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ORIGINAL REPORT

Association of Vitamin D Levels With Outcome in Patients With Melanoma After Adjustment For C-Reactive Protein

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ABSTRACT

Purpose

To evaluate for an association between 25-hydroxyvitamin D levels (vitamin D) and outcome measures in patients with melanoma after evaluation is controlled for systemic inflammatory response (SIR) on the basis of simultaneous C-reactive protein (CRP) measurement.

Materials and Methods

Plasma samples from 1,042 prospectively observed patients with melanoma were assayed for vitamin D and CRP. The associations of demographics and CRP with vitamin D were determined, followed by a determination of the association between vitamin D and stage and outcome measures from the date of blood draw. The vitamin D level was considered sufficient if it was 30 to 100 ng/mL. Kaplan-Meier and Cox regression analyses were performed.

Results

The median vitamin D level was 25.0 ng/mL. The median follow-up time was 7.1 years. A lower vitamin D was associated with the blood draw during fall/winter months ($P < .001$), older age ($P = .001$), increased CRP ($P < .001$), increased tumor thickness ($P < .001$), ulcerated tumor ($P = .0105$), and advanced melanoma stage ($P = .0024$). On univariate analysis, lower vitamin D was associated with poorer overall (OS; $P < .001$), melanoma-specific survival (MSS; $P = .0025$), and disease-free survival (DFS; $P = .0466$). The effect of vitamin D on these outcome measures persisted after adjustment for CRP and other covariates. Multivariable hazards ratios per unit decrease of vitamin D were 1.02 for OS (95% CI, 1.01 to 1.04; $P = .0051$), 1.02 for MSS (95% CI, 1.00 to 1.04; $P = .048$), and 1.02 for DFS (95% CI, 1.00 to 1.04; $P = .0427$).

Conclusion

Lower vitamin D levels in patients with melanoma were associated with poorer outcomes. Although lower vitamin D was strongly associated with higher CRP, the associations of lower vitamin D with poorer OS, MSS, and DFS were independent of this association. Investigation of mechanisms responsible for these associations may be of value to patients with melanoma.

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INTRODUCTION

Vitamin D deficiency has been associated with risks of morbidity and mortality from diabetes and cardiovascular disease, as well as with several cancers, including melanoma.[1-4] Vitamin D has anti-inflammatory properties, has antiproliferative effects on melanoma cells, can inhibit tumor growth[5] and tumor invasiveness,[6] and promotes melanoma cell DNA repair.[7] However, investigations of dietary vitamin D intake or blood levels of vitamin D with melanoma risk have yielded inconsistent results.[8-11] Vitamin D deficiency has been associated with advanced melanoma stage[12]; conversely, elevated vitamin D has been associated with thinner tumors and longer survival.[13] These findings are intriguing but require validation.

Factors that influence blood vitamin D levels have also been associated with melanoma risk or patient outcome. For example, although sun exposure promotes vitamin D synthesis[14,15] it conversely increases the risk of developing melanoma.[16] Markers of the systematic inflammatory response (SIR), specifically C-reactive protein (CRP), have been associated with survival in patients with melanoma[12,17]; our recent investigation has

provided validated evidence that elevated CRP independently predicts poorer melanoma-specific survival (MSS)[18]; in that investigation, we did not evaluate vitamin D levels. Because CRP levels are inversely associated with vitamin D levels in blood, and because levels of vitamin D, an acute-phase reactant, decline with inflammation,[19] measured vitamin D levels in patients with melanoma could reflect SIR. Coordinated investigation of vitamin D and CRP in outcomes of patients with melanoma has not been undertaken previously. We therefore conducted a hospital-based investigation in which blood samples were collected after diagnosis to examine the relationship between blood vitamin D levels and outcomes in patients with melanoma, and we accounted for important confounders, including SIR as assessed by simultaneous CRP measurement.

MATERIALS AND METHODS

Study Design

This study is part of an ongoing prospective investigation that includes patients with all stages of invasive cutaneous melanoma. Individuals gave written informed consent, and the protocol was approved by the institutional review board at The University of Texas MD Anderson Cancer Center. Peripheral blood samples were collected at study entry from 3,189 non-Hispanic white patients with melanoma and cancer-free controls recruited between August 1997 and August 2009.

Data were collected from patient records and maintained in the Melanoma Informatics, Tissue Resource, and Pathology Core Resource. Disease stage was determined according to the 2009 edition of the American Joint Committee on Cancer Cancer Staging Manual.[20] Primary outcome measures were overall survival (OS; time from date of blood draw to date of death, or censored as date of last follow-up if still alive at conclusion of follow-up), MSS (time from date of blood draw to date of death as a result of melanoma, or censored as date of last follow-up/death if death not a result of melanoma), and disease-free survival (DFS; time from date of blood draw to date of relapse of melanoma, or censored as date of last follow-up if without relapse).

Experiments

Heparinized plasma samples were stored at −80°C. A total of 1,042 patients had sufficient plasma stocks for testing (Appendix Fig A1, online only). Sequential blood draws were performed in 66 patients at follow-up. Vitamin D levels were tested as 25-hydroxyvitamin D (25-OH D, including 25-OH D2 and 25-OH D3) and were measured via enzyme immunoassay (AC-57F1; Immunodiagnostic Systems, Gaithersburg, MD). The minimum detection level was 2 ng/mL. CRP was tested via enzyme-linked immunosorbent assay (catalog no. DCRP00; R&D Systems, Minneapolis, MN). The minimum detection level was 0.010 ng/mL. All tests were batch processed in the same laboratory. Determinations of vitamin D and CRP levels were performed by personnel blinded to the clinical status of the patient. Among 1,042 patients, 44 (4.2%) had blood drawn before definitive locoregional surgery, 84 (8.1%) had blood drawn within 2 weeks after surgery, and 914 (87.7%) had blood drawn more than 2 weeks after surgery. All blood samples were drawn before initiation of systemic therapy.

Statistical Analysis

To determine whether patient demographics, including blood draw season, affected vitamin D, we performed Spearman's ρ test and analysis of variance for correlations between continuous variables and vitamin D, and we used the Wilcoxon rank-sum test to assess associations between dichotomous categorical variables and vitamin D. We included standard clinical features associated with patient outcome. In addition, we investigated the potential influence of storage time on vitamin D. Consistent with standard clinical practice and prior reports,[13,21] we used raw vitamin D as a predictor to investigate the relationship between continuous vitamin D and OS, MSS, and DFS. In contrast, because of the highly skewed nature of the distribution of CRP and to be consistent with our previous analysis,[22] we used log-transformed CRP (log CRP) as a predictor in the multivariable model. We dichotomized vitamin D at 20 ng/mL (by convention, levels $<$ 20 ng/mL were considered deficient). We also classified patients (again, by convention) into levels of less than 20 ng/mL (deficiency), 20 to 30 ng/mL (insufficiency), or at least 30 ng/mL[23,24] (sufficiency) and evaluated—with or without adjustment for age at blood draw, sex, disease stage at blood draw, log CRP, and blood draw season (spring/summer [May to October], fall/winter, [November to April]) —whether these categories were associated with clinical outcomes. To determine the best threshold of vitamin D for predicting OS, we performed recursive partitioning of vitamin D and recorded the split that led to the smallest P values in the log-rank test by using the party module in R software v 2.15.0.[24a] Survival analysis was executed with the functions coxph and survfit from the R package survival.

All analyses (except for recursive partitioning) were performed using SAS Enterprise Guide 4.3 (SAS Institute, Cary, NC). Kaplan-Meier survival analysis was performed, Cox regression models were built to estimate hazard ratios (HRs), and adjusted survival curves were plotted.[25] All P values were two sided. P values less than .05 were considered statistically significant.

