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The association between circulating levels of vitamin D and inflammatory markers in the first 2 years after colorectal cancer diagnosis

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Abstract

Background: Calcitriol, the active form of vitamin D, may inhibit colorectal cancer (CRC) progression, which has been mechanistically linked to an attenuation of a pro-inflammatory state. The present study investigated the associations between circulating 25 hydroxy vitamin D_3 (25(OH)D_3) levels and inflammatory markers (IL10, IL8, IL6, TNFα and hsCRP) in the 2 years following CRC diagnosis.

Methods: Circulating 25(OH)D_3 levels and inflammatory markers were assessed at diagnosis, after 6, 12 and 24 months from 798 patients with sporadic CRC participating in two prospective cohort studies. Associations between 25(OH)D_3 levels and individual inflammatory markers as well as a summary inflammatory *z*-score were assessed at each time point by multiple linear regression analyses. To assess the association between 25(OH)D_3 and inflammatory markers over the course of 2 years, linear mixed model regression analyses were conducted.

Results: Higher 25(OH)D_3 levels were associated with lower IL6 levels at diagnosis, at 6 months after diagnosis and over the course of 2 years (β −0.06, 95% CI −0.08 to −0.04). In addition, 25(OH)D_3 levels were inversely associated with the summary inflammatory *z*-score at diagnosis and over the course of 2 years (β −0.17, 95% CI −0.25 to −0.08). In addition, a significant inverse association between 25(OH)D_3 levels and IL10 was found over the course of 2 years. Intra-individual analyses showed an inverse association between 25(OH)D_3 and IL10, IL6 and TNFα. No statistically significant associations between 25(OH)D_3 and IL8 and hsCRP levels were observed.

Conclusions: Serum 25(OH)D_3 levels were inversely associated with the summary inflammatory *z*-score and in particular with IL6 in the years following CRC diagnosis. This is of potential clinical relevance as IL6 has an important role in chronic inflammation and is also suggested to stimulate cancer progression. Further observational studies should investigate whether a possible 25(OH)D_3-associated reduction of inflammatory mediators influences treatment efficacy and CRC recurrence.

Keywords: 25(OH)D_3, colorectal cancer, cytokines, inflammatory markers, interleukin 6

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Introduction

Inflammation is defined as one of the enabling hallmarks of cancer.[1] It is estimated that over 20% of all cancers develop as a direct consequence of systemic low grade inflammation.[2,3] Different inflammatory markers, among which cytokines, can stimulate cancer progression and enhance tumour invasion and metastasis in many cancers including colorectal cancer (CRC).[4,5] In addition, intrinsic inflammatory processes by the tumour itself are involved in the majority of colorectal tumours.[5] Higher levels of inflammatory markers are also associated with advanced disease[6,7] and worse CRC outcomes.[8,9] The use of non-steroidal anti-inflammatory drugs, in particular aspirin, has been shown to reduce CRC risk and potentially CRC recurrence.[10] Given the important role of inflammation in the development and progression of CRC, preventing or reversing systemic low-grade inflammation is considered a relevant and promising approach to improve CRC prognosis.

The active form of vitamin D, 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$), showed anti-inflammatory actions in several cancer models, including CRC models.[11,12] As a consequence, modulation of inflammatory responses by vitamin D could probably result in improved cancer prognosis. Vitamin D is derived from the production of vitamin D_3 in the skin, *via* a non-enzymatic two-step process induced by UVB radiation and heat, or obtained from the diet.[13] In the liver, vitamin D is converted by 25-hydroxylase into 25 hydroxy vitamin D_3 ($25(OH)D_3$), the main circulating form of vitamin D and the most reliable measurement of an individual's vitamin D status.[14] Finally, $25(OH)D_3$ is converted into its active form $1,25(OH)_2D_3$ by 1-α hydroxylase mainly in the kidney.[13] Two main mechanisms by which $1,25(OH)D_3$ exerts anti-inflammatory responses are suggested. First, $1,25(OH)_2D_3$ may inhibit nuclear factor kappa B (NFκB) signaling.[11] NFκB is an important transcription factor involved in the regulation of innate immune responses and inflammation, activation results in the production of pro-inflammatory cytokines.[15] NFκB is suggested to be involved in CRC progression.[16] Second, $1,25(OH)_2D_3$ may suppress p38 stress kinase signalling through the upregulation of mitogen-activated protein kinase phosphate 5, resulting in an inhibition of pro-inflammatory cytokine production, such as interleukin (IL)-6, IL8 and tumour necrosis factor alpha (TNFα).[11]

Despite the suggested mechanistic basis described above, only a few studies directly examined the associations between circulating vitamin D levels and levels of inflammatory markers.[17–20] Results of a recent study in CRC patients showed a weak correlation between $25(OH)D_3$ and C-reactive protein (CRP) levels.[20] In addition, results of a study in severely obese individuals showed an inverse association between $25(OH)D_3$ levels and high-sensitivity C-reactive protein (hsCRP), IL6 and TNFα levels. In addition, a study in colorectal adenoma patients found a non-statistically significant decline in hsCRP, IL6, IL1β and TNFα levels and a significantly lower summary inflammatory *z*-score after vitamin D supplementation.[17] To the best of the authors' knowledge, only one study investigated the association between $25(OH)D_3$ levels and inflammatory markers in CRC patients before and after cancer treatment.[20] However, in this study only CRP was examined. The increasing insight that specific inflammatory pathways are involved in cancer progression[5,21] merits more detailed assessment of individual cytokines. It is important to obtain more insight into the systemic inflammatory status before and after cancer treatment and the question whether circulating vitamin D levels are associated with the systemic inflammatory status in CRC patients. The aim of this study was to investigate the association between serum 25(OH)D3 levels and plasma inflammatory markers involved in CRC progression[21,22] before and after treatment for CRC.

