Independent Association of Low Serum 25-Hydroxyvitamin D and 1,25-Dihydroxyvitamin D Levels With All-Cause and Cardiovascular Mortality

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Background: In cross-sectional studies, low serum levels of 25-hydroxyvitamin D are associated with higher prevalence of cardiovascular risk factors and disease. This study aimed to determine whether endogenous 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D levels are related to all-cause and cardiovascular mortality.

Methods: Prospective cohort study of 3258 consecutive male and female patients (mean [SD] age, 62 [10] years) scheduled for coronary angiography at a single tertiary center. We formed quartiles according to 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D levels within each month of blood drawings. The main outcome measures were all-cause and cardiovascular deaths.

Results: During a median follow-up period of 7.7 years, 737 patients (22.6%) died, including 463 deaths from cardiovascular causes. Multivariate-adjusted hazard ratios (HRs) for patients in the lower two 25-hydroxyvitamin D quartiles (median, 7.6 and 13.3 ng/mL [to convert 25-hydroxyvitamin D levels to nanomoles per liter, multiply by 2.496]) were higher for all-cause mortality (HR, 2.08; 95% confidence interval [CI], 1.60-2.70; and HR, 1.53; 95% CI, 1.17-2.01; respectively) and for cardiovascular mortality (HR, 2.22; 95% CI, 1.57-3.13; and HR, 1.82; 95% CI, 1.29-2.58; respectively) compared with patients in the highest 25-hydroxyvitamin D quartile (median, 28.4 ng/mL). Similar results were obtained for patients in the lowest 1,25-dihydroxyvitamin D quartile. These effects were independent of coronary artery disease, physical activity level, Charlson Comorbidity Index, variables of mineral metabolism, and New York Heart Association functional class. Low 25-hydroxyvitamin D levels were significantly correlated with variables of inflammation (C-reactive protein and interleukin 6 levels), oxidative burden (serum phospholipid and glutathione levels), and cell adhesion (vascular cell adhesion molecule 1 and intercellular adhesion molecule 1 levels).

Conclusions: Low 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D levels are independently associated with all-cause and cardiovascular mortality. A causal relationship has yet to be proved by intervention trials using vitamin D.

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STUDIES[1,2] HAVE DEMONstrated that low levels of vitamin D represent a problem of global dimension. A recent Workshop Consensus for Vitamin D Nutritional Guidelines[3] estimated that about 50% and 60% of the older populations in North America and the rest of the world, respectively, do not have satisfactory vitamin D status. The consensus further concluded that the situation is similar in younger subjects. Reasons for this remain unclear but are likely related to factors such as urbanization, demographic shifts, decreased outdoor activity, air pollution and global dimming, and decreases in the cutaneous production of vitamin D with age. The amount of vitamin D from dietary sources is generally viewed as too insignificant in many regions of the world to have an effect on vitamin D status at the population level.[3]

The minimum desirable serum level of 25-hydroxyvitamin D has been suggested to be 20 to 30 ng/mL (to convert 25-hydroxyvitamin D levels to nanomoles per liter, multiply by 2.496) according to the consensus conference[3] and to a study[4] in which the analysis was expanded to cover potential beneficial effects of vitamin D for multiple health outcomes. Low levels of 25-hydroxyvitamin D are clearly related to compromised bone mineral density,[5] to falls[6] and fractures,[7] and to diminished lower extremity function. In addition to higher incidences of cancer[8] and immune dysfunction,[9] low levels of 25-hydroxyvitamin D have been re-

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cently linked to the presence of cardiovascular disease,[10,11] hypertension,[12] and the metabolic syndrome.[13] Results of recent nationwide investigations showed an association of low 25-hydroxyvitamin D levels with important cardiovascular risk factors[11,14] and further supported the findings of preclinical and clinical investigations that demonstrated positive effects of vitamin D and its analogues on fibrinolysis, blood lipids, thrombogenicity, endothelial regeneration, and smooth muscle cell growth.[15] Together, these findings strongly suggest that 25-hydroxyvitamin D has beneficial effects, some involving the cardiovascular system, that are independent of calcium metabolism.

