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

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/ijc.33043

Running title: Vitamin D, genotypes, and colorectal cancer survival

Keywords: 25-hydroxyvitamin D, single nucleotide polymorphism, cohort studies, survival analysis, gene-environment interaction

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; $1,25(OH)_2D$, 1,25-dihydroxyvitamin D; BMI, body mass index; CI, confidence interval; CLIA, chemiluminescence immunoassay; CPS-II, Cancer Prevention Study-II; CRC, colorectal cancer; GC, vitamin D-binding protein; EPIC, European Prospective Investigation into Cancer and Nutrition; GC, group-specific component; HR, hazard ratio; RCT, randomized clinical trial; US, United States; VDR, vitamin D receptor

Article category: Research Article

Novelty and Impact: Lower circulating vitamin D is associated with higher mortality risk among colorectal cancer (CRC) patients; however, it is unknown whether this association differs by inherited vitamin D-binding protein (GC) isoforms that impact vitamin D metabolism. In this study, vitamin D deficiency, relative to sufficiency, was associated with over 2-fold higher CRC-specific mortality risk, but only among those with the Gc2-encoding genotype, identifying a vulnerable subgroup of patients who may particularly benefit from higher vitamin D.

ABSTRACT

Lower pre-diagnostic circulating 25-hydroxyvitamin D (25[OH]D)—considered the best marker of total vitamin D exposure—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 (GC) isoform Gc2 (encoded by *GC* rs4588*C>A, Thr436Lys), which may substantially affect vitamin D metabolism and modify associations of 25(OH)D with colorectal neoplasm risk. Pre-diagnostic 25(OH)D-mortality associations according to Gc2 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 United States (Cancer Prevention Study-II) and Europe (European Prospective Investigation into Cancer and Nutrition). 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 1.44–3.49) among cases with the Gc2 isoform, and 0.94 (95% CI 0.68–1.22) among cases without Gc2 ($P_{interaction}$ = 0.0002). The corresponding HRs for all-cause mortality were 1.80 (95% CI 1.24–2.60) among those with Gc2, and 1.12 (95% CI 0.84–1.51) among those without Gc2 ($P_{interaction}$ = 0.004). Our findings suggest that the association of pre-diagnostic vitamin D status with mortality among CRC patients may differ by functional GC isoforms, and patients who inherit the Gc2 isoform (*GC* rs4588*A) may particularly benefit from higher circulating 25(OH)D for improved CRC prognosis.

INTRODUCTION

Colorectal cancer (CRC) is the second leading cause of cancer death among men and women combined globally.[1] 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 (collective term for D_2 and D_3, 25[OH]D) is considered the best marker of total vitamin D exposure and is used clinically to assess vitamin D status.[3] Lower 25(OH)D concentrations are associated with higher mortality risk among CRC patients in observational studies;[4-7] however, it is unknown whether this association differs depending on functional variants in the gene (*GC*, formerly known as group-specific component) encoding for the vitamin D-binding protein (GC, also known as DBP), which may impact vitamin D bioavailability and metabolism. Investigation of interaction between 25(OH)D and functional *GC* variants could be important for: 1) identifying subgroups of individuals in which adequate 25(OH)D may be particularly beneficial, and 2) providing biologic insight into vitamin D metabolism and CRC progression.[8]

Nearly 90% of circulating 25(OH)D is bound to the GC protein, which delivers vitamin D to target tissues and helps maintain stable 25(OH)D stores.[9, 10] The two missense variants *GC* rs4588 and rs7041 encode for three common protein isoforms—Gc1s, Gc1f, and Gc2.[11] We recently reported that associations of 25(OH)D concentrations with risk of incident, sporadic colorectal adenoma[12] and CRC[13] were stronger among individuals with the Gc2 isoform than among those with only Gc1 isoforms. Relative to the Gc1 isoforms (distinguished by the rs7041