RESULTS

Demographic and Clinical Data

To determine whether vitamin D levels changed with time from diagnosis, we examined for an association between vitamin D and time from diagnosis to blood draw. Our data demonstrated a small, nonsignificant decrease in vitamin D with increasing time from diagnosis (Pearson correlation test $r^2 = -0.01145$ [$P = .712$]; Spearman correlation test $r^2 = -0.05302$ [$P = .087$]). To evaluate whether vitamin D levels changed over time in individual patients with melanoma, 66 patients for whom serial blood samples were available underwent testing. There was no association between vitamin D levels and time between blood draws (median, 11.4 months apart; range, 0.3 to 65.8 months apart; Spearman correlation test $r^2 = -0.00434$ [$P = .9724$]). The median blood draw time from diagnosis was longer in patients with stage III/IV disease (5.3 months with stage I/II v 12 months with stage III/IV; $P < .001$; data not shown), which reflected the referral nature of this population in particular, and median vitamin D levels were lower in patients with stage III/IV disease (25.58 ng/mL with stage I/II v 23.94 ng/mL with stage III/IV; $P = .002$; Appendix Table A1, online only). However, the lack of dependence of vitamin D on time from diagnosis to blood draw and the lack of association of vitamin D with interval between blood draws suggested that lower vitamin D levels in patients with stage III/IV disease were related to the disease stage itself rather than to the longer blood draw interval. Because vitamin D levels did not seem to decline significantly over time after a diagnosis of melanoma, we did not include time from diagnosis to blood draw as a covariate in our analyses. In addition, because we found no relationship between the length of time from surgery to blood draw and vitamin D level (Spearman correlation test $r^2 = -0.02444$; $P = .43$), we did not include time from surgery to blood draw as a covariate in our analyses.

Table 1. Demographic and Clinical Characteristics in 1,042 Patients With Melanoma

Characteristic	Data Set (N = 1,042)
Age at blood draw, years	
Median	54.8
Interquartile range	44.2-66.0
No. (%) female	452 (43.4)
Tumor thickness, mm	
Median	1.2
Interquartile range	0.7-2.4
No./No. evaluated (%) with ulceration	176/840 (21.0)
No./No. evaluated (%) with ≥ 1 mitosis per mm^2	455/654 (69.6)
No. (%) with SLN biopsy performed	695 (66.7)
No./No. with biopsy performed (%) with positive SLN	135/695 (19.4)
No. (%) with stage III/IV at blood draw,	349 (33.5)
No. (%) by spring/summer season	515 (49.4)
Time from diagnosis to blood draw, years	
Median	0.6
Interquartile range	0.1-2.4
Storage time from blood draw to Vitamin D testing, years	
Median	5.1
Interquartile range	3.7-6.7
Vitamin D level, ng/mL	
Median	25.0
Interquartile range	20.1-30.6
No. (%) with vitamin D < 20 ng/mL	256 (24.6)
No. (%) with vitamin D ≥ 20 and < 30 ng/mL	497 (47.7)
No. (%) with vitamin D ≥ 30 ng/mL	289 (27.7)
CRP, mg/L	
Median	1.7
Interquartile range	0.7-4.4
Follow-up time from blood draw to disease relapse or censoring, years	
Median	7.2
Interquartile range	4.4-9.1
Follow-up time from blood draw to death or censoring, years	
Median	7.1
Interquartile range	3.8-9.0
No./No. evaluated (%) with recurrence among all patients	148/881(16.8)
No./No. evaluated (%) with recurrence among patients with stage I/II disease	71/692 (10.3)
No. (%) of deaths	294 (28.2)
No. (%) of deaths as a result of melanoma	191 (18.3)

Abbreviations: CRP, C-reactive protein; SLN, sentinel lymph node.

Demographic and clinical information is provided in Table 1. Blood was stored for a median of 5.1 years before testing. The distribution of raw vitamin D levels was slightly skewed (skewness = 1.80, kurtosis = 11.03; Appendix Fig A2, online only), whereas the distribution of raw CRP levels was highly skewed (skewness = 8.23, kurtosis = 101.70; data not shown). The median vitamin D level was 25.0 ng/mL, and 24.6% of patients had a vitamin D level less than 20 ng/mL. The median follow-up time from blood draw to last contact or death was 7.1 years (interquartile range, 3.8 to 9.0 years).

Correlations Between Clinical Factors and Vitamin D Levels

The median vitamin D level was higher in blood drawn in spring/summer than in fall/winter (26.39 ng/mL *v* 23.59 ng/mL; nonparametric $P < .001$; Appendix Table A1; Appendix Fig A3, online only). Consistent with other reports,[26,27] decreased vitamin D was not significantly associated with blood storage time (nonparametric $r^2 = -0.06$; $P = .0581$) but was associated with higher patient age ($r^2 = -0.10$; $P = .001$), increased primary tumor thickness ($r^2 = -0.15$; $P < .001$), and increased CRP ($r^2 = -0.19$; $P < .001$). Patients with ulcerated primary tumors had lower vitamin D levels than those without ulcerated tumors ($P = .0105$), and patients with advanced-stage disease at blood draw had lower vitamin D levels than those with earlier-stage disease ($P = .0024$; Appendix Table A1). We included these factors in the multivariable model.

Association of Vitamin D Levels With Patient Outcome

Univariate analysis demonstrated an association between lower vitamin D and poorer OS (HR, 1.03 per unit decrease of vitamin D; 95% CI, 1.01 to 1.04; $P < .001$), MSS (HR, 1.03 per unit decrease of vitamin D; 95% CI, 1.01 to 1.04; $P = .0025$), and DFS (HR, 1.02 per unit decrease of vitamin D; 95% CI, 1.00 to 1.04; $P = .0466$; Table 2; Appendix Table A2, online only). Older age at blood draw, male sex, advanced stage at blood draw, and higher log-transformed CRP were also associated with poorer OS, MSS, and DFS (all $P < .05$; Appendix Table A2). Although blood draw season was not associated with OS ($P = .7468$) or MSS ($P = .2957$), it was associated with DFS on univariate analysis ($P = .0367$; Appendix Table A2) and multivariable analysis ($P = .0139$; Table 2). After adjustment for age, sex, disease stage, blood draw season, and log-transformed CRP, vitamin D levels remained significantly associated with outcome measures of OS (HR, 1.02 per unit decrease of vitamin D; 95% CI, 1.01 to 1.04; $P = .005$) MSS (HR, 1.02 per unit decrease of vitamin D; 95% CI, 1.00 to 1.04; $P = .048$), and DFS (HR, 1.02 per unit decrease of vitamin D; 95% CI, 1.00 to 1.04; $P = .04327$; Table 2). In addition to the evaluation of DFS in patients with all stages of disease (similar to the approach reported previously by Newton-Bishop et al[13]), we performed DFS subset analyses in patients with stage I/II disease and stage III/IV disease for completeness (Appendix Tables A3 and A4, online only). These stratified analyses show borderline effects or nonsignificant results for early- or late-stage cancers. This likely reflects the smaller number of patients and events in these subsets, so the effect estimates and significance of findings were more variable. One additional possibility considered was a nonlinear relationship of vitamin D on risk, which might confound the relationships of vitamin D with outcomes according to stage; we had assumed that vitamin D risk increases linearly with hazard for recurrence or death. To evaluate potential nonlinear effects of vitamin D on the three outcomes, we also modeled quadratic terms (data not shown). In all cases, the quadratic terms provided no significant or substantial improvement to the linear hazard models.