The mediator of these effects is thought to be 1,25-dihydroxyvitamin D, produced by the kidney, by extrarenal tissues (such as the vasculature), and by immune and gastrointestinal cells that express 1α-hydroxylase, which converts 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D.[16] Locally produced 1,25-dihydroxyvitamin D may act in an autocrine or a paracrine manner via activation of the vitamin D receptor, which is found in many different cell types throughout the body.[16] Because the serum level of 25-hydroxyvitamin D is roughly 1000-fold higher and the affinity for vitamin D receptor 100-fold lower compared with 1,25-dihydroxyvitamin D, direct effects of 25-hydroxyvitamin D on gene transcription or activation by intracellular mitochondrial 1α-hydroxylase may be operative.[17] Therefore, low circulating levels of 25-hydroxyvitamin D could in theory damage target tissues in the following 2 ways: (1) by low substrate availability for renal and extrarenal 1α-hydroxylase and decreased local intracellular conversion to 1,25-dihydroxyvitamin D and (2) by a diminished direct effect on vitamin D receptor activation.

Apart from intervention studies[18-20] in hemodialysis patients demonstrating decreased mortality associated with calcitriol treatment, as well as a recent prospective cohort study[21] that found an association between mortality and elevated parathyroid hormone (PTH) levels but not between mortality and 25-hydroxyvitamin D level, there are no large longitudinal studies addressing associations of endogenous serum vitamin D levels with overall and cardiovascular mortality. To focus on this research question, we followed up a large cohort of patients referred for coronary angiography who underwent detailed baseline examinations. This allowed us to adjust our analysis for possible confounders of the association between vitamin D levels and mortality.

METHODS

STUDY POPULATION

The Ludwigshafen Risk and Cardiovascular Health (LURIC) study[22] is a prospective cohort trial designed to evaluate the effect of genetic polymorphisms and plasma biomarkers on cardiovascular health. Patients were recruited between July 1, 1997, and January 14, 2000, at the Herzzentrum Ludwigshafen (Cardiac Center Ludwigshafen) in southwest Germany (49° 29 minutes north latitude). The rationale and design of this study, baseline characteristics of the population, and definitions used for diagnosis of diabetes mellitus and hypertension have been published previously.[22] In brief, our study population comprised 3316 patients of white race/ethnicity referred for coronary angiography in a tertiary care medical center. Serum levels of 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D were determined in 3258 patients (98.3% of the entire study population; mean [SD] age, 62 [10] years), and the analysis was restricted to this group. Study participants had to demonstrate a stable clinical condition except for acute coronary syndrome. Exclusion criteria were any acute illness other than acute coronary syndrome, any predominant noncardiac chronic disease, and a history of malignant neoplasm(s) within the past 5 years. Seventy-eight patients (2.4%) reported taking vitamin supplements on a regular basis, which usually contained B complex vitamins or vitamin D_3. Because 25-hydroxyvitamin D levels (mean [SD], 22.1 [11.3] ng/mL) were only slightly higher in users of vitamin D preparations compared with the remaining cohort (mean [SD], 17.2 [9.1] ng/mL) and because age, PTH levels, and 1,25-dihydroxyvitamin D levels did not differ significantly, we decided to include these patients in the present analysis. Written informed consent was obtained from each participant, and the study was approved by the institutional review board at the Ärztekammer Rheinland-Pfalz (Medical Association of Rheinland-Pfalz).

Coronary angiography was commonly indicated because of clinical symptoms or results of noninvasive tests that suggested myocardial ischemia. Coronary artery disease (CAD) was defined as the presence of at least 50% stenosis of at least 1 of 15 segments of the 3 major coronary arteries, based on maximal luminal narrowing. We used the Charlson Comorbidity Index, which has been shown to be a valid and reliable instrument to assess comborbidity,[23] to form 3 groups of patients with 0 score points (group 0), 1 score point (group 1), and 2 or more score points (group 2). A questionnaire with a scoring system ranging from 1 to 11 was used to classify the mean physical activity levels, and study participants were grouped into the following 3 categories of physical activity: below average (score, 1-3), average (score, 4-7), and above average (score, ≥8).