Methods

Study population

In total, 798 CRC patients were included, recruited between October 2013 and November 2016, from two prospective cohort studies in the Netherlands: the COLON study ($n=564$) and the EnCoRe study ($n=234$). These patients donated blood samples at diagnosis and several time points before and after treatment.

The design of the COLON study[23] [ClinicalTrials.gov identifier: NCT03191110] as well as that of the EnCoRe study[24] [trialregister.nl identifier: NTR7099] has been described earlier. In short, newly diagnosed CRC patients were recruited directly after diagnosis in 14 hospitals and were followed during and after treatment. Men and women above the age of 18 were eligible. In the COLON study patients with stage I-IV CRC

were eligible. In the EnCoRe study patients with stage IV of disease were not recruited. Non-Dutch speaking patients, or patients with a history of CRC or (partial) bowel resection, chronic inflammatory bowel disease, hereditary CRC syndromes (e.g. Lynch syndrome, familial adenomatous polyposis, Peutz-Jegher), dementia or another mental condition obstructing participation were excluded from the study. All patients signed informed consent and the COLON study was approved by the Committee on Research involving Human Subjects, region Arnhem-Nijmegen, the Netherlands (2009-349). The EnCoRe study was approved by the Medical Ethics Committee of the University Hospital Maastricht and Maastricht University, the Netherlands (METC 11-3-075).

Blood collection

For the COLON study, blood samples were obtained during a regular clinical visit in the hospital at diagnosis, and at 6 months and 2 years after diagnosis. For patients receiving chemotherapy blood samples were also drawn in the hospital 1 year after diagnosis. For the EnCoRe study, blood samples were obtained during a home visit before the start of treatment and at 6 weeks, 6 months, 1 year and 2 years after the end of treatment. All blood samples were centrifuged and aliquoted into serum and plasma and immediately stored in a freezer at −80°C until analysis.

To be able to harmonize the data of both cohorts for analyses at several time points after CRC diagnosis, we selected the time point in the EnCoRe cohort closest to either 6 months (mean 7.6 months), 1 year (mean 12.6 months) and 2 years (mean 23.8 months) after diagnosis as the second, third and fourth time points for these analyses (Supplemental Figure S1).

Serum vitamin D levels

Serum 25(OH)D_3 levels were measured for all participants by liquid chromatography tandem mass spectrometry (LC-MS/MS) in the Canisius Wilhelmina Hospital, Nijmegen, the Netherlands.[25] The inter-assay coefficients of variation were 5.3%, 3.1% and 2.9% at 25(OH)D_3 concentrations of 39.0, 92.5 and 127.0 nmol/l, respectively and were calculated from quality control (QC) data over at least 30 days from the same lot that was used for measurement of the study samples. Serum 25(OH)D_3 is the main circulating form of vitamin D and the most reliable measurement of an individual's vitamin D status.[14]

Plasma inflammatory cytokines

Plasma levels of IL-1β, IL6, IL8, IL10, IL-12p70 and TNFα were determined using a custom-made multiplex assay using electrochemiluminiscence detection (Meso Scale Diagnostics, Rockville, MD, USA). The analyses were performed following the manufacturers' instructions, and assay plates were analysed on a QuickPlex SQ 120 plate reader (Meso Scale Diagnostics). Each sample plate contained a calibration curve and three manufacturers' QC samples with different levels of cytokines. Calibrators, QCs and study samples were analysed in duplicate. Control samples were not masked because the laboratory technician performs the initial quality assessment, and therefore needs to identify the QCs. However, the study samples were blinded.

Cytokines were previously shown to remain stable in plasma for a period up to 2 years of storage at −80°C.[26] Therefore, only samples stored for less than 2 years were analysed. In addition, to exclude any residual influence of storage time on cytokine levels, levels for each individual were measured after storage for a fixed time period; in this way storage time did not influence relative levels of cytokines in each individual over time. For example, all samples of patient *X* were analysed after approximately 3 months of storage and all samples of patient *Y* after approximately 4 months of storage. In total, we had four analysis rounds that is, February 2016, May 2016, January 2017 and December 2017, consisting of in total 70 assay plates.

The quality of the multiplex cytokine data was monitored by evaluating the inter-batch reproducibility of the manufacturers' QC samples for which target values were provided. IL12p70 and IL1β were excluded for further analyses because the plasma levels were undetectable in most of the samples. Inter- and intra-batch coefficients of variation for IL10, IL8, IL6 and TNFα were $<8\%$, and reported values deviated no more than 15% from the assigned target values.

The results for individual samples within the calibration range with a coefficient of variation above 40% were considered too imprecise to be further processed. Levels measured between the lower

detection limit of a specific plate and the lowest point of the calibration line (0.4%) were imputed as the lowest detection limit of all plates. Non-detectable levels (0.1%) were imputed as the lowest detection limit of all plates divided by 2.

hsCRP was measured at diagnosis, 6 months after diagnosis and 1 year after diagnosis, using an immuno-MALDI mass spectrometry method[27] (BEVITAL, Bergen, Norway). The inter-assay coefficient ranged between 3% and 6%.

Summary inflammatory z-score. The summary inflammatory *z*-score (including IL10, IL8, IL6, TNFα and hsCRP) was calculated as follows.[17] First, a normalized *z*-score for each individual biomarker value, with a mean of zero and standard deviation of 1.0 was calculated as $z_j = (x_{ij} - \mu_j)/\sigma_j$, where x_{ij} is a participant's (i) inflammation marker value at a given visit (j), and μ_j and σ_j are the study population mean and standard deviation at given visits, respectively. The combined score was calculated by summing the *z*-scores of each inflammatory marker [inflammatory *z*-score $= z_{score}(LnIL10) + z_{score}(LnIL8) + z_{score}(LnIL6) + z_{score}(LnTNF\alpha) + z_{score}(LnCRP)]$. This summary inflammatory *z*-score was calculated to cluster conceptually related markers of low-grade inflammation and improve statistical efficiency.