Because vitamin D levels less than 20 ng/mL are considered deficient, we evaluated this level as a clinical cutoff. On univariate analysis, patients with vitamin D levels less than 20 ng/mL had poorer OS (HR, 1.44; 95% CI, 1.13 to 1.85; $P = .0036$) and MSS (HR, 1.37; 95% CI, 1.00 to 1.87; $P = .0475$) than those with a vitamin D level of 20 ng/mL or greater, but the DFS in patients with vitamin D levels less than 20 ng/mL (HR, 1.12; 95% CI, 0.77 to

Table 2. Association Between Blood Levels of Vitamin D and OS, MSS, and DFS in 1,042 Patients With Melanoma

Variable by survival and analysis type	Analysis by Vitamin D Level (ng/mL)							
	Continuous (per-unit decrease)		< 16 *v* ≥ 16		< 20 *v* ≥ 20		< 20 *v* 20-30 *v* ≥ 30*	
	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
OS (n = 1,042)								
Univariate analysis								
Vitamin D	1.03 (1.01 to 1.04)	< .001	2.00 (1.50 to 2.66)	< .001	1.44 (1.13 to 1.85)	.004	1.69 (1.23 to 2.33)	.0013
							1.27 (0.95 to 1.71)	.1127
Multivariate analysis								
Vitamin D	1.02 (1.01 to 1.04)	.0051	1.85 (1.38 to 2.48)	< .001	1.40 (1.08 to 1.81)	< .001	1.46 (1.05 to 2.04)	.0247
						< .001	1.07 (0.79 to 1.44)	.6788
Logarithmic CRP	1.17 (1.07 to 1.27)	< .001	1.16 (1.07 to 1.27)	< .001	1.18 (1.08 to 1.28)	< .001	1.18 (1.08 to 1.28)	< .001
Age in 5-year units	1.22 (1.17 to 1.27)	< .001	1.22 (1.17 to 1.27)	< .001	1.22 (1.17 to 1.27)	< .001	1.22 (1.17 to 1.27)	< .001
Male *v* female sex	1.75 (1.36 to 2.26)	< .001	1.76 (1.36 to 2.27)	< .001	1.76 (1.37 to 2.28)	< .001	1.76 (1.37 to 2.28)	< .001
Stage III/IV *v* I/II	3.68 (2.90 to 4.67)	< .001	3.71 (2.92 to 4.70)	< .001	3.69 (2.91 to 4.68)	< .001	3.68 (2.90 to 4.67)	< .001
Spring/summer *v* fall/winter	1.10 (0.87 to 1.39)	.4048	1.09 (0.87 to 1.37)	.4651	1.10 (0.87 to 1.40)	.4104	1.11 (0.88 to 1.40)	.3902
MSS (n = 1,042)								
Univariable analysis								
Vitamin D	1.03 (1.01-1.04)	.0025	1.76 (1.22 to 2.53)	.0026	1.37 (1.00 to 1.87)	.048	1.71 (1.14 to 2.56)	.0097
							1.39 (0.96 to 2.02)	.0794
Multivariable analysis								
Vitamin D	1.02 (1.00-1.04)	.048	1.52 (1.04 to 2.22)	.029	1.31 (0.94 to 1.81)	.111	1.40 (0.91 to 2.14)	.1265
							1.10 (0.75 to 1.60)	.6284
Logarithmic CRP	1.22 (1.10 to 1.35)	< .001	1.22 (1.10 to 1.36)	< .001	1.23 (1.11 to 1.36)	< .001	1.23 (1.10 to 1.36)	< .001
Age in 5-year units	1.08 (1.02 to 1.34)	.0046	1.08 (1.02 to 1.34)	.0039	1.08 (1.02 to 1.34)	.005	1.08 (1.02 to 1.34)	.0048
Male *v* female sex	1.57 (1.15 to 2.13)	.0043	1.56 (1.15 to 2.12)	.0047	1.57 (1.15 to 2.15)	.004	1.57 (1.15 to 2.15)	.0043
Stage III/IV *v* I/II	8.36 (5.89 to 11.9)	< .001	8.41 (5.93 to 11.9)	< .001	8.43 (5.94 to 12.0)	< .001	8.39 (5.91 to 11.9)	< .001
Spring/summer *v* fall/winter	1.16 (0.87 to 1.55)	.3202	1.13 (0.85 to 1.51)	.4056	1.15 (0.86 to 1.54)	.356	1.16 (0.86 to 1.56)	.3299
DFS (n = 881)								
Univariable analysis								
Vitamin D	1.02 (1.00 to 1.04)	.0466	1.62 (1.04 to 2.53)	.0335	1.12 (0.77 to 1.62)	.564	1.42 (0.89 to 2.28)	.1427
							1.44 (0.96 to 2.16)	.0791
Multivariable analysis								
Vitamin D	1.02 (1.00 to 1.04)	.0427	2.00 (1.27 to 3.16)	.003	1.29 (0.87 to 1.90)	.204	1.53 (0.94 to 2.49)	.0857
							1.29 (0.86 to 1.94)	.2255
Logarithmic CRP	1.02 (0.90 to 1.16)	.711	1.03 (0.91 to 1.16)	.6616	1.04 (0.92 to 1.18)	.555	1.03 (0.91 to 1.17)	.6303
Age in 5-year units	1.12 (1.05 to 1.19)	< .001	1.12 (1.06 to 1.19)	< .001	1.12 (1.05 to 1.19)	< .001	1.12 (1.05 to 1.19)	< .001
Male *v* female sex	1.42 (1.00 to 2.03)	.0502	1.44 (1.01 to 2.05)	.0449	1.43 (1.00 to 2.04)	.052	1.42 (0.99 to 2.03)	.0553
Stage III/IV *v* I/II	4.77 (3.43 to 6.64)	< .001	4.94 (3.55 to 6.89)	< .001	4.79 (3.44 to 6.67)	< .001	4.74 (3.40 to 6.60)	< .001
Spring/summer *v* fall/winter	1.51 (1.09 to 2.10)	.0139	1.50 (1.08 to 2.08)	.0154	1.49 (1.07 to 2.08)	.018	1.51 (1.09 to 2.11)	.0144

Abbreviations: CRP, C-reactive protein; DFS, disease-free survival; MSS, melanoma-specific survival; OS, overall survival.
*Single Cox regression analysis using three categories of vitamin D levels, with ≥ 30 ng/dL as the reference group.

1.62; $P = .5644$) was similar to that in patients with a vitamin D of 20 ng/mL or greater (Table 2; Appendix Table A2). In multivariable analysis, we observed similar significance patterns (Table 2), as we did in univariate and multivariable analyses when we stratified vitamin D levels into three standard, clinically defined groups (deficiency, insufficiency, and sufficiency; Table 2).

To determine the optimal cutoff value of vitamin D for predicting OS in patients with melanoma, we applied recursive partition in R using the party function and obtained the smallest *P* value in log-rank testing (Appendix Fig A4, online only) at an optimal cutoff value of 16 ng/mL. We used this cutoff point in the analysis of MSS and DFS. Compared with patients who had a vitamin D level of 16 ng/mL or greater, patients who had a vitamin D level less than 16 ng/mL were 2.0 times more likely to die as a result of all-cause disease (OS; 95% CI, 1.50 to 2.66; $P < .001$), 1.76 times more likely to die as a result of melanoma (MSS; 95% CI, 1.22 to 2.53; $P = .0026$), and 1.62 times more likely to have disease recurrence (DFS; 95% CI, 1.04 to 2.53; $P = .0335$) on univariate analysis. All three associations remained significant on multivariable analysis (Table 2; Figs 1 and 2).

