrade the extracellular matrix, allowing the tumor to migrate and metastasize (41). Treatment with an IL6 receptor antibody reduced the expression of the MMPs (42). The synthesis of IL6 is regulated by a variety of factors. Activation of NF- κ B (26) or p38 MAPK (43) results in increased IL6 expression. There are also factors that downregulate IL6 expression, including mitogen-activated protein kinase phosphatase 5 (MKP5). MKP5 inactivates p38 MAPK, leading to reduced IL6 synthesis (44).

Several reports have shown that vitamin D may influence the regulation of IL6 synthesis (45). *In vitro* studies have shown that treatment of prostate cancer cells with calcitriol increases MKP5 expression, and reduces IL6 release (44, 46). A study in a mouse model of colon cancer showed that an increase in dietary vitamin D decreased p38 MAPK activation (7-fold) in leukocytes. The decrease was accompanied with decreased inflammatory cell infiltrates and a reduced expression of proinflammatory cytokines. However, no change in p38 MAPK in colonic epithelial cells was observed in these mice (36).

IL8

IL8 is a cytokine secreted by tumor and immune cells as a result of the activation of NF- κ B. After secretion it binds to its receptor and elicits several tumor promoting effects (47). Treatment of colon cancer cells with IL8 resulted in increased proliferation *in vitro*, and showed increased migration, angiogenesis, and metastases *in vivo* (28). The effect of calcitriol on IL8 production is still inconclusive. A study in colon cancer cells showed that the biologically active calcitriol, but not vitamin D3, reduced the production of IL8 (48). Furthermore, treatment of prostate cancer cells with calcitriol resulted in a reduced secretion of IL8 and suppressed angiogenesis (49). Thus, it seems that vitamin D might affect IL8 in cancer progression, but this needs further investigation in colorectal cancer.

IL17

IL17 is a family of cytokines produced by immune cells and specific intestinal cells known as Paneth cells. There are six subtypes in the IL17 family, of which IL17A and IL17F are best studied. Different IL17 cytokines may have different effects on initiation and promotion of tumorigenesis (31). An *in vivo* study using colon cancer cells inoculated in mice showed that IL17F had antitumorigenic effects, possibly via inhibiting tumor angiogenesis (50). However, IL17A is associated with protumorigenic effects. Elimination of IL17A in a colon cancer mouse model reduced tumor development and the number of circulating tumor cells, it prevented metastases and decreased the expression of several inflammatory cytokines, such as IL6, IL1 β , and COX2 (51, 52). Treatment of colon cancer cells with IL17A increased the expression of MMP9 and tumor cell mobility (52).

Several studies suggest that calcitriol affects the synthesis of IL17. It inhibits IL17A and IL17F production in immune cells (53). Moreover, it was shown in patients with Behcet's disease and asthma that calcitriol reduced IL17 levels (54, 55). However, the effects of calcitriol on IL17 levels remain to be demonstrated in colorectal cancer.

TGF β 1

TGF β 1 has both tumor promoting and tumor suppressing activity. Increased TGF β 1 levels have been associated with the

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development of metastases (56). In contrast, TGF β 1 also activates apoptosis, inhibits tumor growth, and the production of the proinflammatory and protumorigenic cytokine IL6 (39, 56). However, *in vitro*, most colon cancer cells are resistant to the antiproliferative actions of TGF β 1. A study in colon cancer cells showed that calcitriol was able to inhibit proliferation, whereas TGF β 1 alone had no effect. However, the combined treatment of calcitriol and TGF β 1 resulted in an additional reduction of proliferation. This suggests that calcitriol sensitizes colon cancer cells to the antitumor effects of TGF β 1 (57).

Inflammatory markers for which conflicting evidence exist on their association with colorectal cancer progression and/or calcitriol

For some inflammatory markers there is only limited or conflicting evidence regarding their potential bridging role between vitamin D and colorectal cancer progression. For example, limited data from mechanistic studies suggest that, in contrast to the inflammatory markers described above, both IL10 and IL12 reduce colorectal cancer progression (58, 59). At the same time, calcitriol seems to decrease levels of these cytokines (60, 61). Together, this may represent a mechanism by which calcitriol might promote tumor progression.

Another frequently measured inflammatory marker is C-reactive protein (CRP). The synthesis of this acute-phase protein is induced by different mediators including TNF α , IL1 β , and IL6. It has been suggested that CRP promotes breast cancer progression (62). However, the role of CRP in colorectal cancer progression has not been reported. It may also behave as bystander, because CRP is regarded as a general inflammation marker, being responsive to different proinflammatory stimuli.