Data collection

Information on demographics (age, gender, education), menopausal status and lifestyle (smoking, use of non-steroidal anti-inflammatory drugs (NSAIDs)) was obtained using self-administered questionnaires in both cohorts at the same time as the blood samples were collected. Information on height, weight, and waist and hip circumference was collected using self-administered questionnaires in the COLON study. In the EnCoRe study, these measurements were performed during home visits. Physical activity was assessed using the Short QUestionnaire to ASsess Health-enhancing physical activity (SQUASH) in both cohorts.[28]

Habitual dietary intake in the month (COLON study) or year (EnCoRe study) preceding diagnosis was assessed using a semi-quantitative food frequency questionnaire. During follow-up habitual dietary intake was assessed with the same semi-quantitative food frequency questionnaire in the COLON study and 7-day food diaries in the EnCoRe study. Average daily intake of macro- and micro-nutrients was calculated using the 2011 Dutch food composition table (NEVO-table, 2011).

Clinical data, such as stage of disease, tumour location (colon/rectum), tumour differentiation, histological type, date of start treatment, type of treatment (surgery, neo-adjuvant/adjuvant chemotherapy, radiation therapy) and presence of comorbidities (diabetes, endocrine disorders, cardiovascular, infectious, gastro-intestinal, muscular and joint, neurologic, pulmonary and urogenital diseases) for both cohorts were derived from the Dutch ColoRectal Audit (DCRA). This nationwide audit was initiated by the association of surgeons of the Netherlands to monitor, evaluate and improve CRC care.[29]

Data analyses

Levels on inflammatory markers (IL10, IL8, IL6, TNFα and hsCRP) were natural log-transformed to obtain normally distributed data.

Patient characteristics at diagnosis were described as numbers with percentages or medians with interquartile range (IQR) for the total study population and stratified by vitamin D status (insufficiency serum $25(OH)D_3 < 50$ nmol/l and sufficiency serum $25(OH)D_3 \geq 50$ nmol/l).[30] In addition, levels of serum $25(OH)D_3$ and plasma inflammatory markers at diagnosis and at the follow-up time points were described as medians with IQR. Sensitivity analyses were done for patients who donated blood samples at all measurement points during the study period.

The association between serum $25(OH)D_3$, continuous per 10 nmol/l, and inflammatory markers was assessed using multivariable linear regression analyses. This was done for each inflammatory marker separately as well as for the summary inflammatory *z*-score.

Based on the literature, the following covariates were added to the model: age, gender, season of blood collection, use of NSAIDs, body mass index (BMI), hours of moderate to vigorous physical activity and stage of disease at diagnosis.[7,17,19,31] In addition, having comorbidities at diagnosis (yes/no) changed the regression coefficient substantially (>10%) and was therefore added to the model as well. All models were adjusted for cohort. The use of statins,

smoking status and dietary factors, such as vitamin D, calcium, magnesium, dietary fat, alcohol and fibre, did not influence the regression coefficient and were thus not taken into account in the models.

Mixed model regression was used to determine the overall mean association between 25(OH)D_3 levels and inflammatory markers over time. Linear mixed models take into account both the individual changes in serum vitamin D levels (random effects) and the average change in the population (fixed effects) by using all available measurements and including patients with incomplete data.[32] Time was added as a continuous variable. As fixed effects, we included cohort, season of blood collection, age, gender, use of NSAIDs, BMI, physical activity, having comorbidities, stage of disease and physical activity × time. As random effects we included subject. The unstructured co-variance model was used. Inter- and intra-individual associations were disaggregated by adding centred person-mean values to the model to estimate inter-individual associations and individual deviations from the person-mean value to estimate intra-individual associations.[33] It is important to disaggregate intra (within) and inter (between) person associations, since results obtained from group-level data cannot always be directly translated to individuals.[33]

Stratified analyses were done for stage of disease (I, II, III, IV), gender, and cohort (COLON, EnCoRe). A sensitivity analysis was done including only those patients with hsCRP values <10 mg/L, thus excluding those with acute inflammation. In addition, a sensitivity analyses was done excluding inflammatory markers measured at diagnosis when studying the overall mean associations, since inflammatory markers measured at time of diagnosis may be influenced by the procedure performed to make the diagnosis.

To interpret the beta coefficient of the regression line, the exponential of the beta was taken (EXP^β), since the outcome variable was natural log transformed. These interpreted betas (expressed in percentages) are reported in the text in the results section. In addition, all results described in the result sections are derived from the adjusted models/model 2.

Statistical analyses were performed in SAS 9.4 (SAS Institute, Cary NC). p-values <0.05 were considered statistically significant.

Results

Patient' characteristics

We included 798 CRC patients of whom 252 (32%) were female (Table 1). Median age was 66.9 (IQR 62.2–73.0) years. Two-thirds of the patients had colon cancer. At presentation, only 7% of the patients were in stage IV of disease, 28% presented with stage I of disease, 26% with stage II and 39% with stage III.

Patient characteristics for the total population and stratified by vitamin D status are shown in Table 1. Patients who had sufficient ($\geqslant$50 nmol/l) vitamin D levels ($n=448$) were more often female, were more often diagnosed with stage I disease, used more frequent NSAIDs, used more frequent vitamin D supplements and were more physically active compared with patients who had insufficient levels.

Circulating levels of 25(OH)D_3 and inflammatory markers

Blood levels of 25(OH)D_3 were higher 2 years after diagnosis (64, IQR 49–83 nmol/l) compared with levels at diagnosis (54, IQR 41–70 nmol/l) (Table 2). Levels of IL10, IL8, IL6 and TNFα, did not substantially change over time. Levels of hsCRP were slightly lower 1 year after diagnosis (1.7 mg/l, IQR 0.7–4.4) compared with levels at diagnosis (2.5 mg/l, IQR 1.1–6.0). Comparable results were found when only patients were included who donated blood at three or more time points (Supplemental Table S1).