genotype), the Gc2 isoform (determined by the rs4588 genotype) is associated with an approximately 2- to 4-fold lower 25(OH)D binding affinity[14] and 2- to 3-fold higher vitamin D-pathway induction by 25(OH)D *in vitro*[15], 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 Gc2 isoform than among those without it. We investigated whether associations of 25(OH)D with CRC-specific and all-cause mortality differed by Gc2 isoform among 1,281 CRC patients in two large prospective cohort studies in the United States (US) 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 (CPS-II) prospective cohort studies. Details of the study populations and data collection were published previously for EPIC[16] and CPS-II.[17] Briefly, EPIC recruited over 520,000 men and women from the general population in 10 western European countries from 1992 to 1998[18], and CPS-II recruited 184,194 men and women across 21 US states from 1992 to 1993.[17] 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[18] and CPS-II,[19] respectively. Detailed descriptions of case selection and exclusions for these studies are described elsewhere.[4, 18, 19] Of these 1,546 CRC cases, we further excluded 7 non-white CPS-II cases, 142 EPIC cases and 44 CPS-II cases with missing genotyping information, 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,[4, 18] and 1999–2007 in CPS-II.[20] 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.[4] 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.[20] CRC-attributable deaths were determined using the International Classification of Diseases for Oncology (ICD-O) 10th revision codes C18.0-18.7 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[19] (Heartland Assays, Ames, IA), and the OCTEIA enzyme immunoassay (Immuno Diagnostic Systems, Boldon, UK) in EPIC.[18] 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.[21] Each assay batch included National Institute of Standards and Technology 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[22] and EPIC[23] were reported previously. Individuals with the *GC* rs4588 CC, CA, and AA genotypes were classified as having Gc1-1, Gc1-2, and Gc2-2 isoform combinations (or phenotypes), respectively.[11] These

genotypes perfectly predict the expected amino acid changes of the circulating protein isoforms as determined in previous proteomic analyses.[24] 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[19, 21]). 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 seasonal variation in 25(OH)D.

CRC-specific mortality was the primary endpoint, and all-cause mortality was the secondary endpoint. Our primary exposure was circulating 25(OH)D categorized *a priori* according to clinical guidelines for vitamin D status set by the Institute of Medicine (IOM, now the National Academy of Medicine): <30 nmol/L (deficient), 30 – <50 nmol/L (insufficient), and ≥50 nmol/L (sufficient). For our primary analysis, effect modification by Gc2 was evaluated using a dominant inheritance model given the low frequency of Gc2-2 homozygotes. As a secondary analysis, we coded Gc2 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 Gc2-encoding alleles; here, 25(OH)D was dichotomized at 50 nmol/L to maximize statistical efficiency.

A Cox proportional hazards model, stratified by country of cancer diagnosis, was 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 Gc2 isoform. Age between diagnosis and censorship or death was used as the time-scale, which may better control for age and reduce bias.[25] Covariates included year of diagnosis (continuous), sex, tumor site (colon, rectum, missing/not specified), body mass index (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 the final model 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.[26] 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 Gc2 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 Gc2 isoform using Fine and Gray's competing-risks regression.[27]

All statistical tests were two-sided; a *P*-value <0.05 or a 95% confidence interval 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 Gc2

Associations of 25(OH)D concentrations with mortality among all participants and according to Gc2 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 Gc2, and

non-statistically significantly 6% lower among cases without Gc2 ($P_{interaction}$ = 0.0002). There was a dose-response association trend between lower (poorer) vitamin D status and higher mortality risk among those with Gc2 (P_{trend} = <0.0001 and 0.0002 for CRC-specific and overall mortality, respectively), but not among those without Gc2 (P_{trend} = 0.69 and 0.49 for CRC-specific and overall mortality, respectively). This pattern of effect modification by Gc2 was similar in both EPIC and CPS-II (Supplementary Table S1).

Associations of 25(OH)D concentrations with CRC-specific and all-cause mortality among all participants and according to Gc2 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 Gc1-1 cases, statistically significantly 54% higher among Gc1-2 cases, and non-statistically significantly 150% higher among Gc2-2 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 Gc1-1, Gc1-2, and Gc2-2 cases, but did not statistically significantly differ by Gc2 ($P_{interaction}$ = 0.09). The pattern of effect modification by number of Gc2-encoding alleles for CRC-specific mortality was similar in EPIC and CPS-II (Supplementary Table S2).

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 Gc2 isoform, but not among those without Gc2 (**Figure 1**). Among individuals with Gc2, 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 accounting for competing causes of death.

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 Gc2 isoform did not statistically significantly differ according to sex, stage, tumor site, or calcium intake; however, the observed effect-modification pattern by Gc2 was slightly more pronounced among rectal cancer cases, stage I-II cases, and individuals with above-median dietary calcium intake (Supplementary Table S3). In sensitivity analyses, our effect-modification findings were slightly stronger when we excluded metastatic CRC cases (Supplementary Table S4) or cases diagnosed within 1 or 3 years of their pre-diagnostic blood draw (Supplementary Table S5). There was also a similar pattern of effect modification by Gc2 when we categorized 25(OH)D using study-specific 25(OH)D tertile cut-points (Supplementary Table S6), 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, but only among those with the common Gc2-encoding *GC* rs4588*A functional variant that may affect 25(OH)D binding affinity, bioavailability, and vitamin D-pathway activation.[11, 28] 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 the association of 25(OH)D concentrations with mortality among CRC patients by GC vitamin D-binding protein isoform.