LABORATORY ANALYSIS

A fasting venous blood sample was obtained in the morning before coronary angiography. Selected variables were measured after samples were snap frozen and stored at –80°C. A summary of methods and test kits used for variables relevant to this study has been published.[22] The estimated glomerular filtration rate was calculated according to the 4-variable model of the Modification of Diet in Renal Disease study.[24]

Serum levels of 25-hydroxyvitamin D were assayed on a weekly basis using a radioimmunoassay (DiaSorin SA, Antony, France) with intra-assay and interassay coefficients of variation of 8.6% and 9.2%, respectively. In a random sample of 100 study participants, 25-hydroxyvitamin D level was also determined using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) with isotopic-labeled internal standard and 2 fragment mass to charge ratios of 401.4 to 382.2 (quantifier) and 401.4 to 365.3 (qualifier). Another fragment (mass to charge ratio, 413.5 to 395.4) was used to monitor 25-hydroxyvitamin D_2 level, but none could be detected in any of the samples. A highly significant correlation was noted between 25-hydroxyvitamin D levels obtained by radioimmunoassay and by LC-MS/MS ($r=0.875$, $P<.001$). Levels of 1,25-dihydroxyvitamin D (Nichols Institute Diagnostika GmbH, Bad Nauheim, Germany) were measured by radioimmunoassay on a multicrystal counter (Berthold LB2014, DiaSorin SA). Intra-assay and

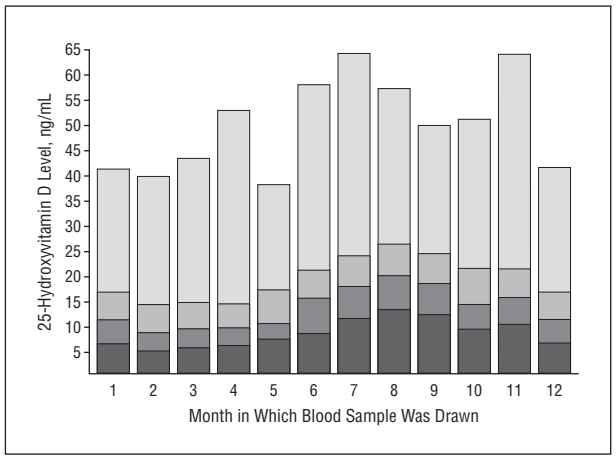

Figure 1. Monthly 25-hydroxyvitamin D level changes in the 25-hydroxyvitamin D quartiles. Black bars indicate 25-hydroxyvitamin D quartile 1 (lowest levels); light gray bars, quartile 4 (highest levels). To convert 25-hydroxyvitamin D levels to nanomoles per liter, multiply by 2.496.

interassay coefficients of variation were below 10% for all described laboratory procedures.

FOLLOW-UP

Information on mortality was obtained from local registries. We used death certificates to classify the deceased into those who died from cardiovascular vs noncardiovascular causes. This classification was done independently by 2 experienced clinicians who were blinded to any data on the study participants except the already-mentioned information that was required to classify the causes of death. In the event of disagreement or uncertainty concerning the cause of death, the decision was made by one of us who is a principal investigator of the LURIC study (W.M.). Eighteen patients could not be contacted for follow-up, and for 23 of the deceased we had insufficient information to classify the cause of death. The latter study participants were included in the analysis of all-cause mortality but were excluded from any statistical procedure regarding cardiovascular mortality.

STATISTICAL ANALYSIS

Because serum vitamin D levels fluctuate by month throughout the year, we decided for the purpose of this analysis not to use absolute vitamin D levels but instead to categorize individual patients' 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D levels into quartiles that were obtained on the basis of 202 to 358 vitamin D measurements of study patients each month. To address other possibilities of 25-hydroxyvitamin D categorization and to allow for comparisons with the quartile-based approach already mentioned, these results are also briefly mentioned in the "Comment" section.