Associations between 25(OH)D_3 levels and inflammatory markers

A 10 nmol/l higher 25(OH)D_3 was associated with a 6.8% (95% CI –8.7 to –3.9) lower IL6 level at diagnosis, a 4.9% (95% CI –7.0 to –2.0) lower IL6 level 6 months after diagnosis and a 5.8% (95% CI –12.2 to –0.1) lower IL6 level 1 year after diagnosis (Table 3). A statistically non-significant association between 25(OH)D_3 and IL6 was found 2 years after diagnosis (–3.0%, 95% CI –6.8 to 0,0). No associations were observed between 25(OH)D_3 and IL10, IL8, TNF-α and hsCRP at the separate time points. At diagnosis, but not at follow-up time points, a statistically significant inverse association was found between 25(OH)D_3 and the summary inflammatory z-score (β –14.0%, 95% CI –25.2 to –2.0).

Table 1. Baseline characteristics of colorectal cancer patients stratified by vitamin D status.

	Total population ***n*** **= 798**	**Vitamin D insufficient (<50 nmol/L)** ***n*** **= 333**	**Vitamin D sufficient (⩾50 nmol/L)** ***n*** **= 448**
Age (years)	66.9 (62.2–73.0)	66.9 (61.8–73.8)	67.0 (62.8–72.5)
Gender (female)	252 (32)	91 (27)	155 (35)
Education level[a]			
Low	313 (41)	123 (38)	183 (42)
Medium	243 (32)	103 (32)	136 (32)
High	210 (27)	95 (30)	112 (26)
Unknown	32	12	17
Season of blood collection[b]			
Spring	223 (29)	129 (39)	94 (21)
Summer	227 (29)	46 (14)	181 (41)
Autumn	140 (18)	50 (15)	90 (20)
Winter	185 (24)	106 (32)	79 (18)
Unknown	23	2	4
Serum 25(OH)D_3 (nmol/l)	53.9 (40.8–70.2)	38.5 (28.9–44.5)	67.2 (58.1–79.8)
Unknown	17		
Interleukin 10 (pg/ml)	0.3 (0.2–0.4)	0.3 (0.2–0.4)	0.2 (0.2–0.4)
Unknown	67	24	28
Interleukin 8 (pg/ml)	5.8 (4.3–8.2)	5.9 (4.4–8.5)	5.7 (4.2–8.1)
Unknown	29	5	9
Interleukin 6 (pg/ml)	1.0 (0.7–1.7)	1.1 (0.8–1.8)	1.0 (0.7–1.6)
Unknown	31	5	11
Tumour necrosis factor α (pg/ml)	2.1 (1.7–2.6)	2.1 (1.7–2.6)	2.0 (1.6–2.6)
Unknown	29	6	8
C-reactive protein (μg/ml)	2.5 (1.1–6.0)	2.9 (1.2–6.9)	2.4 (1.1–5.3)
Unknown	112	40	56
Type of cancer			
Colon	512 (64)	204 (61)	298 (67)
Rectal	286 (36)	129 (39)	150 (33)
Tumour stage			
I	209 (28)	77 (24)	132 (31)
II	200 (26)	84 (26)	116 (27)
III	293 (39)	139 (43)	153 (36)
IV	52 (7)	21 (7)	30 (7)
Unknown	44	12	17

(Continued)

Table 1. (Continued)

	Total population *n* = 798	Vitamin D insufficient (<50 nmol/L) *n* = 333	Vitamin D sufficient (≥50 nmol/L) *n* = 448
Type of treatment			
Surgery only	419 (54)	167 (52)	247 (58)
Surgery + chemotherapy	171 (22)	68 (21)	97 (23)
Surgery + radiotherapy	97 (13)	53 (17)	41 (10)
surgery + chemo radiation	69 (9)	30 (10)	37 (9)
unknown	34	11	19
Comorbidities (yes)	592 (74)	248 (74)	331 (74)
Daily use of NSAIDs (yes)	157 (20)	58 (17)	98 (22)
BMI (kg/m^2)	26.5 (24.4–29.5)	26.9 (24.5–30.6)	26.2 (24.3–29.1)
Unknown	5	2	3
Smoking			
Current	98 (13)	39 (12)	57 (13)
Former	463 (59)	193 (59)	263 (60)
Never	221 (28)	97 (29)	117 (27)
Unknown	16	4	11
Moderate to vigorous physical activity (hours/week)[c]	11.5 (5.0–20.0)	10.0 (4.3–19.0)	12.8 (6.0–21.2)
Unknown	18	4	12
Dietary intake			
Dietary vitamin D (µg/day)	3.2 (2.2–4.2)	3.1 (2.2–4.2)	3.2 (2.2–4.3)
Total vitamin D (µg/day)[d]	3.8 (2.6–6.2)	3.4 (2.4–4.6)	4.3 (2.9–8.5)
Calcium (mg/day)	873 (655–1101)	880 (651–1116)	871 (656–1089)
Fibre (g/day)	21.4 (16.8–26.5)	20.9 (16.6–26.5)	21.6 (17.2–26.3)
Total fat (g/day)	71.3 (55.1–91.9)	71.6 (54.3–92.1)	70.8 (55.4–90.2)
EPA (g/day)	0.06 (0.03–0.10)	0.05 (0.03–0.10)	0.07 (0.04–0.12)
DHA (g/day)	0.08 (0.04–0.15)	0.07 (0.03–0.13)	0.08 (0.04–0.16)
Unknown	22	7	14
Use of vitamin D supplements (yes)	210 (26)	50 (15)	160 (36)
Unknown	12	3	9

Values presented are median (quartile 1 – quartile 3) or number (percentage).
BMI, body mass index; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; NSAIDs, non-steroidal anti-inflammatory drugs.
[a]Low education was defined as primary school and lower general secondary education; medium as lower vocational training and higher general secondary education; high as high vocational training and university.
[b]Spring: March–May; summer: June–August; autumn: September–November; winter: December–February.
[c]Activities with a Metabolic Equivalent score (MET score) ≥ 3 were defined as moderate to vigorous physical activity.
[d]Total vitamin D intake from diet and supplements.