Findings from observational studies suggest an association of circulating 25(OH)D concentrations—including those measured before diagnosis[4, 5] and after diagnosis[7]—with CRC-specific mortality. Furthermore, findings from some studies indicate that 25(OH)D may be a clinically relevant prognostic factor and add value to predictive survival models for CRC patients.[7, 29] However, our findings suggest that the utility of 25(OH)D as prognostic factor among CRC patients in the US and Europe may critically depend on inherited genotypes encoding common, functional GC isoforms. If our findings are confirmed, they would support *GC* genotyping, which could be easily and affordably obtained in clinical settings, for guiding vitamin D-related therapy and survival stratification.

Evidence from randomized clinical trials (RCTs) of vitamin D supplementation improving survival of CRC patients is limited. In a US phase-II, multi-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 0–0.90, $P = 0.02$), which was the primary outcome, although no significant treatment effect was observed for overall survival.[30] Importantly, findings from a larger RCT (n=2,259) suggest that the effects of vitamin D supplementation on increasing 25(OH)D concentrations[31] and reducing colorectal adenoma risk[32] are stronger among individuals with the Gc2-encoding variant. Specifically, the effect of vitamin D supplementation on adenoma risk was statistically significantly 18% lower with each Gc2-encoding-rs4588 variant inherited (interaction relative risk = 0.82, 95% CI 0.69–0.98, $P_{interaction} = 0.03$).[32] These findings are consistent with ours, and collectively suggest that future trials should consider potential differences in supplementation effects according to Gc2 isoform. If confirmed, this effect modification could be important clinically, and for public health, given the high prevalence of the Gc2-encoding allele (40 – 50% with European ancestry[33]) and vitamin D concentrations <50 nmol/L in the US and Europe (26 – 76%, depending on age and country[3, 34]).

The Gc2 isoform is encoded by the missense *GC* rs4588*C>A variant resulting in a Threonine (Gc1)→Lysine (Gc2) amino acid substitution at residue 436.[11] Although the physiologic consequences of the isoforms have not been fully elucidated, the Gc2-encoding variant is strongly associated with lower circulating 25(OH)D concentrations and higher odds of vitamin D insufficiency.[35-37] This association may be mediated by lower GC protein concentration (20 – 30% lower among Gc2 homozygotes relative to Gc1 homozygotes in studies that did not use the isoform-biased monoclonal R&D assay[24, 38-40]) since GC mediates the renal

reabsorption of 25(OH)D and prolongs its circulating half-life.[41] Gc2 may also have lower 25(OH)D binding affinity than Gc1 isoforms[14], which, in addition to lower circulating GC, could lead to higher levels of bioavailable and free 25(OH)D available to target tissues.[9, 14, 24, 28] This may underlie the higher induction of vitamin D target genes by 25(OH)D in cultured monocytes and colon cancer cell lines with Gc2 relative to cells cultured with Gc1 isoforms.[15, 42] Importantly, 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$(OH)_2$D form, which may play an important role in CRC progression via modulating pathways involved in cell proliferation, inflammation, angiogenesis, and metastasis.[2, 43] Taken together, we hypothesize that individuals with the Gc2 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 Gc2 individuals' reduced capacity to maintain adequate 25(OH)D concentrations.

Supporting this hypothesis are other studies that reported a similar pattern of effect modification by Gc2 in relation to 25(OH)D and risk of colorectal neoplasms. 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 lower risk of incident, sporadic colorectal adenoma, but only among those with Gc2 (OR among Gc1-2/Gc2-2 = 0.51, 95% CI 0.33–0.81; OR among Gc1-1 = 1.11, 95% CI 0.68–1.82; $P_{interaction}$ = 0.05).[12] Additionally, in a pooled nested case-control study using EPIC, CPS-II, and Nurses' Health Study data (n=3,359), 25(OH)D concentrations ≥50

relative to <30 nmol/L were associated with a statistically significant 53% lower CRC risk among individuals with Gc2, but non-statistically significant 12% lower risk among individuals without Gc2 ($P_{heterogeneity}$ = 0.01).[13]

Our study strengths include its prospective design, long follow-up, and use of data from two independently conducted cohort studies of participants in the US and 10 European countries. Additional strengths include using seasonally-adjusted 25(OH)D concentrations (limiting exposure misclassification) and calibrating 25(OH)D measurements to a standard assay to permit estimating hazards using absolute clinical cut-points.