To determine if vitamin D and CRP have joint effects on melanoma outcomes, we tested for an interaction between logarithmic CRP and continuous or categorical vitamin D, with or without adjustment for other covariates, on OS, MSS, and DFS. No significant joint effects were found, with the exception of an effect on OS by categorical vitamin D at a cutoff of 16 ng/mL on univariate analysis ($P = .048$ for the interaction; additional data not shown).

DISCUSSION

In this study, we simultaneously examined vitamin D and CRP levels in a hospital-based cohort of patients with melanoma in relation to demographics, tumor histopathology, disease stage, and clinical outcome measures. We found that a lower level of vitamin

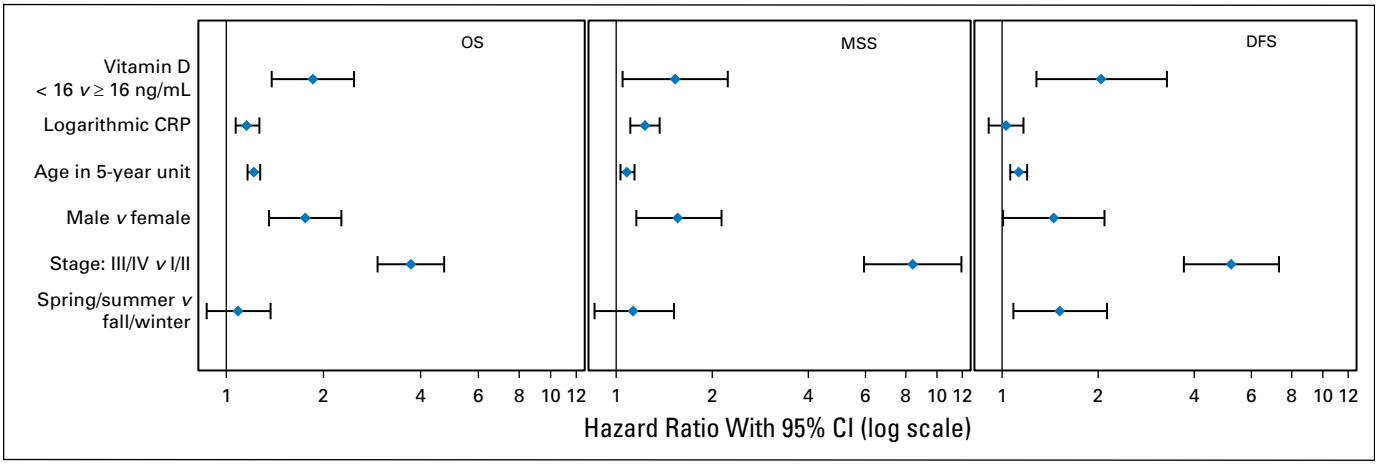

Fig 1. Multivariable-adjusted forest plots of hazard ratios of vitamin D (dichotomized at 16 ng/mL) and significant covariates for overall survival (OS), melanoma-specific survival (MSS), and disease-free survival (DFS) in 1,042 patients with melanoma. CRP, C-reactive protein.

D was associated with higher CRP, fall/winter months of blood draw, thicker and more ulcerated primary tumors, and advanced melanoma stage. We also found that a lower level of vitamin D was independently associated with poorer OS, MSS, and DFS. We confirmed in a population of patients with melanoma a finding from other populations[19,28]: vitamin D levels are inversely correlated with CRP levels. Therefore, we included CRP as a measure of SIR and as a potential confounder of associations

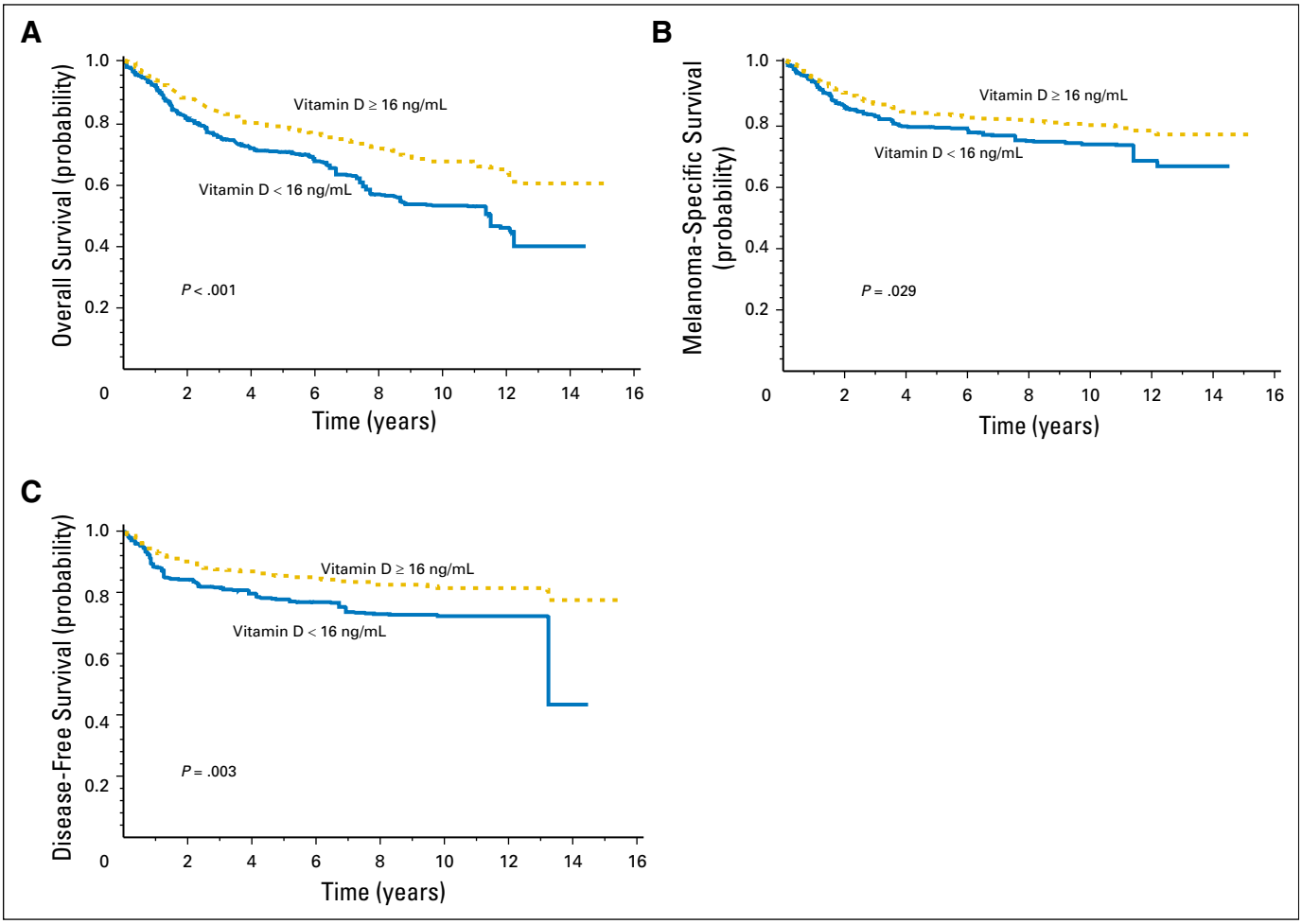

Fig 2. Multivariable-adjusted survival probabilities by vitamin D levels dichotomized at 16 ng/mL, with adjustment for logarithmic C-reactive protein, age, sex, stage, and blood draw season. Vitamin D effects on overall survival (A); melanoma-specific survival (B); and disease-free survival (C).

between vitamin D and patient outcome measures.[29] To our knowledge, this is the first evaluation to link coordinated measurements of these two important inflammatory biomarkers with long-term clinical outcomes in a large population of patients with cancer. Newton-Bishop et al[13] observed a significant association between higher vitamin D level and lower Breslow primary tumor thickness as well as lower rates of relapse and death in a two-stage study; however, no evaluation of the contribution of SIR to these associations was included.[13] The current study identified associations between vitamin D levels and melanoma disease severity and clinical outcomes similar to those reported by Newton-Bishop et al[13]; we extend their findings by reporting an independent association of low vitamin D with an increased risk of melanoma-related death (ie, MSS). Although our results demonstrated significant associations between vitamin D and OS, MSS, and DFS in the combined population of patients with all disease stages, we found borderline effects or nonsignificant associations on stratified stage-specific subgroup analyses. This likely reflects the smaller number of patients and events in these smaller subsets, so the effect estimates and significance were more variable. When we investigated alternative hypotheses, we found no evidence for a nonlinear relationship of vitamin D on risk, and we also observed no interaction between stage and vitamin D levels. These findings emphasize that additional confirmation should be sought in large, prospective series.

