Genetic predictors of circulating 25-hydroxyvitamin D and prognosis after colorectal cancer

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Conflict of interest

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Abstract:

Background:

Low serum 25-hydroxyvitamin D (25(OH)D) concentrations in colorectal cancer (CRC) patients have been consistently associated with higher mortality in observational studies. It is unclear whether low 25(OH)D levels directly influence CRC mortality. To minimize bias, we use genetic variants associated with vitamin D levels to evaluate the association with overall and CRC-specific survival.

Methods:

Six genetic variants have been robustly identified to be associated with 25(OH)D levels in genome-wide association studies. Based on data from the International Survival Analysis in Colorectal Cancer Consortium (ISACC) the individual genetic variants and a weighted genetic risk score were tested for association with overall and CRC-specific survival using Cox proportional hazards models in 7 657 stage I-IV CRC patients of which 2 438 died from any cause and 1 648 died from CRC.

Results:

The 25(OH)D decreasing allele of single nucleotide polymorphism (SNP) rs2282679 (*GC*) was associated with poorer CRC-specific survival, although not significant after multiple-testing correction. None of the other five SNPs showed an association. The genetic risk score showed non-significant associations with increased overall (HR=1.54, 95% CI:0.86-2.78) and CRC-specific mortality (HR=1.76, 95% CI:0.86-3.58). A significant increased risk of overall mortality was observed in women (HR=3.26, 95% CI:1.45-7.33, p-value for heterogeneity=0.01) and normal-weight individuals (HR=4.14, 95% CI:1.50-11.43, p-value for heterogeneity=0.02).

Conclusions:

Our results provided little evidence for an association of genetic predisposition of lower vitamin D levels with increased overall or CRC-specific survival, although power might have been an issue.

Impact:

Further studies are warranted to investigate the association in specific subgroups.

Introduction:

Colorectal cancer (CRC) belongs to the most common cancer types (third) and is the second leading cause of cancer related death globally (1). Despite improved therapy regimens which have led to increased survival after diagnosis, survival time is still limited for advanced stages (2).

One of the factors with potential prognostic relevance is 25(OH)D levels since the vitamin D receptor is highly expressed in the colon (3,4). The most stable and therefore most reliable indicator of circulating vitamin D is 25(OH)D; it is influenced by both dietary intake and skin synthesis by sun exposure (5). Low levels of 25(OH)D have been consistently found associated with reduced overall (hazard ratio (HR): 0.68, 95% confidence interval (CI): 0.55–0.85) and CRC-specific survival (HR: 0.67, 95% CI: 0.57–0.78), as evidenced in a recent meta-analysis (6). Therefore, vitamin D status could be a potential modifiable factor for improving prognosis in CRC patients.

It is unclear whether a genetic predisposition of higher/lower vitamin D levels is involved in mechanisms leading to better survival or whether low vitamin D levels are primarily an indicator for poor health (7). Studies assessing post-diagnostic vitamin D concentrations need to be interpreted with caution as lower vitamin D levels in patients with poorer health could also be due to behavior changes after diagnosis and treatment e.g. less sun exposure. The association between postdiagnostic vitamin D and survival after CRC in observational studies could thus reflect confounding or reverse causation.

Previous genome-wide association studies (GWAS) have identified six single nucleotide polymorphisms (SNPs) associated with circulating 25(OH)D levels at genome-wide significance (p-value$<5x10^{-8}$) (8,9). The four SNPs with the strongest influence on vitamin D levels have been replicated by several studies (8,9,32). As genetic variants are randomly allocated during gamete formation independent of environmental factors, confounding should not play a role. Therefore, determining associations using vitamin D-related genetic variants could help minimize confounding bias and reverse causation.

We aimed to estimate the relationship of these six vitamin D related genetic variants with overall and CRC-specific survival in studies collaborating in the International Survival Analysis in Colorectal Cancer Consortium (ISACC).

Materials and Methods:

Study population and genotype data:

This analysis is based on 7 657 CRC patients from 10 studies with available genotyping and follow-up data participating in the ISACC Consortium. The studies are Darmkrebs: Chancen der Verhuetung durch Screening (DACHS) (10,11), Diet, Activity and Lifestyle Study (DALS) (12), Cancer Prevention Study II Nutrition cohort (CPSII) (13), Health Professionals Follow-up Study (HPFS) (14), Nurses' Health Study (NHS) (15-17), Physicians` Health Study (PHS) (18); Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial (PLCO) (19,20), Post-Menopausal Hormone- Seattle Colon Cancer Family Registry Study (PMH-CCFR), VITamins And Lifestyle Study (VITAL) (21) and Woman's Health Initiative (WHI) (22) (Supplementary Table 1). Nine of the ten studies are also included in the Genetics and Epidemiology of Colorectal Cancer Consortium (GECCO) (23) and one study, CPS II, is part of the Colorectal Cancer Transdisciplinary consortium (CORECT) (24). Details on the consortia and studies have been described previously (13,23,24) and study descriptions are provided in the supplementary information (Supplementary Table 1). Participant overlap between the studies has been excluded. All participants provided written, informed consent and studies were approved by their respective institutional review boards. Demographic and lifestyle factors were queried by in-person interviews or self-filled questionnaires. A multistep data harmonization procedure was carried out centrally for pooled analyses (25). Details of assessment of survival in the individual studies have been previously published (10,12-14,17,21,26-29). In short, vital status was obtained either using active follow-up with confirmation of death by review of death certificates or medical records or the studies used linkage to state death records or state cancer registries. Alive patients were censored at the date of last follow-up or data linkage.

Genotype data and imputation

All included studies provided genotype information. See previously published reports for details on genotyping, quality assurance and imputation (23,24,30). Imputation was conducted using the imputation panel of the Haplotype Reference Consortium (31). Exclusion of SNPs was based on call rate (<98% in GECCO; <95% in CORECT), Hardy-Weinberg equilibrium in controls (p-value<$1x10^{-}$

^{4}) or low minor allele frequency (≤1%). Patients were assigned values of 0, 1, or 2 for having 0 (wild-type homozygous), 1 (heterozygous) or 2 (homozygous for the risk allele) alleles associated with lower vitamin D levels for each SNP. For imputed SNPs, patients received continuous values between 0 and 2.

SNP selection and Genetic Risk Score

Six SNPs, which were found associated with 25(OH)D levels at genome-wide significance (p-value<5x10-8) in a GWAS of European populations, were selected (8,9,32) (Table 1). Two of these SNPs have been recently discovered by the SUNLIGHT Consortium (8), which also confirmed the other four previously identified SNPs (9). The six SNPs explain around 2.8% of the variance in circulating 25(OH)D levels (8).

Linkage disequilibrium between the individual SNPs was checked, and no strong correlation was detected (R^2<0.01). A weighted genetic risk score (GRS) consisting of the six SNPs was calculated for each person as the sum of the number of vitamin D decreasing alleles weighted by their effect on vitamin D levels as reported in Jiang et al. (8). As Jiang et al. used natural log transformed vitamin D levels as outcome in their GWAS, units of the GRS are not easily interpretable. The risk score ranges between 0 and 0.432. Results are reported per one unit increase in risk score.

According to Milaneschi et al. (33) a one unit increase in this risk score is associated with a change of 5.29nmol/l (95% CI:4.18-6.39).

Statistical analysis:

The association of the individual SNPs with overall survival and CRC-specific survival was assessed using cox proportional hazard models. Analyses were adjusted for age, sex and principal components (PCs) of genetic ancestry to control for potential confounding due to population substructure (PCs were calculated using the EIGENSTRAT method (https://reich.hms.harvard.edu/software)). Three PCs were used for Analysis of GECCO studies used three PC and 10 PCs were used for CPSII from the CORECT consortium. Analyses were conducted for GECCO and CORECT studies separately. Fixed-effects meta-analysis was employed to get summary results for GECCO and CORECT. Bonferroni correction was used to account for multiple testing of six single SNP tests (p<0.05/6=0.0083).

Additional stratified analyses were carried out by sex, cancer site (colon/rectum), stage (stage 1-local, stage 2,3-regional, stage 4-distant) and BMI categories (self-reported pre-diagnostic BMI) in kg/m² (BMI 18.5–24.9 for normal weight, 25-30 for overweight, >30 for obese, excluding the 1% with BMI<18.5). Heterogeneity was assessed using likelihood ratio tests which compare the models including / excluding interaction term.

To calculate time-to-event, date of diagnosis was considered as the starting point and follow-up time was censored at death or end of follow-up, whichever occurred first. For calculation of CRC-specific survival, censoring was done at the time of death for all patients who died from other causes than CRC The reverse Kaplan-Meier method was employed for calculation of median follow-up time (34).

Results:

Of 7 657 CRC patients included for this analysis, 2 438 died from any cause and 1 648 died from CRC after a median follow-up time of 54.8 month (interquartile range: 27.7-73.6 months). Supplementary Table 2 shows selected characteristics of the study population and numbers per GECCO/CPSII. Among patients included, 54.6% were women and 45.4% were men.