Our study has several limitations. The CPS-II sample size was small; however, the direction of the HRs within strata and the pattern of effect modification 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 Gc2-2 genotype. There may have been some misclassification of vitamin D status related to using the DiaSorin immunoassay; however, this assay is one of the most commonly used in clinical settings, and is highly concordant (r^2>0.95) with liquid chromatography-mass spectrometry.[44] 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.[45, 46] 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 geographic 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.[47] We did not collect tumor microenvironment data, such as degree and type of tumor infiltrating lymphocytes—important histologic prognostic features of CRC.[48] Given the putative immunomodulatory functions of vitamin D[2,15], future research is warranted to investigate whether and how vitamin D and GC isoforms may influence, or interact with, immune cells in the CRC tumor microenvironment. 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 GC vitamin D-binding protein isoforms, such that CRC patients with the Gc2 isoform may particularly benefit from a sufficient vitamin D status.

Financial support: This research was supported by the National Cancer Institute (NCI) of the National Institutes of Health (NIH) (F30 CA236231 to D.C.G.; R03 CA183016 to V.F.), the Anne and Wilson P. Franklin Foundation (to R.M.B.), and the World Cancer Research Fund (WCRF) (WCRF 2011-443; to M.J.). The American Cancer Society funds the creation, maintenance and updating of the Cancer Prevention Study-II (CPS-II) cohort. The coordination of European Prospective Investigation into Cancer and Nutrition (EPIC) cohort is financially supported by the European Commission (Directorate-General for Health and Consumers, DG-SANCO) and the International Agency for Research on Cancer (IARC). The national EPIC cohorts are supported by: Danish Cancer Society (Kræftens Bekæmpelse) (Denmark); Ligue Contre le Cancer, Institut Gustave Roussy, Mutuelle Générale de l'Education Nationale, and Institut National de la Santé et de la Recherche Médicale (INSERM) (France); Deutsche Krebshilfe, Deutsches Krebsforschungszentrum (DKFZ), and Bundesministerium für Bildung und Forschung (Germany); Hellenic Health Foundation (Greece); Associazione Italiana per la Ricerca sul Cancro (AIRC) and AIRE-ONLUS Ragusa, AVIS Ragusa, Sicilian Government (Italy); Dutch Ministry of Public Health, Welfare and Sports (VWS), Netherlands Cancer Registry (NKR), ZonMw, and Statistics Netherlands (The Netherlands); Health Research Fund (FIS), Regional Governments of Andalucía, Asturias, Basque Country, and Murcia, Instituto de Salud Carlos III (ISCIII) (RD06/0020), and the Catalan Institute of Oncology (Spain); Swedish Cancer Society (Cancerfonden), Swedish Scientific Council, and Regional Government of Skåne and Västerbotten (Sweden); Cancer Research UK (C570/A16491 for EPIC-Oxford) and the Medical Research Council (1000143 for EPIC-Norfolk and MR/M012190/1 for EPIC-Oxford) (UK).

Disclosure of Potential Conflicts of Interest: None declared

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Data Accessibility: Data are available by application to the EPIC Steering Committee (https://epic.iarc.fr/access/) and the American Cancer Society's Behavioral & Epidemiology Research Group (https://www.cancer.org/content/dam/cancer-org/research/epidemiology/cancer-prevention-study-data-access-policies.pdf).

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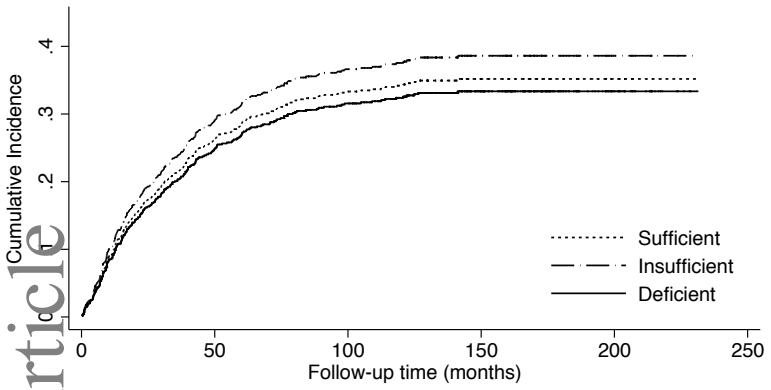
Figure Legends:

Figure 1. Adjusted cumulative incidence curves for CRC-specific mortality 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 Gc2 (*GC* rs4588*CC) and (B) patients with Gc2 (*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.