Table 2. Serum 25(OH)D_3 levels and plasma inflammatory marker levels in colorectal cancer patients at diagnosis and several time points after diagnosis.

	n	25(OH)D_3	*n*	IL10 (pg/ml)	*n*	IL8 (pg/ml)	*n*	IL6 (pg/ml)	*n*	TNFα (pg/ml)	*n*	hsCRP** (μg/ml)	*n*	Inflammatory *z*-score*
At diagnosis	781	53.9 (40.8–70.2)	731	0.2 (0.2–0.4)	769	5.8 (4.3–8.2)	767	1.0 (0.7–1.7)	769	2.1 (1.7–2.6)	686	2.5 (1.1–6.0)	643	−0.5 (−2.3–1.8)
Six months after diagnosis	641	50.9 (37.2–69.7)	593	0.3 (0.2–0.5)	633	5.1 (4.0–6.8)	632	1.0 (0.7–1.8)	636	2.4 (2.0–3.0)	568	1.8 (0.9–4.1)	514	−0.5 (−2.4–1.6)
One year after diagnosis	294	52.3 (38.6–72.3)	226	0.3 (0.2–0.4)	244	4.7 (3.5–6.3)	242	0.9 (0.6–1.5)	244	2.2 (1.7–2.8)	185	1.7 (0.7–4.4)	170	−0.6 (−2.2–2.1)
Two years after diagnosis	467	64.1 (49.0–82.8)	397	0.2 (0.2–0.3)	418	5.7 (4.4–7.2)	404	0.8 (0.5–1.3)	419	1.9 (1.6–2.5)			381	0.0 (−1.8–1.6)

Values were presented as median (IQR).
*The inflammatory *z*-score was calculated as $z = (x - \mu)/\sigma$, where x is a participant's cytokine value at a given visit, and μ and σ are the study population mean and standard deviation at that visit, respectively. The combined score was calculated by summing the *z*-scores of each inflammatory marker.
**hsCRP was only measured at diagnosis, 6 months and 1 year after diagnosis.

Table 3. Association between serum 25(OH)D_3 and plasma inflammatory markers at diagnosis and several time points after diagnosis.

	n	IL10 β (95% CI)	*n*	IL8 β (95% CI)	*n*	IL6 β (95% CI)	*n*	TNFα β (95% CI)	*n*	hsCRP* β (95% CI)	*n*	Inflammatory *z*-score** β (95% CI)
25(OH)D_3 (continuous per 10 nmol/l)												
At diagnosis												
Model 1	729	−0.02 (−0.05 to 0.00)	767	−0.01 (−0.03 to 0.01)	765	−0.06 (−0.08 to −0.03)	767	−0.00 (−0.01 to 0.01)	685	−0.03 (−0.07 to 0.01)	642	−0.14 (−0.26 to −0.02)
Model 2	685	−0.02 (−0.05 to 0.01)	722	−0.00 (−0.03 to 0.02)	720	−0.07 (−0.09 to −0.04)	722	0.00 (−0.01 to 0.01)	647	−0.04 (−0.09 to 0.01)	605	−0.15 (−0.29 to −0.02)
Six months after diagnosis												
Model 1	587	−0.01 (−0.04 to 0.01)	625	−0.00 (−0.03 to 0.01)	624	−0.06 (−0.08 to −0.03)	628	0.00 (−0.01 to 0.02)	560	−0.06 (−0.10 to −0.02)	509	−0.16 (−0.29 to −0.04)
Model 2	549	0.00 (−0.03 to 0.03)	583	0.00 (−0.02 to 0.02)	581	−0.05 (−0.07 to −0.02)	585	0.01 (−0.01 to 0.02)	523	−0.03 (−0.08 to 0.02)	479	−0.09 (−0.22 to 0.05)
One year after diagnosis												
Model 1	226	−0.00 (−0.04 to 0.04)	244	−0.02 (−0.06 to 0.02)	242	−0.03 (−0.08 to 0.02)	244	−0.00 (−0.03 to 0.02)	184	−0.05 (−0.13 to 0.02)	170	−0.12 (−0.33 to 0.09)
Model 2	199	0.00 (−0.05 to 0.06)	212	0.01 (−0.04 to 0.05)	211	−0.06 (−0.13 to 0.00)	212	0.00 (−0.03 to 0.03)	155	−0.05 (−0.15 to 0.05)	146	−0.14 (−0.41 to 0.14)
Two years after diagnosis												
Model 1	397	−0.00 (−0.03 to 0.03)	418	−0.00 (−0.02 to 0.01)	404	−0.05 (−0.08 to −0.02)	418	−0.01 (−0.02 to 0.00)			361	−0.08 (−0.18 to 0.02)
Model 2	357	0.01 (−0.02 to 0.05)	376	−0.00 (−0.02 to 0.02)	364	−0.03 (−0.07 to 0.00)	376	−0.01 (−0.03 to 0.01)			343	−0.04 (−0.15 to 0.07)

Model 1, crude model; Model 2, adjusted for cohort, season of blood collection, age, gender, use of NSAIDs, BMI, physical activity, having comorbidities and stage of disease.
To interpret the beta coefficient of the regression line, the exponential of the beta should be taken (EXP^β), since a natural log transformation was done on the outcome variable.
*hsCRP was only measured at diagnosis, 6 months and 1 year after diagnosis.
**The inflammatory *z*-score was calculated as $z = (x - \mu)/\sigma$, where x is a participant's cytokine value at a given visit, and μ and σ are the study population mean and standard deviation, respectively. The combined score was calculated by summing the *z*-scores of each inflammatory marker.