The association of the vitamin D associated SNPs with overall and with CRC-specific survival were similar, see Table 2. None of the six SNPs were statistically significantly associated with overall survival. There was a significant association between SNP rs2282679 and CRC specific survival (HR: 1.08, 95% CI: 1.00-1.16) (Table 2), which did not remain significant when applying a Bonferroni corrected p-value of <0.0083. None of the other five SNPs showed significant associations with CRC specific survival. Results per GECCO/CPSII studies are shown in Supplementary Table 3 for overall survival and CRC-specific survival.

The GRS representing genetically determined lower levels of vitamin D was not significantly associated with risk of death after CRC diagnosis (HR per one unit of GRS: 1.54, 95% CI: 0.86-2.78) (Table 3). For CRC-specific survival, a similar non-significant association was found for lower genetically determined levels of vitamin D (HR per one unit of GRS: 1.76, 95% CI: 0.86-3.58).

Exploration of effect heterogeneity yielded differential associations for overall survival according to sex and BMI (Table 3). A higher GRS was significantly associated with increased overall mortality (HR: 3.26, 95% CI: 1.45-7.33) in women and not in men (HR: 0.68, 95% CI: 0.29-1.62). In addition, the association of higher GRS with increased overall mortality was only significant in normal weight patients (HR: 4.14, 95% CI: 1.50-11.43) but not obese patients (HR: 1.67, 95% CI: 0.53-5.26). In overweight patients the SNP association was in the opposite direction (HR: 0.55, 95% CI: 0.21-1.45). No significant differential associations were found for CRC-specific survival, but associations in the subgroups were generally similar to that for overall survival in magnitude and direction (Table 3).

Discussion

In this large study, we investigated the association between six genetic variants and a GRS associated with vitamin D levels and overall and CRC-specific survival. The single SNPs and GRS were not significantly associated with overall/CRC-specific survival. We found significant effect heterogeneity by sex and BMI in the association of GRS for vitamin D and overall survival.

Recent large observational studies reported inverse associations between serum 25(OH)D levels and survival after CRC (4,35,36). Except for one study that used pre-diagnostic 25(OH)D levels (35) all studies used post-diagnostic vitamin D levels which could already have been influenced by the disease or a poor health status after treatment (4,36). We and others have thus employed vitamin D related genetic variants as instrumental variables to evaluate the association with CRC mortality in order to minimize bias due to reverse causation and confounding.

A recent study that investigated only rs2282679 (*GC* gene) found a non-significant association with reduced overall survival in 489 CRC patients, corroborating our findings. Additionally, they reported a significant association of the vitamin D lowering allele with poorer disease-free survival (37), however, CRC-specific survival was not investigated. A further study that investigated three SNPs rs2282679 (*GC*), rs10741657 (*CYP2R1*) and rs12785878 (*DHCR7*) in association with time to recurrence in patients with stages II and III colon cancer found only rs2282679 to be significantly associated with decreased time to recurrence in the subgroup of patients who underwent only surgery (38).

Mendelian randomization (MR) studies on vitamin D levels have also been conducted. These MR studies were not conducted specifically in CRC patients and CRC-specific death was not investigated as outcome in any of these studies. A former large MR study of 95 766 participants investigated the association of SNPs associated with lower vitamin D levels (using a GRS score of two SNPs in the *DHCR7* gene and two SNPs in the *CYP2R1* gene explaining around 1% in variation in vitamin D levels) with all-cause mortality and cancer-specific mortality and found that the genetic instrument was associated with increased all cause and cancer-specific mortality (39). In contrast to that, a study in women of 33 682 participants and 3 985 cancer cases found no significant association of a five SNP

instrument and overall or cancer mortality (40). A recent study using UK Biobank data (438 870 participants and 6 998 cancer-specific deaths) did not find evidence for genetically determined low vitamin D levels (using five vitamin D related variants, four of them overlapping with the variants used in our study) and increased cancer mortality (41). The variance in vitamin D levels explained by a genetic instrument using the previously known four SNPs, which were confirmed by Jiang et al., was estimated to be approximately 5% (42). Jiang et al. estimated that the six SNPs used in our study explain around 2.8% of genetic variation; 7.5% of variation was explained by all common GWAS variants (8).