We found no significant association between time from diagnosis to blood draw and vitamin D levels. Furthermore, serial blood draw data showed no association between consecutive blood draw interval and vitamin D level. We concluded that there was no demonstrable direct evidence for reverse causality in this patient population (that is, that sun-avoiding behavior in patients diagnosed with melanoma led to a decline in vitamin D levels after diagnosis).

Importantly, after adjustment for CRP, vitamin D remained an independent predictor of OS, MSS, and DFS. This suggests that, although blood levels of vitamin D and CRP are highly correlated with each other, each independently predicts clinical outcome in patients with melanoma. Therefore, an investigation of the roles of each of these biomarkers is important in clinical studies of outcomes in patients with melanoma and in clinical trials of novel therapies. Furthermore, these data suggest that interventions to increase vitamin D or to reduce SIR and CRP could ultimately benefit patients with melanoma.

Our study has several limitations. First, our patients were recruited from a population referred to a large cancer hospital and might not be representative. Second, we did not collect patient-specific sun exposure data. Although blood draw season provides an indirect measure of UV radiation exposure proximate to blood draw, it does not accurately estimate long-term sun exposure. We adjusted for blood draw season in the analyses, as has been done in other similar studies.[8,30] Third, we did not include measures of vitamin D intake. Few studies have assessed the correlation between vitamin D intake and melanoma risk or mortality.[26,31] The potential influence of vitamin D supplementation on melanoma risk remains controversial,[14] although vitamin D levels are correlated with vitamin D intake.[32,33] We believe that investigations of the potential role of vitamin D supplementation in melanoma risk are reasonable to pursue but were beyond the scope of this study. Last, we did not include additional potential confounders, such as body mass index (BMI), details of surgery, or systematic treatment variables, in our analyses. We excluded BMI, because systematic review has indicated only a weak inverse link between serum 25-OH D levels and BMI in adults.[34] We did not adjust for timing of surgery, because our data revealed no relationship between time from surgery to blood draw and vitamin D levels. We did not attempt to adjust for systemic therapies, because of the following: (1) patients underwent initial blood draw before administration of any systemic therapy; (2) multiple different systemic therapies were provided to patients with advanced disease, which made analysis of small subsets problematic; (3) treatments available during the study period had limited efficacy; and (4) patients in this study were treated before introduction of more effective, modern targeted therapies or immune checkpoint inhibitors. We recognize that it will be important to include systemic treatment variables, for example, in future investigations.

In summary, the current investigation is the first, to our knowledge, to report a significant, independent association between lower vitamin D levels and poorer melanoma survival after adjustment for the influence of the SIR through simultaneous measurement of CRP. A coordinated investigation of the mechanisms responsible for the independent association of these two important inflammatory biomarkers with outcomes in patients with melanoma is likely to be clinically relevant and may have implications for other cancers. Additional investigation is needed to determine whether supplementation of vitamin D and/or interventions to reduce maladaptive systemic inflammation could be beneficial to patients with melanoma.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Association of Vitamin D Levels With Outcome in Patients With Melanoma After Adjustment For C-Reactive Protein

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Appendix

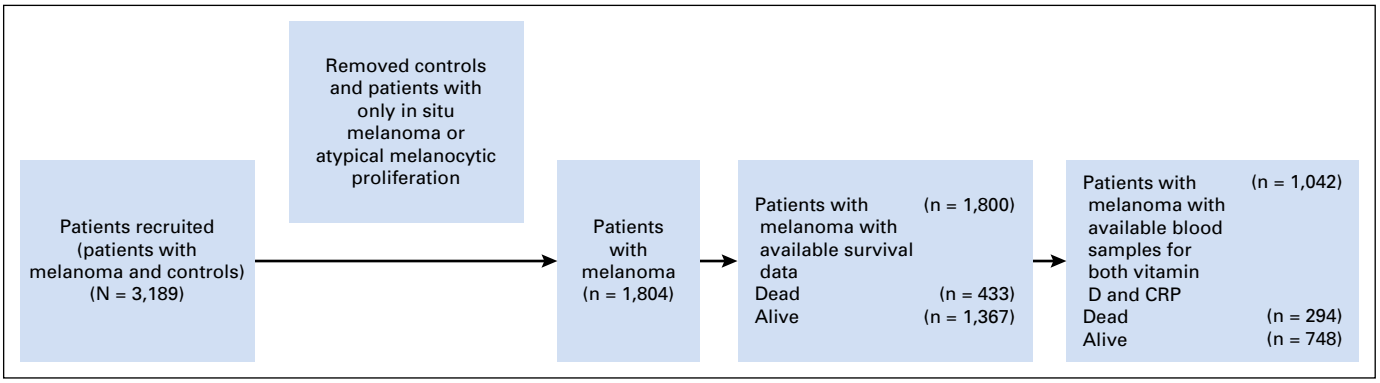

Fig A1. Flow diagram of population data sets used in this study. CRP, C-reactive protein.

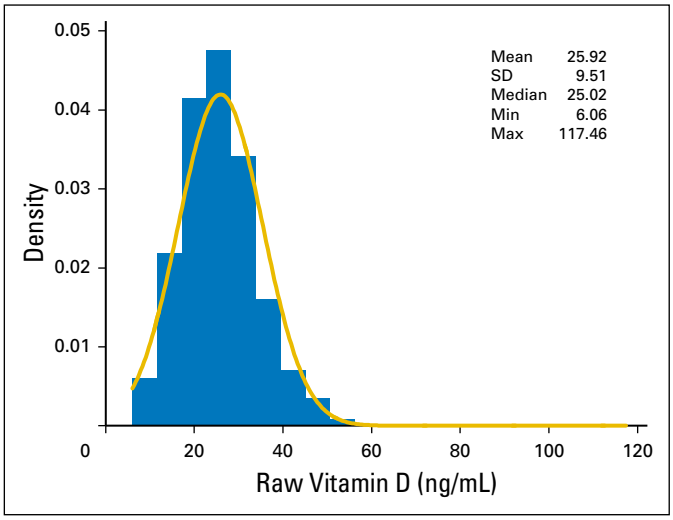

Fig A2. Distribution of raw vitamin D levels in 1,042 patients with melanoma. Max, maximum; Min, minimum; SD, standard deviation.