When combining all data using mixed models, statistically significant inverse associations between 25(OH)D_3 and IL6, IL10 and the summary inflammatory *z*-score were observed (Table 4). A 10 nmol/l higher 25(OH)D_3 level was associated with a 5.8% (95% CI –7.7 to –3.9) lower IL6 level, a 2.0% (95% CI –3.9 to –0.1) lower IL10 level and a 15.6% (95% CI –21.1 to –7.7) lower inflammatory summary *z*-score.

When investigating the association within and between individuals, we found stronger associations within individuals compared with between individuals for IL10, IL6, TNF-α and the inflammatory *z*-score. A significant association between 25(OH)D_3 levels and TNFα was found within individuals (β –0.02, 95% CI –0.03 to –0.00) but not between individuals (β 0.00, 95% CI –0.01 to 0.01). In addition, an association between 25(OH)D_3 levels and IL10 was found within individuals (β –0.04, 95% CI –0.06 to –0.01) but not between individuals (β 0.00, 95% CI –0.02 to 0.03).

Stratified analyses. Stratified analyses for stage of disease showed slightly stronger associations between 25(OH)D_3 and IL6 in stage II (–3.9%, 95% CI –7.7 to 1.0), III (–5.8%, 95% CI –8.6 to –3.9) and IV (–4.9%, 95% CI –11.3 to 1.0) compared with stage I (–2.0% 95%CI –4.9; 0.1). Comparable results were observed for the summary *z*-score (Table 5). No differences between men and women were observed (Table 5). Finally, similar results were found in the COLON study and the EnCoRe study regarding IL10, IL8, IL6 and TNFα (Table 5). However, an association between 25(OH)D_3 and hsCRP was found in the EnCoRe study (–6.8%, 95% CI –11.3 to –2.0) but not in the COLON study (–0.1%, 95% CI –3.9 to 3.0). Consequently, the association between 25(OH)D_3 and the summary inflammatory *z*-score was stronger in the EnCoRe study (–24.4%, 95% CI –34.4 to –13.1) compared with the COLON study (–10.4%, 95% CI –18.1 to –2.0). Similar associations were observed when excluding patients with hsCRP levels >10 µg/ml and when excluding baseline measurements.

Discussion

Circulating levels of pro-inflammatory cytokines were generally low at diagnosis and during follow up in prospectively analysed CRC patients. A statistically significantly inverse association between

Table 4. Mean association over time between serum 25(OH)D_3 and plasma inflammatory markers in colorectal cancer patients.

	N/O*	IL10 β (95% CI)	N/O	IL8 β (95% CI)	N/O	IL6 β (95% CI)	N/O	TNFα β (95% CI)	N/O	hsCRP# β (95% CI)	N	Inflammatory z-score** β (95% CI)
Serum 25(OH)D_3 (continuous per 10 nmol/l)												
Unadjusted model	793/2090	–0.03 (–0.04 to –0.01)	793/2207	0.00 (–0.01 to 0.01)	793/2190	–0.05 (–0.07 to –0.04)	793/2211	–0.01 (–0.02 to –0.00)	793/1624	–0.03 (–0.06 to –0.01)	793/1509	–0.18 (–0.25 to –0.10)
Adjusted model	738/1924	–0.02 (–0.04 to –0.00)	738/2031	0.00 (–0.00 to 0.02)	738/2015	–0.06 (–0.08 to –0.04)	738/2034	–0.01 (–0.01 to 0.00)	738/1499	–0.03 (–0.06 to 0.00)	738/1395	–0.17 (–0.25 to –0.08)
Intra-individual[a]	738/1924	–0.04 (–0.06 to –0.01)	738/2031	0.00 (–0.01 to 0.02)	738/2015	–0.08 (–0.10 to –0.05)	738/2034	–0.02 (–0.03 to –0.00)	738/1499	–0.01 (–0.06 to 0.03)	738/1395	–0.25 (–0.37 to –0.13)
Inter-individual[b]	738/1924	0.00 (–0.02 to 0.03)	738/2031	–0.00 (–0.02 to 0.02)	738/2015	–0.05 (–0.07 to –0.02)	738/2034	0.00 (–0.01 to 0.01)	738/1499	–0.04 (–0.09 to –0.00)	738/1395	–0.11 (–0.22 to –0.00)

Adjusted for cohort, season of blood collection, age, gender, use of NSAIDs, BMI, physical activity, stage of disease and time × physical activity.
*N/O, number of patients/number of observations.
**The inflammatory z-score was calculated as $z = (x - \mu)/\sigma$, where x is a participant's cytokine value at a given visit, and μ and σ are the study population mean and standard deviation, respectively. The combined score was calculated by summing the z-scores of each inflammatory marker.
#hsCRP was only measured at diagnosis, 6 months and 1 year after diagnosis.
To interpret the beta coefficient of the regression line, the exponential of the beta should be taken (EXP^β), since a natural log transformation was done on the outcome variable.
[a]The beta coefficient represents the association between 25(OH)D_3 levels and inflammatory markers within individuals.
[b]The beta coefficient represents the association between 25(OH)D_3 levels and inflammatory markers between individuals.

Table 5. Association between serum 25(OH)D_3 and plasma inflammatory markers stratified by stage of disease, type of cancer, gender and cohort.