We found sex-differences in the association of the GRS with overall survival. Some observational studies have also observed differences by sex (43) or significant associations of vitamin D levels with cancer incidence (44) or mortality only in women (45) but not in men. In the present study, the GRS conferring lower vitamin D levels was found associated with worse prognosis only in women. One reason for this could be stronger associations of vitamin D levels with diseases that are more common in women than in men, like breast cancer (46) and osteoporosis (47). But whether the vitamin D related SNPs are also associated with breast cancer mortality or mortality after osteoporosis (e.g. as consequence of fractures) is unclear. A lookup in the BCAC consortium including 42 124 patients with 3 733 breast cancer-specific deaths showed that rs2282679 is not associated with breast cancer specific mortality (http://bcac.ccge.medschl.cam.ac.uk/). A SNP in the vitamin D receptor gene, which was previously reported to be significantly associated with higher breast cancer specific mortality in 498 breast cancer patients (48), has not been found associated with vitamin D levels in GWAS. In addition, our finding of higher mortality with a GRS for lower 25(OH)D levels only in normal weight people is interesting and it is consistent with results of a recent RCT in the VITAL study of vitamin D supplementation and all cancer incidence (49) which found lower all-cancer incidence only in normal weight participants receiving vitamin D compared to placebo and not in overweight or obese participants. It is known that overweight and obese people have lower levels of vitamin D compared to normal weight people (7) which could be due to sequestration of vitamin D in adipose tissues or dilution of ingested vitamin D (7). In this case higher levels of 25(OH)D would be needed for protection in overweight/obese individuals. Also there is a difference in direction of point estimates

when comparing the overweight and obese groups. Since the confidence intervals are large, these differences could be due to chance because of power issues for the subgroup analyses and therefore needs to be investigated in further larger studies.

Regarding mortality after CRC diagnosis, RCTs of vitamin D supplementation are also being conducted. Two recent meta-analysis reported that vitamin D supplementation reduced all-cancer death by 16% (50) and 13% (51), CRC death was not specifically investigated. One small RCT has been published (52) on vitamin D supplementation in CRC patients which was conducted in Croatia and randomized 71 metastatic CRC patients to either standard chemotherapy or standard chemotherapy plus 2000IU vitamin D. No difference in overall or progression-free survival between groups was observed (52). A further RCT of 139 metastatic CRC patients reported in a conference abstract a longer progression-free survival in patients who received high vitamin D supplementation compared to low vitamin D supplementation (53). A more recent RCT found suggestive evidence that high dose compared to standard-dose vitamin D supplementation combined with chemotherapy could improve survival for patients with advanced or metastatic CRC (54). Supplementation for CRC patients might have a different effect on mortality after CRC compared to genetically determined differences in vitamin D levels. And this RCT suggests that higher than physiologic levels may lead to improvement in survival.

Strengths of our study include the large sample size, which is the largest published sample size among CRC survivors to date for investigation of the association of vitamin D related genetic variants with overall and CRC-specific survival. We used the latest published set of SNPs discovered in GWAS to be associated with vitamin D levels. The association of vitamin D related SNPs with CRC-specific survival has been investigated for the first time. Moreover, all included studies carried out a comprehensive follow-up with long follow-up duration. Our study has also some limitations. As this study is based on populations of European descent, the generalizability to other populations is limited. The advantage is that we were able to minimize bias due to confounding by population stratification. We did not have serum measurements of vitamin D levels available for all included studies; therefore we were not able to analyze the strength of the association of the SNPs with vitamin D levels in our

study. Although our study has a large sample size, power to conduct a Mendelian randomization study is still limited due to the moderate association of the SNPs with decreasing vitamin D levels.

In conclusion, this is the first study to examine the most recent set of vitamin D related SNPs discovered in GWAS with respect to overall and also CRC-specific mortality among CRC patients. We did not find evidence for an association of genetically determined lower vitamin D levels and higher mortality. The potential effect heterogeneity by sex and BMI requires confirmation. Further larger studies are warranted to investigate the association of vitamin D levels and survival after CRC also in specific subgroups.

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Tables

Table 1: Single nucleotide polymorphisms identified in genome-wide association analyses for circulating 25-hydroxyvitamin D concentrations

SNP	Chr	Gene	Effect allele[a]	beta[b]	Standard error
rs2282679	4	GC	G	-0.089	0.0023
rs10741657	11	CYP2R1	G	-0.031	0.0022
rs12785878	11	DHCR7	G	-0.036	0.0022
rs6013897	20	CYP24A1	A	-0.026	0.0027
rs10745742	12	AMDHD1	C	-0.017	0.0022
rs8018720	14	SEC23A	C	-0.017	0.0029

Abbreviations: Chr, chromosome; SNP, single nucleotide polymorphism.
[a]effect allele of the vitamin D level decreasing allele.
[b] beta value and standard error as reported in Jiang et al. (8) for the association of the genetic variants with natural log transformed vitamin D levels.