Inflammation and Vitamin D in Patients with Colorectal Cancer

The above-mentioned inflammatory pathways are of clinical significance in patients with colorectal cancer. Levels of several inflammatory mediators involved in these pathways are generally elevated in patients with colorectal cancer and associated with clinical outcomes. For example, COX2 is found to be overexpressed in patients with colorectal cancer (63, 64). And indeed, patients with colorectal cancer using COX2 inhibitors, such as NSAIDs, show a decreased risk of recurrence and overall mortality (65). NF- κ B expression is increased in more advanced tumor stages (66). Furthermore, patients with metastatic colorectal cancer with tumors not expressing NF- κ B had a longer time to progression, better survival, and a better response to chemotherapy compared with patients with tumors expressing NF- κ B (67).

Serum levels of TNF α , IL1 β , IL6, IL8, and IL17A are higher in patients with colorectal cancer compared with healthy controls (39, 68–70). IL6 is additionally associated with increased tumor size, tumor stage, metastases, and decreased survival (39). IL17A levels also positively correlate with tumor size (70), the percentage of circulating tumor cells, and risk of recurrence (52). IL17F is, however, not detected in the serum of patients with colorectal cancer (70), whereas higher serum levels of IL8 are observed in patients with stage IV colorectal cancer compared with stage II and stage III patients (28, 47). Human data on levels of IL10 and IL12 are conflicting. A study in 18 patients with colon cancer found no

difference in IL10 levels between patients and controls (71). Others showed that serum levels of IL10 were elevated in patients with colon cancer compared with controls (72). In a study of 20 patients with colorectal cancer, IL10 concentrations were lower in patients compared with controls (73). No differences were observed for IL12 and TGF β 1 levels in this study (73). Preoperative CRP levels were inversely associated with survival of patients with colorectal cancer (74, 75). Because of the limited number of studies and contrasting findings on IL10, IL12, and CRP, the involvement of these inflammatory markers would need further investigation in both mechanistic and human colorectal cancer studies.

One clinical trial investigated the effects of vitamin D supplementation on inflammatory markers in colorectal adenoma—a precursor lesion of colorectal cancer—patients (76). In this randomized, double-blind, placebo-controlled, 2 \times 2 factorial trial, colorectal adenoma patients ($n = 92$) were supplemented with 2 g calcium and/or 800 international units vitamin D3 per day versus placebo over 6 months. Plasma levels of inflammatory markers and serum vitamin D levels (25-hydroxyvitamin D) were measured at baseline and after 6 months. Vitamin D levels significantly increased during the study period in groups receiving vitamin D supplementation (from 54.9 through 73.6 nmol/L). In the vitamin D supplementation group, levels of CRP, TNF α , IL1 β , IL6, and IL8 decreased and in the group receiving calcium and vitamin D, levels of IL1 β , IL6, and IL8 decreased, although changes within both groups were not statistically significant. After 6 months, a combined inflammatory z -score (including CRP, TNF α , IL1 β , IL6, IL8, and IL10) significantly decreased with 77% in the vitamin D group and with 33% in the combined group compared with baseline levels. A major limitation of the study is the small sample size, which may explain the non-significant results for the separate inflammatory markers. In addition, the dose of vitamin D was not high enough to increase vitamin D levels to levels considered sufficient according to the authors (80 nmol/L; ref. 76). To the best of our knowledge, this is the only study in a condition related to colorectal cancer, and not even in patients with colorectal cancer themselves, that investigated vitamin D and inflammatory markers simultaneously. This lack of studies underlines the relevance to include measurement of inflammatory markers in future epidemiologic studies.

Recommendations to Study Vitamin D and Inflammation in Patients with Colorectal Cancer

The mechanistic studies described above underline that inflammatory processes may play a role in the association between vitamin D and colorectal cancer progression. Whether inflammation is a mediator or just a bystander in the association between vitamin D and progression in patients with colorectal cancer remains to be studied. We therefore provide several recommendations on how this question could be addressed in future epidemiologic studies. Although epidemiologic studies by themselves are not able to answer the question whether vitamin D and colorectal cancer progression are causally associated, inclusion of one or more mediators improves causal inference.

A main limitation of the prospective cohort studies performed so far on vitamin D status and survival of colorectal

cancer is the single measurement of vitamin D levels. Although a single measurement of vitamin D appears to be an accurate estimate for long-term vitamin D status in cancer-free subjects (77), it is possible that vitamin D status changes after diagnosis of cancer. Vitamin D levels decrease, for example, after an acute inflammatory stimulus, such as surgery (78). To gain insight into fluctuations of vitamin D levels during disease progression or recovery, vitamin D levels should preferably be measured before and at multiple times after treatment of colorectal cancer. Furthermore, inflammatory markers should be measured simultaneously with vitamin D levels to see to what extent changes in vitamin D levels are associated with changes in inflammatory processes.

Several methodologic issues need to be considered when measuring inflammatory markers in large epidemiologic studies.