	N/O*	IL10 β (95% CI)	N/O	IL8 β (95% CI)	N/O	IL6 β (95% CI)	N/O	TNFα β (95% CI)	N/O	hsCRP	N/O	Inflammatory *z*-score** β (95% CI)
Serum 25(OH)D3 **Stage of disease**												
Stage I	206/539	−0.00 (−0.03 to 0.02)	206/577	0.02 (0.00 to 0.04)	206/573	−0.02 (−0.05 to 0.00)	206/576	−0.00 (−0.01 to 0.01)	206/426	−0.03 (−0.08 to 0.02)	206/392	−0.11 (−0.24 to 0.03)
Stage II	196/510	0.00 (−0.03 to 0.03)	196/537	−0.01 (−0.03 to 0.01)	196/531	−0.04 (−0.08 to −0.01)	196/539	0.00 (−0.01 to 0.02)	196/397	−0.04 (−0.09 to 0.02)	196/369	−0.18 (−0.23 to −0.02)
Stage III	287/767	−0.04 (−0.07 to −0.01)	287/806	−0.01 (−0.02 to 0.01)	287/801	−0.06 (−0.09 to −0.04)	287/807	−0.01 (−0.02 to 0.00)	287/665	−0.01 (−0.05 to 0.04)	287/623	−0.17 (−0.28 to −0.05)
Stage IV	49/108	−0.06 (−0.13 to 0.02)	49/111	0.06 (0.00 to 0.11)	49/110	−0.05 (−0.12 to 0.01)	49/112	−0.00 (−0.03 to 0.03)				
Gender												
Female	235/598	−0.01 (−0.04 to 0.01)	235/639	0.01 (−0.01 to 0.03)	235/633	−0.06 (−0.09 to −0.04)	235/641	0.00 (−0.01 to 0.02)	235/473	−0.05 (−0.10 to −0.01)	235/439	−0.17 (−0.30 to −0.04)
Male	503/1326	−0.02 (−0.04 to 0.00)	503/1392	−0.00 (−0.01 to 0.01)	503/1382	−0.04 (−0.06 to −0.02)	503/1393	−0.01 (−0.02 to −0.00)	503/1026	−0.01 (−0.05 to 0.03)	503/956	−0.15 (−0.25 to −0.06)
Cohort												
COLON	537/1327	−0.02 (−0.04 to 0.00)	537/1396	0.01 (−0.01 to 0.02)	537/1381	−0.05 (−0.07 to −0.03)	537/1399	−0.00 (−0.01 to 0.00)	537/997	−0.00 (−0.04 to 0.03)	537/930	−0.11 (−0.20 to −0.02)
EnCoRe	201/597	−0.02 (−0.05 to 0.00)	201/635	−0.00 (−0.02 to 0.02)	201/634	−0.05 (−0.09 to −0.02)	201/635	−0.00 (−0.02 to 0.01)	201/502	−0.07 (−0.12 to −0.02)	201/465	−0.28 (−0.42 to −0.14)
Only patients who had hsCRP levels < 10 mg/l												
	717/1758	−0.02 (−0.04 to −0.00)	717/1857	0.00 (−0.01 to 0.01)	717/1841	−0.04 (−0.06 to −0.03)	717/1323	−0.01 (−0.01 to 0.00)	717/1449	−0.00 (−0.02 to 0.02)	717/1229	−0.14 (−0.21 to −0.07)

Models are adjusted for cohort, season of blood collection, age, gender, use of NSAIDs, physical activity, stage of disease and time × physical activity

*N/O, number of patients/number of observations.

**The inflammatory *z*-score was calculated as $z = (x - \mu)/\sigma$, where x is a participant's cytokine value at a given visit, and μ and σ are the study population mean and standard deviation, respectively. The combined score was calculated by summing the *z*-scores of each inflammatory marker.

To interpret the beta coefficient of the regression line, the exponential of the beta should be taken (EXP^β), since a natural log transformation was done on the outcome variable.

$25(OH)D_3$ levels and IL6 levels was observed at diagnosis, 6 months and 1 year after diagnosis and when combining all time points. At diagnosis and when combining all time points also a significantly inverse association between $25(OH)D_3$ and the summary inflammatory *z*-score was observed.

Plasma levels of cytokines were generally low in CRC patients. We found a median level of 1.0 pg/ml for IL6, 5.8 pg/ml for IL8, 0.2 pg/ml for IL10 and 2.0 pg/ml for TNFα at diagnosis. Other studies measuring inflammatory markers in CRC patients found higher levels, with median IL6 levels ranging between 2.8 and 35.7 pg/ml,[34–38] median IL8 levels ranging between 25 and 114 pg/ml,[35,38,39] median IL10 levels ranging between 7 and 24 pg/ml[37,38,40] and median TNFα levels ranging between 16 and 272 pg/ml.[35,38] However, the study of Hopkins *et al.* found levels comparable with ours in colorectal adenoma patients (median IL6 level 1.1 pg/ml, median IL8 level 5.5 pg/ml, median IL10 level 0.5 pg/ml and median TNF level 3.4 pg/ml).[17] Differences between levels of inflammatory markers could be explained by the methods used to assess levels of inflammatory markers. The study of Hopkins and colleagues used a comparable method, namely high-sensitivity multiplex enzyme-linked immunosorbent assay (ELISA), while other studies measured inflammatory markers using a method based on flow cytometry or sandwich ELISA. Furthermore, Hopkins *et al.* measured inflammatory markers in plasma, as we did, while the other studies mentioned previously measured inflammatory markers in serum. A recent study concluded that plasma is a more sensitive matrix for detecting changes in low levels of cytokines.[41] Furthermore, that study found a higher non-specific background in serum compared with plasma cytokines.[41] The different biological specimen (plasma *versus* serum) and laboratory methods that are used make it hard to compare absolute values. To improve comparability of different studies, method harmonization is required.