Table 2: Association between the vitamin D decreasing allele of vitamin D related genetic variants and overall and CRC-specific mortality after CRC diagnosis

SNP	No (total)	No (events[a])	HR[b]	95% CI	p-value
Overall mortality					
rs2282679	7657	2438	1.06	1.00-1.13	0.07
rs10741657	7657	2438	0.99	0.93-1.05	0.75
rs12785878	7657	2438	1.05	0.98-1.11	0.17
rs6013897	7657	2438	0.95	0.89-1.02	0.19
rs10745742	7657	2438	1.00	0.95-1.06	0.94
rs8018720	7657	2438	0.98	0.91-1.05	0.57
CRC-specific mortality					
rs2282679	7657	1648	1.08	1.00-1.16	<0.05
rs10741657	7657	1648	0.99	0.92-1.06	0.73
rs12785878	7657	1648	1.04	0.96-1.13	0.31
rs6013897	7657	1648	0.97	0.88-1.05	0.44
rs10745742	7657	1648	1.00	0.93-1.07	0.97
rs8018720	7657	1648	0.97	0.89-1.07	0.57

Abbreviations: CI, confidence interval, GRS, genetic risk score; HR, hazard ratio; No, number; SNP, single nucleotide polymorphism.
[a]Median follow-up time: 54.8 months (interquartile range: 27.7-73.6)
[b]Adjusted for age, sex, genotyping phase and 3 PC for GECCO, and 10 PC for CPSII, respectively.

Table 3: Association between GRS for vitamin D levels and mortality after CRC diagnosis stratified according subgroups

		Overall mortality[b]				CRC-specific mortality[c]				
Group	**N (total)**	**N (events)**	**HR[a]**	**95% CI**	**p. het[d]**	**N (total)**	**N (events)**	**HR[a]**	**95% CI**	**p. het[d]**
GRS	7657	2438	1.54	0.86-2.78		7657	1648	1.76	0.86-3.58	
Sex										
Female	4182	1278	3.26	1.45-7.33	0.01	4179	912	3.32	1.28-8.60	0.15
Male	3473	1160	0.68	0.29-1.62		3466	736	0.78	0.27-2.29	
Cancer site										
Colon	5824	1870	1.49	0.76-2.92	0.98	5816	1230	1.34	0.58-3.06	0.64
Rectum	1755	535	1.46	0.41-5.16		1753	395	3.16	0.74-13.20	
Stage										
Stage 1 / local	2325	356	3.88	0.79-19.00	0.39	2324	95	11.07	0.56-217.56	0.65
Stage 2,3 / regional	4010	1124	1.27	0.54-3.01		4004	710	1.52	0.52-4.48	
Stage 4 / distant	939	788	2.04	0.69-6.02		936	735	1.67	0.55-5.13	
BMI										
Normal	2549	824	4.14	1.50-11.43	0.02	2545	563	3.78	1.12-12.78	0.51
Overweight	3190	961	0.55	0.21-1.45		3185	645	0.93	0.28-3.01	
Obese	1771	594	1.67	0.53-5.26		1770	306	1.32	0.33-5.28	

Abbreviations: CI, confidence interval, GRS, genetic risk score; HR, hazard ratio; No, number; p.het, p-value for heterogeneity; SNP, single nucleotide polymorphism.

[a] Adjusted for age, sex, genotyping phase and 3 PC for GECCO, and 10 PC for CPSII, respectively.

[b] weighted genetic risk score for overall survival is computed out of the sum of vitamin D decreasing alleles of the six vitamin D associated SNPs multiplied by their effect on vitamin D levels (e.g. beta from Table 1). The HR indicates risk of death per 1 unit change in GRS. The risk score ranges between 0 and 0.432. One unit change in GRS is associated to a change in vitamin D levels of 5.29nmol/l according to Milaneschi et al (33).

[c] weighted genetic risk score for CRC-specific survival is computed out of the sum of vitamin D decreasing alleles of the six vitamin D associated SNPs multiplied by their effect on vitamin D levels (e.g. beta from Table 1). The HR indicates risk of death per 1 unit decrease in GRS. The risk score ranges between 0 and 0.432. One unit change in GRS is associated to a change in vitamin D levels of 5.29nmol/l according to Milaneschi et al. (33).

[d] p-value calculated using likelihood ratio tests comparing the model with and without interaction term.

Data and Materials Availability

Genotyping data of the GECCO studies are available at the database of Genotypes and Phenotypes (dbGaP) for download at the accession number: phs001078.v1.p1.

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Genetic predictors of circulating 25-hydroxyvitamin D and prognosis after colorectal cancer

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