First, blood samples are generally collected over an extended timespan. Improper storage may result in time-dependent degradation of cytokines and in particular of lipid-derived mediators. Scarce data suggest that stability differs per cytokine and that most cytokines can be stably measured with ELISAs for up to 2 years (79). To investigate changes over time, it would be preferable to analyze all samples of one patient in the same batch on the same day. If samples are collected over several years, however, degradation of cytokines will be larger in the older samples. In order to have similar degradation in all samples, the interval between blood collection and analysis should be kept constant, but this will result in interassay variability. Thus, it is clear that a conflicting situation arises when dealing with stability and interassay variability. The best solution depends on specific circumstances that differ for each study.

Second, circulating cytokine concentrations are normally low. Cytokines, such as IL6, TNF α , IL8, IL1 β , IL17, IL10, and IL12, can be measured in blood samples using single or multiplex ELISAs (13), but levels may be close to the lower limit of sensitivity. It is therefore recommended to evaluate the assay's sensitivity to establish whether it is suited to measure circulating cytokines in serum or plasma.

Third, several parameters of inflammation are primarily present within cells and are thus not expected to be present in the circulation. For example, NF- κ B, Wnt, and STAT3 are transcription factors and can only be analyzed in immune cells. TGF β 1 is only detectable in blood platelets, whereas COX2 can only be detected in tissue. Products of COX2, such as PGE₂, are detectable in blood samples, for example, using LC/MS-MS (13). However, PGs and other lipid-derived mediators are chemically far less stable than cytokines, demanding specific measures in terms of sample collection and storage. This makes these mediators often impractical to include when samples are to be collected at multiple sites under variable conditions. Thus, soluble peptides and proteins, such as cytokines and CRP, are inflammatory markers that will be most accessible to be measured in blood plasma collected in large epidemiologic studies. It is important to note that plasma markers of inflammation may not exclusively reflect processes within the tumor and in the tumor microenvironment, but may also be linked to systemic inflammation or other inflammatory sites of inflammation. Therefore, data on circulating markers of inflammation have to be interpreted with caution. It can be hypothesized that measuring inflammation and vitamin D status in the tumor itself may provide a better picture of the interaction between

vitamin D, inflammation, and colorectal cancer survival. Tumor tissues can be collected through, for example, biopsy and can be characterized by their vitamin D status and markers of inflammation, after which their relation with colorectal cancer survival can be investigated. This approach, called molecular pathologic epidemiology (80), in which tumors are subdivided according to (molecular pathology) markers, is increasingly being used to understand the development of cancer among the general population as well as the progression of cancer in patients.

The next step is to statistically analyze whether inflammation is a mediator in the association between vitamin D and colorectal cancer progression. Mediation analysis is a statistical technique that allows to better understand underlying mechanisms, by evaluating to which extent the association can be explained by the mediator. It increases the biologic plausibility of a theory and strengthens the evidence that exposure and outcome are causally related (81). Mediation analysis is relatively new in epidemiology and continues to be improved for applications in medical research. The basic idea of mediation analysis is to disentangle (i) the direct effect from exposure to outcome, (ii) the indirect effect via the mediator, and (iii) the interaction effect between exposure and mediator (82). Next to the standard exposure–outcome confounding, other sources of bias, such as mediator–outcome confounding, should be taken into account when applying mediation analysis (83). An additional challenge is how to include multiple inflammatory markers as a mediator in the model. The advantage of including all inflammatory markers in the model as single variables is that the effect of single markers on each other is taken into account. However, a more powerful approach is to include the inflammatory markers as a score. For example, Hopkins and colleagues calculated an "inflammation z-score" to study inflammation in colorectal adenoma patients (76). When taking together all markers into one score, it is very important to make sure that all markers mediate the association in the same direction.

Conclusion

In conclusion, mechanistic studies have shown that the COX2 and NF- κ B pathways and the cytokines, TNF α , IL1 β , IL6, IL8, IL17, and TGF β 1 are involved in colorectal cancer progression and that these are also regulated by vitamin D. The evidence for IL10, IL12, and CRP is less clear, but these markers may also be interesting in the relation between vitamin D and colorectal cancer progression. Future epidemiologic studies should measure both vitamin D and a comprehensive set of inflammatory markers before and preferably multiple times after treatment of colorectal cancer. The following important issues should be taken into account. First, multiple measurements over time of vitamin D and inflammatory markers will give more insight into fluctuations during disease progression or recovery. It also allows to study whether changes in vitamin D levels are associated with changes in inflammatory markers. Second, measuring cytokines in blood samples requires special attention for blood collection and storage. Finally, statistical methods such as mediation analysis can provide more information on the extent to which inflammation is a mediator between vitamin D and colorectal cancer progression. When taking these issues into account, future epidemiologic studies may contribute to answer the question whether vitamin D

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influences progression of colorectal cancer by modulating inflammation.

Disclosure of Potential Conflicts of Interest

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