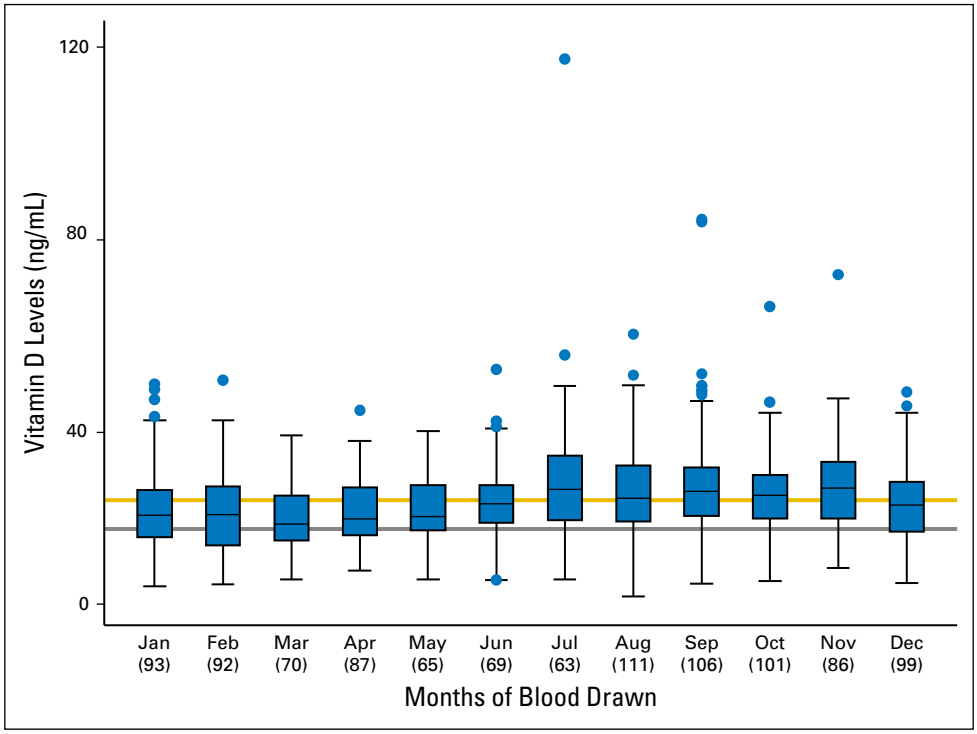

Fig A3. Vitamin D levels by month of blood collection in 1,042 patients with melanoma. Numbers in parentheses under the month of blood collection indicate the number of patients. The horizontal line in each month box indicates the median vitamin D value. The lower limit of the box represents the first quartile (Q1), and the upper limit of the box represents the third quartile (Q3) vitamin D value. The lower bar represents the lower limit (Q1 − 1.5 [Q3-Q1]), and the upper bar represents the upper limit (Q3 + 1.5 [Q3-Q1]). Blue dots represent outliers. The gray line signifies a vitamin D level of 20 ng/ML, and the gold line is the mean vitamin D level of 25.92 ng/mL.

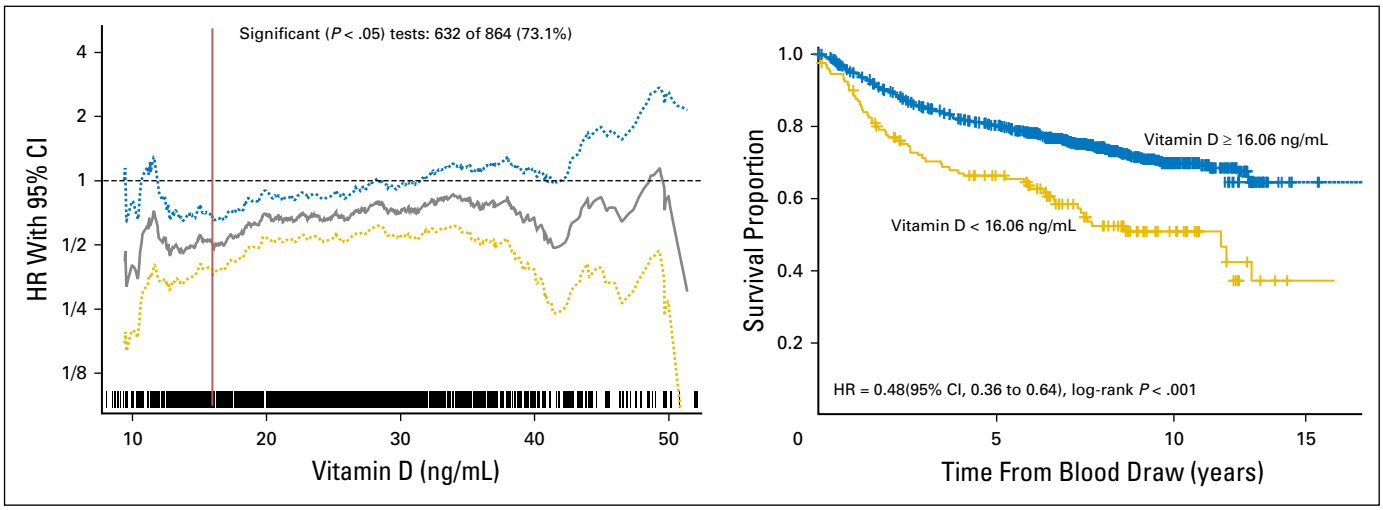

Fig A4. Recursive partitioning was performed to obtain the optimal vitamin D level cutoff to predict overall survival. Hazard ratios (HRs; solid line) and 95% CIs (dotted line) were calculated for each vitamin D level association with overall survival. The optimal cutoff at 16.06 ng/mL (rounded to 16 ng/mL in the text) was defined as the vitamin D level with the most significant split on log-rank test (left); a Kaplan curve was plotted based on this split (right).

Table A1. Association Between Blood Level of Vitamin D and Demographic and Clinical Characteristics in 1,042 Patients With Melanoma

Clinical characteristic	No. of Patients	Vitamin D Level (ng/mL)		*P**
		Median	Interquartile Range	
Storage time, years	1,042	25.03	20.10-30.65	.0581†
Season				< .001
Fall/winter	527	23.59	18.7-29.4	
Spring/summer	515	26.39	21.6-31.9	
Sex				.1846
Female	452	24.94	18.9-30.6	
Male	590	25.09	20.8-30.8	
Age at blood draw, years	1,042	25.03	20.10-30.65	.001†
Tumor thickness	928	25.09	20.16-30.82	< .001†
Tumor thickness level, mm				< .001
≤ 1	409	26.51	21.0-32.2	
1-2	251	25.20	20.4-29.9	
2-4	176	23.89	18.6-28.3	
> 4	92	21.86	16.0-30.1	
Ulceration				.0105
Absent	664	25.49	20.6-31.3	
Present	176	24.32	17.8-29.5	
Mitotic rate				.2704
0	199	25.63	20.3-31.7	
> 0	455	25.11	20.2-30.8	
Positive SLN				.4922
No	560	25.33	20.3-30.9	
Yes	135	25.06	20.2-29.8	
Stage at blood draw				.0024
I, I/II, or II	693	25.58	20.4-31.3	
III or IV	349	23.94	19.1-29.3	
Logarithmic CRP	1,042	25.03	20.10-30.65	< .001*

Abbreviations: CRP, C-reactive protein; SLN, sentinel lymph node.
*Spearman's ρ test for continuous variables; Wilcoxon test for categorical variables.
†Negative coefficients (storage time, −0.06; age, −0.10; thickness, −0.15; logarithmic CRP, −0.19).