A



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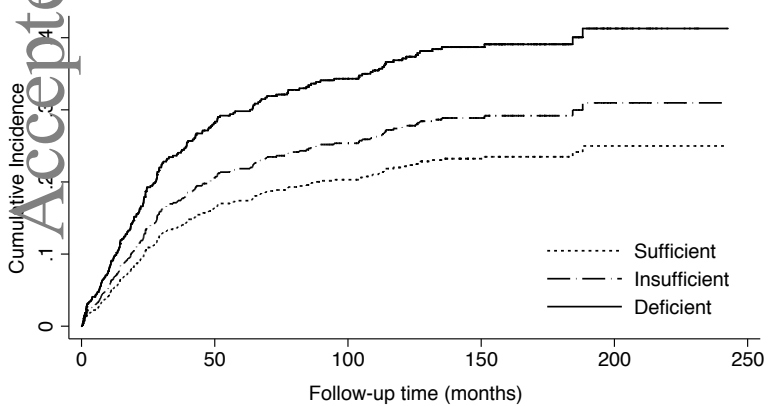

Table 1. Selected characteristics of CRC cases according to pre-diagnostic vitamin D status[a] in the EPIC and CPS-II cohorts (n = 1,281)

	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*[b]**
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[c], %										
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.

[a]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.

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

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

Table 2. Multivariable-adjusted associations of pre-diagnostic vitamin D status[a] with CRC-specific and all-cause mortality among all CRC cases and according to vitamin D-binding protein (GC) 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 GC strata	**No. total**	**No. died**	**HR (95% CI)[b]**	**No. total**	**No. died**	**HR (95% CI)[b]**	**No. total**	**No. died**	**HR (95% CI)[b]**	***P*$_{trend}$[c]**	***P*$_{interaction}$[d]**
CRC-specific mortality											
All CRC cases	322	106	1.00 (Ref)	593	241	1.09 (0.83–1.43)	366	136	1.33 (1.03–1.72)	0.02	
No Gc2 (*GC* rs4588*CC)	187	72	1.00 (Ref)	309	114	1.11 (0.78–1.57)	164	70	0.94 (0.68–1.22)	0.69	
Gc2 (*GC* rs4588*CA or AA)	135	34	1.00 (Ref)	284	127	1.29 (0.81–2.06)	202	66	2.24 (1.44–3.49)	<0.0001	0.0002
All-cause mortality											
All CRC cases	322	146	1.00 (Ref)	593	301	1.13 (0.90–1.43)	366	188	1.36 (1.09–1.70)	0.005	
No Gc2 (*GC* rs4588*CC)	187	93	1.00 (Ref)	309	148	1.26 (0.93–1.72)	164	93	1.12 (0.84–1.51)	0.49	
Gc2 (*GC* rs4588*CA or AA)	135	53	1.00 (Ref)	284	153	1.09 (0.75–1.61)	202	95	1.80 (1.24–2.60)	0.0002	0.004

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

[a]According to Institute of Medicine 2011 recommendations.

[b]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.

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

[d]$P_{interaction}$ between vitamin D status and GC isoform calculated using a likelihood ratio test.

Table 3. Multivariable-adjusted associations of pre-diagnostic vitamin D status[a] with CRC-specific and all-cause mortality among all CRC cases and according to vitamin D-binding protein (GC) 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 GC strata	**No. total**	**No. died**	**HR (95% CI)[b]**	**No. total**	**No. died**	**HR (95% CI)[b]**	***P*$_{interaction}$[c]**
CRC-specific mortality							
All CRC cases	322	106	1.00 (Ref)	959	377	1.22 (0.97–1.52)	
Gc1-1 (*GC* rs4588*CC)	187	72	1.00 (Ref)	473	184	0.96 (0.72–1.29)	
Gc1-2 (*GC* rs4588*CA)	120	32	1.00 (Ref)	390	149	1.54 (1.02–2.32)	0.003
Gc2-2 (*GC* rs4588*AA)	15	2	1.00 (Ref)	96	44	2.50 (0.56–11.1)	
All-cause mortality							
All CRC cases	322	146	1.00 (Ref)	959	489	1.21 (1.00–1.47)	
Gc1-1 (*GC* rs4588*CC)	187	93	1.00 (Ref)	473	241	1.06 (0.83–1.37)	
Gc1-2 (*GC* rs4588*CA)	120	48	1.00 (Ref)	390	194	1.33 (0.94–1.86)	0.09
Gc2-2 (*GC* rs4588*AA)	15	5	1.00 (Ref)	96	54	1.13 (0.41–3.05)	

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

[a]According to Institute of Medicine 2011 recommendations.

[b]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.

[c]$P_{interaction}$ between vitamin D status and GC isoform calculated using a likelihood ratio test.