In the present study, we observed a statistically significant inverse association between $25(OH)D_3$ levels and IL6 levels. To the best of the authors' knowledge, no other studies assessed the association between $25(OH)D_3$ and IL6 levels and other inflammatory markers in CRC patients. A study in colorectal adenoma patients ($n = 92$) found a non-significant reduction in IL6 levels after supplementation with vitamin D3.[17] Another study in obese individuals ($n = 147$) observed an inverse association between serum $25(OH)D_3$ and IL6 levels.[19] The association between $25(OH)D_3$ levels and IL6 is of potential interest as IL6 has an important role in chronic inflammation[42] and is also suggested to stimulate cancer progression.[22,34,43] IL6 is important in the transmission from beneficial acute inflammation to harmful chronic inflammation.[42] It is involved in the recruitment of macrophages into the tissue leading to chronic inflammation proliferation[42] and probably increases tumorigenesis. In addition, IL6 is also know to stimulate STAT3, which is an oncogene.[22] Stimulation of STAT3 promotes tumour growth by facilitating cell proliferation and inhibition of apoptosis.[22] Finally, higher levels of IL6 were associated with increased expression of matrix metalloproteases favouring tumour escape from apoptosis and metastasis.[44,45] Lowering IL6 levels could, thus, possibly improve CRC prognosis.

Apart from an association between $25(OH)D_3$ and IL6, we did not find associations between $25(OH)D_3$ and the other inflammatory markers. A possible explanation for not finding a significant association here is the central role of IL6, compared to the other inflammatory markers in chronic inflammation.[5,42,46] However, when inter- and intra-individual associations were disaggregated, intra-individual analyses also showed a significant association between $25(OH)D_3$ and TNFα and IL10. Thus, within individuals an increase in $25(OH)D_3$ levels was associated with a decrease in IL6, TNFα and IL10 levels. This indicates that within individuals an increase in $25(OH)D_3$ levels, due to either supplementation or sunlight exposure, may lead to a lower systemic inflammatory status.

This study also showed an inverse association between $25(OH)D_3$ and the summary inflammatory *z*-score. In line with our findings a study in colorectal adenoma patients ($n = 92$) also found a statistically significant lower inflammatory *z*-score after supplementation with vitamin D3.[17] In both studies a summary inflammatory *z*-score was used assuming equal contribution of each inflammatory marker. However, the role of cytokines in the progression of cancer is complex,[22] as several cytokines probably act in synergy[47] or antagonistically. In addition, it could be that some cytokines

are more important in the progression of cancer. Thus, although knowledge is currently lacking, adding a weighting factor for each cytokine might be more appropriate.

Although we found an inverse association between vitamin D levels and levels of inflammatory markers, the associations were relatively small, as a 10 nmol/l higher $25(OH)D_3$ level was associated with a 6% lower IL6 levels and a 16% lower summary *z*-score. Previous studies showed that inflammatory cytokines are associated with quality of life and cancer recurrence and survival.[8] Lowering the inflammatory status is a promising way to control cancer. However, the question remains whether this can be achieved by increasing $25(OH)D_3$ levels. To further investigate this, a well-powered intervention study focussing on the effects of increasing vitamin D levels on inflammation would be needed. Above that, further studies should elucidate underlying mechanisms involved in inflammation and the progression of CRC and the role of vitamin D in this. One way to examine this is by investigating differences in expression of genes involved in inflammatory processes in CRC tumour tissues in patients with high vitamin D levels compared with patients with low vitamin D levels.

The present study has some limitations. First, plasma levels of inflammatory markers, especially cytokines, were relatively low. This could limit the ability to detect associations. However, we found associations between $25(OH)D_3$ and IL6 levels as well as between $25(OH)D_3$ levels and the summary inflammatory *z*-score. Second, since vitamin D and inflammatory markers were measured at the same time points, we cannot conclude from this study whether vitamin D decreases inflammatory markers or the other way around. However, results of previous studies, in other populations, showed a decrease in inflammatory markers after vitamin D supplementation.[17,18] In addition, the anti-inflammatory effects of vitamin D are well studied.[11,21,48]

The present study also has some important strengths. First, this prospective study measured serum $25(OH)D_3$ and a set of inflammatory markers simultaneously before and after treatment in CRC patients. Another strength is the use of a multiplex assay, allowing to measure several cytokines at once in a single small plasma sample. Above that, our multiplex assay with chemiluminescence detection has very low detection limits, which is essential since many cytokines exist in very low levels in the peripheral blood. It should be mentioned that testing several inflammatory markers (five in total) raises the risk of false-positive findings. However, we are confident that the associations observed between $25(OH)D_3$ and IL6 are not chance findings. As we found these associations very consistently throughout our study, at several time points (at diagnosis and during follow-up), in both cohorts and in almost all strata of the stratified analyses. Furthermore, in order to exclude pre-analytical artefacts due to for example, degradation, we eliminated samples collected more than 2 years before, since several cytokines including IL6 and IL10 degrade after 2 years of storage at –80°C.[26] Finally, due the availability of detailed data on diet and other clinical and lifestyle factors, we could adjust for the most plausible confounders, although residual confounding can never be fully excluded.

To conclude, serum $25(OH)D_3$ levels were inversely associated with plasma IL6 levels and a summary inflammatory *z*-score in CRC patients at different time points before and after treatment. Further intervention studies, investigating the effect of increasing vitamin D levels on inflammatory mediators in CRC patients are needed.

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Author contributions

EW, MJLB, HJWdW, RFW, DEK, MPW, EK and FJBvD contributed to the design and the conceptualization of this study. EW, HJWdW, HvB, AJMRG, BH, ETPK, JvdO and MvZ contributed to recruitment of participants and the data collection. Statistical data analyses were done by EW. The manuscript was drafted by EW and FJBvD, and all authors critically read and revised the manuscript. All authors approved the final version of the manuscript.

Availability of data and material

Since the data consist of identifying cohort information, some access restrictions apply and therefore cannot be made publicly available. Data will be shared with permission from the acting committee of the COLON Study. Requests for data can be sent to Dr. Fränzel van Duijnhoven, Division of Human Nutrition and Health, Wageningen University & Research, The Netherlands. Email: franzel.vanduijnhoven@wur.nl.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Supplemental material

Supplemental material for this article is available online.

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