Table A2. Univariable Analysis of Clinical Factors Associated With OS, MSS, and DFS in 1,042 Patients With Melanoma

Associated variable	OS				MSS			DFS			
	No. of Patients	No. of Deaths	Univariate *P*	Hazard Ratio (95% CI)	No. of Melanoma-Specific Deaths	Univariate *P*	Hazard Ratio (95% CI)	No. of Patients	Recurrence	Univariate *P*	Hazard Ratio (95% CI)
Age at blood draw	1,042	294	< .001	1.04 (1.03 to 1.05)	191	< .001	1.02 (1.01 to 1.03)	881	148	< .001	1.02 (1.01 to 1.03)
Tumor thickness	1,042	294	< .001	1.11 (1.09 to 1.13)	191	< .001	1.11 (1.09 to 1.14)	881	148	< .001	1.11 (1.08 to 1.14)
Sex											
Female	452	87	—	1.00	62	—	1.00	383	47	—	1.00
Male	590	207	< .001	1.99 (1.55 to 2.55)	129	< .001	1.71 (1.27 to 2.32)	498	101	< .0017	1.74 (1.23 to 2.46)
Stage											
I/II	693	117	—	1.00	41	—	1.00	692	71	—	1.00
III/IV	349	177	< .001	4.15 (3.28 to 5.25)	150	< .001	9.59 (6.79 to 13.60)	189	77	< .001	4.88 (3.53 to 6.74)
Mitotic rate											
0	199	25	—	1.00	11	—	1.00	196	13	—	1.00
≥ 1	455	144	< .001	2.78 (1.82 to 4.25)	100	< .001	4.33 (2.32 to 8.08)	394	86	< .001	3.54 (1.97 to 6.34)
Ulceration											
Absent	664	125	—	1.00	61	—	1.00	615	64	—	1.00
Present	176	91	< .001	3.82 (2.92 to 5.01)	71	< .001	5.78 (4.10 to 8.14)	143	57	< .001	4.94 (3.46 to 7.07)
Season											
Fall/winter	527	148	—	1.00	90	—	1.00	452	65	—	1.00
Spring/summer	515	146	.7468	1.04 (0.83 to 1.31)	101	.2927	1.16 (0.88 to 1.55)	429	83	.0367	1.41 (1.02 to 1.96)
Continuous vitamin D	1,042	294	< .001	1.03 (1.01 to 1.04)	191	.0025	1.03 (1.01 to 1.04)	881	148	.0466	1.02 (1.00 to 1.04)
Logarithmic CRP	1,042	294	< .001	1.31 (1.20 to 1.43)	191	< .001	1.42 (1.27 to 1.58)	881	148	.0385	1.14 (1.01 to 1.30)
Two categories of vitamin D, ng/mL											
≥ 20	786	202	—	1.00	134	—	1.00	679	111	—	1.00
< 20	256	92	.0036	1.44 (1.13 to 1.85)	57	.0475	1.37 (1.00 to 1.85)	202	37	.5644	1.12 (0.77 to 1.62)
Three categories of vitamin D, ng/mL											
≥ 30	289	64	—	1.00	40	—	1.00	257	33	—	1.00
20-30	497	138	.1127	1.27 (0.95 to 1.71)	94	.0794	1.39 (0.96 to 2.02)	422	78	.0791	1.44 (0.96 to 2.16)
< 20	256	92	.0013	1.69 (1.23 to 2.33)	57	.00970	1.71 (1.14 to 2.56)	202	37	.1427	1.42 (0.89 to 2.28)

Abbreviations: CRP, C-reactive protein

Table A3. Association Between Blood Levels of Vitamin D and OS, MSS, and DFS in Patients With Stage I/II Disease

	Analysis by Vitamin D Level (ng/mL)							
	Continuous (per-unit decrease)		< 16 *v* ≥ 16		< 20 *v* ≥ 20		< 20 *v* 20-30 *v* ≥ 30*	
Variable by survival and analysis type	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
OS (n = 693)								
Univariable analysis								
Vitamin D	1.03 (1.01 to 1.05)	.0041	2.50 (1.61 to 3.86)	< .001	1.66 (1.13 to 2.44)	.010	1.25 (0.78 to 2.01)	.3489
							1.92 (1.17 to 3.15)	.01
Multivariable analysis								
Vitamin D	1.02 (0.99 to 1.04)	.1393	1.89 (1.19 to 3.03)	.0074	1.32 (0.88 to 1.98)	.174	1.20 (0.75 to 1.93)	.4462
							1.49 (0.89 to 2.50)	.1272
Logarithmic CRP	1.11 (0.95 to 1.30)	.1846	1.09 (0.93 to 1.27)	.3011	1.12 (0.96 to 1.31)	.164	1.11 (0.95 to 1.30)	.175
Age in 5-year units	1.38 (1.28 to 1.48)	< .001	1.38 (1.28 to 1.48)	< .001	1.38 (1.28 to 1.48)	< .001	1.38 (1.28 to 1.48)	< .001
Male *v* female sex	2.17 (1.41 to 3.33)	< .001	2.19 (1.43 to 3.37)	< .001	2.20 (1.43 to 3.38)	< .001	2.20 (1.43 to 3.38)	< .001
Stage I/II *v* I	2.13 (1.10 to 4.13)	.0246	2.20 (1.14 to 4.23)	.0189	2.22 (1.15 to 4.28)	.018	2.19 (1.13 to 4.23)	.0201
Stage II *v* I		< .001	2.13 (1.43 to 3.15)	< .001	2.21 (1.49 to 3.27)	< .001	2.19 (1.48 to 3.25)	< .001
Spring/summer *v* fall/winter	2.22 (1.50 to 3.28)	.7319	0.97 (0.67 to 1.42)	.8865	0.94 (0.64 to 1.37)	.739	0.94 (0.65 to 1.38)	.7662
MSS (n = 693)								
Univariable analysis								
Vitamin D	1.01 (0.98 to 1.05)	.5128	0.95 (0.34 to 2.67)	.9223	0.93 (0.44 to 1.95)	.843	1.66 (0.77 to 3.58)	.1984
							1.30 (0.51 to 3.28)	.5808
Multivariable analysis								
Vitamin D	1.00 (0.97 to 1.04)	.9423	0.68 (0.23 to 1.97)	.4734	0.70 (0.32 to 1.53)	.375	1.51 (0.69 to 3.29)	.3016
							0.94 (0.36 to 2.49)	.901
Logarithmic CRP	0.99 (0.76 to 1.30)	.9652	1.02 (0.77 to 1.34)	.8983	1.02 (0.78 to 1.35)	.872	1.01 (0.76 to 1.33)	.9519
Age in 5-year units	1.11 (0.99 to 1.24)	.0758	1.11 (0.99 to 1.24)	.072	1.11 (0.99 to 1.24)	.070	1.11 (0.99 to 1.24)	.0687
Male *v* female sex	1.78 (0.87 to 3.62)	.1141	1.77 (0.87 to 3.60)	.1165	1.73 (0.85 to 3.54)	.131	1.70 (0.83 to 3.48)	.1458
Stage I/II *v* I	2.85 (0.80 to 10.2)	.1069	2.86 (0.80 to 10.2)	.1066	2.82 (0.79 to 10.1)	.111	2.78 (0.78 to 9.94)	.1166
Stage II *v* I	6.43 (3.18 to 13.0)	< .001	6.60 (3.27 to 13.3)	< .001	6.70 (3.30 to 13.6)	< .001	6.58 (3.25 to 13.4)	< .001
Spring/summer *v* fall/winter	1.20 (0.64 to 2.23)	.569	1.18 (0.64 to 2.19)	.595	1.17 (0.63 to 2.17)	.628	1.21 (0.65 to 2.25)	.5546
DFS (n = 692)								
Univariable analysis								
Vitamin D	1.01 (0.98 to 1.03)	.4776	1.58 (0.83 to 3.00)	.165	1.11 (0.64 to 1.90)	.716	1.25 (0.71 to 2.21)	.436
							1.28 (0.66 to 2.47)	.4674
Multivariable analysis								
Vitamin D	1.00 (0.97 to 1.02)	.8973	1.27 (0.65 to 2.51)	.4868	0.92 (0.52 to 1.64)	.787	1.22 (0.69 to 2.17)	.4994
							1.06 (0.52 to 2.13)	.878
Logarithmic CRP	1.04 (0.85 to 1.27)	.7307	1.02 (0.83 to 1.25)	.8393	1.05 (0.85 to 1.28)	.665	1.04 (0.85 to 1.27)	.7207
Age in 5-year units	1.05 (0.97 to 1.14)	.2395	1.05 (0.97 to 1.14)	.2448	1.05 (0.97 to 1.14)	.228	1.05 (0.97 to 1.14)	.2274
Male *v* female sex	1.87 (1.10 to 3.19)	.0216	1.89 (1.10 to 3.22)	.0202	1.86 (1.09 to 3.18)	.024	1.84 (1.08 to 3.15)	.026
Stage I/II *v* I	2.60 (1.05 to 6.40)	.0382	2.58 (1.05 to 6.34)	.0391	2.61 (1.06 to 6.41)	.037	2.58 (1.05 to 6.35)	.0392
Stage II *v* I	4.72 (2.83 to 7.88)	< .001	4.62 (2.76 to 7.74)	<.001	4.79 (2.86 to 8.02)	< .001	4.77 (2.84 to 7.99)	< .001
Spring/summer *v* fall/winter	1.76 (1.08 to 2.85)	.0225	1.77 (1.09 to 2.86)	.02	1.74 (1.07 to 2.81)	.025	1.77 (1.09 to 2.87)	.0209

Abbreviations: CRP, C-reactive protein; HR, hazard ratio.
*Single Cox regression analysis using three categories of vitamin D levels, with ≥ 30 ng/dL as the reference group.

Table A4. Association Between Blood Levels of Vitamin D and OS, MSS, and DFS in Patients With Stage III/IV Disease

	Analysis by Vitamin D Level (ng/mL)							
	Continuous (per-unit decrease)		< 16 *v* ≥ 16		< 20 *v* ≥ 20		< 20 *v* 20-30 *v* ≥ 30*	
Variable by survival and analysis type	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
OS (n = 349)								
Univariable analysis								
Vitamin D	1.01 (1.00 to 1.03)	.1582	1.43 (0.98 to 2.09)	.0647	1.17 (0.85 to 1.62)	.338	1.03 (0.70 to 1.51)	.8862
							1.19 (0.79 to 1.81)	.406
Multivariable analysis								
Vitamin D	1.01 (0.99 to 1.03)	.1841	1.30 (0.87 to 1.92)	.2006	1.20 (0.85 to 1.70)	.295	0.99 (0.67 to 1.46)	.9511
							1.19 (0.76 to 1.87)	.4459
Logarithmic CRP	1.12 (1.01 to 1.25)	.0339	1.13 (1.01 to 1.26)	.0265	1.13 (1.02 to 1.26)	.024	1.13 (1.02 to 1.26)	.0241
Age in 5-year units	1.11 (1.05 to 1.17)	< .001	1.11 (1.05 to 1.17)	< .001	1.11 (1.05 to 1.17)	< .001	1.11 (1.05 to 1.17)	< .001
Male *v* female sex	1.42 (1.03 to 1.96)	.0347	1.40 (1.01 to 1.93)	.0407	1.41 (1.02 to 1.96)	.039	1.41 (1.02 to 1.96)	.0393
Stage IV *v* III	2.60 (1.85 to 3.67)	< .001	2.54 (1.79 to 3.59)	< .001	2.60 (1.84 to 3.66)	< .001	2.60 (1.84 to 3.66)	< .001
Spring/summer *v* fall/winter	1.13 (0.83 to 1.53)	.4431	1.10 (0.82 to 1.49)	.5218	1.12 (0.82 to 1.52)	.480	1.12 (0.82 to 1.52)	.4914
MSS (n = 349)								
Univariate analysis								
Vitamin D	1.02 (1.00 to 1.04)	.0494	1.62 (1.09 to 2.40)	.0174	1.33 (0.94 to 1.87)	.105	1.03 (0.68 to 1.57)	.8874
							1.36 (0.86 to 2.13)	.1863
Multivariable analysis								
Vitamin D	1.02 (1.00 to 1.04)	.1026	1.35 (0.89 to 2.06)	.1604	1.3 (0.90 to 1.89)	.162	1.00 (0.65 to 1.54)	.987
							1.31 (0.80 to 2.14)	.2842
Logarithmic CRP	1.16 (1.03 to 1.30)	.0151	1.16 (1.03 to 1.31)	.0114	1.16 (1.04 to 1.31)	.010	1.16 (1.04 to 1.31)	.0105
Age in 5-year units	1.06 (1.00 to 1.12)	.0631	1.0 (1.00 to 1.12)	.061	1.06 (1.00 to 1.12)	.066	1.06 (1.00 to 1.12)	.066
Male *v* female sex	1.41 (1.00 to 2.01)	.0517	1.39 (0.98 to 1.96)	.0657	1.41 (0.99 to 2.01)	.054	1.42 (0.99 to 2.02)	.0542
Stage IV *v* III	2.91 (2.02 to 4.19)	< .001	2.83 (1.95 to 4.10)	< .001	2.90 (2.01 to 4.17)	< .001	2.90 (2.01 to 4.17)	< .001
Spring/summer *v* fall/winter	1.11 (0.79 to 1.55)	.5472	1.07 (0.77 to 1.49)	.6761	1.10 (0.79 to 1.54)	.578	1.10 (0.78 to 1.55)	.5813
DFS (n = 189)								
Univariable analysis								
Vitamin D	1.03 (1.00 to 1.06)	.0931	1.88 (1.01 to 3.48)	.0448	1.12 (0.67 to 1.88)	.665	1.29 (0.72 to 2.32)	.3968
							1.35 (0.68 to 2.65)	.3909
Multivariable analysis								
Vitamin D	1.03 (1.00 to 1.08)	.0408	2.46 (1.28 to 4.72)	.0068	1.23 (0.70 to 2.16)	.481	1.33 (0.74 to 2.41)	.3409
							1.51 (0.73 to 3.11)	.2622
Logarithmic CRP	1.03 (0.87 to 1.23)	.7106	1.06 (0.89 to 1.26)	.4941	1.07 (0.90 to 1.27)	.442	1.06 (0.89 to 1.26)	.4941
Age in 5-year units	1.15 (1.05 to 1.27)	.0025	1.15 (1.05 to 1.27)	.0015	1.15 (1.05 to 1.27)	.005	1.15 (1.05 to 1.27)	.0044
Male *v* female sex	1.04 (0.64 to 1.68)	.8777	1.01 (0.63 to 1.64)	.9521	0.98 (0.60 to 1.61)	.936	0.97 (0.59 to 1.60)	.9158
Stage IV *v* III	0.66 (0.15 to 2.84)	.5804	0.64 (0.15 to 2.73)	.5448	< .001	.560	0.63 (0.15 to 2.70)	.5294
Spring/summer *v* fall/winter	1.26 (0.80 to 1.98)	.3251	1.19 (0.76 to 1.88)	.4418	1.22 (0.77 to 1.93)	.406	1.23 (0.77 to 1.95)	.3857

Abbreviations: CRP, C-reactive protein; DFS, disease-free survival; HR, hazard ratio; MSS, melanoma-specific survival; OS, overall survival.
*Single Cox regression analysis using three categories of vitamin D levels, with ≥ 30 ng/dL as the reference group.