Baseline characteristics of the 25-hydroxyvitamin D groups are given as percentages for categorical data and as medians with interquartile ranges for continuous variables. Comparisons between groups were performed using the χ^2 test for categorical data and using analysis of variance and analysis of covariance with P for trend and adjustments as indicated for continuous data. If appropriate, continuous variables were logarithmically transformed before use in parametric procedures. Kaplan-Meier curves followed by log-rank test were used to evaluate differences in overall and cardiovascular mortality for 25-hydroxyvitamin D categories. Hazard ratios with 95% confidence intervals (CIs) for all-cause and cardiovascular mortality were calculated using Cox proportional hazards regression models. Hazard ratios for 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D categories were calculated by comparing the data with those of the highest 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D quartiles, respectively. We adjusted for several possible confounders in the Cox proportional hazards regression model by using different models that included important clinical variables, cardiovascular risk factors, or factors related to calcium metabolism. The backward stepwise logistic regression selection method was used, and the results of the final step are given. We tested for plausible interactions between the covariates by adding product terms to our models, and we tested for colinearity. Further assumptions underlying the Cox proportional hazards regression model were evaluated by log minus log survival and partial (Schönfeld) residuals vs survival time plots and were found valid. All statistical tests were 2-sided, and statistical significance was defined as $P<.05$. All data were analyzed using commercially available statistical software (SPSS 15.0; SPSS Inc, Chicago, Illinois).

RESULTS

MORTALITY RATES

After a median follow-up of 7.7 years, 737 persons (22.6% of the study population at baseline) had died. Of these, 463 deaths (62.8%) were from cardiovascular causes, 251 (34.1%) were from noncardiovascular causes, and 23 (3.1%) could not be classified because of insufficient data about the cause of death. Among patients with angiographic CAD, 319 deaths occurred from cardiovascular causes and 148 deaths from noncardiovascular causes.

25-HYDROXYVITAMIN D LEVELS

Significant seasonal changes in serum 25-hydroxyvitamin D levels were found, with the lowest and highest levels appearing in March (mean [SD], 12.0 [6.8] ng/mL) and in August (mean [SD], 22.7 [9.6] ng/mL) ($P<.001$, analysis of variance), corresponding to an 89% difference in the mean levels. When patients were grouped into different 25-hydroxyvitamin D categories (0-10, >10-20, >20-25, >25-30, and >30 ng/mL), PTH levels began to rise significantly with the category of greater than 20 to 25 ng/mL. Respective increases in PTH levels averaged 3 pg/mL (to convert PTH levels to nanograms per liter, multiply by 1.0) (for the >20-25 ng/mL category), 5 pg/mL (for the >10-20 ng/mL category), and 12 pg/mL (for the 0-10 ng/mL category). Because 25-hydroxyvitamin D levels vary with the month of the year, the range of 25-hydroxyvitamin D quartiles fluctuates as well (**Figure 1**).

BASELINE CHARACTERISTICS

Baseline characteristics according to 25-hydroxyvitamin D quartiles are summarized in **Table 1**. On average, patients in the lowest 25-hydroxyvitamin D quartile were older, were more likely to be female, and had more comorbidities. The mean serum PTH levels were

Table 1. Baseline Characteristics According to 25-Hydroxyvitamin D Quartiles

Characteristic	Quartile				
	1st (n = 836)	2nd (n = 802)	3rd (n = 813)	4th (n = 807)	*P* Value[a]
25-Hydroxyvitamin D level, ng/mL, median (IQR)	7.6 (5.8-10.1)	13.3 (10.4-16.8)	18.9 (14.6-22.8)	28.4 (23.6-33.5)	<.001
Age, median (IQR), y	66.3 (58.4-73.4)	64.3 (56.3-70.8)	62.7 (55.6-69.5)	61.5 (55.0-67.6)	<.001
Female sex, %	44.1	28.6	25.0	23.3	<.001
Body mass index, median (IQR)[b]	27.4 (24.5-30.4)	27.3 (24.8-30.1)	27.2 (25.0-29.7)	26.7 (24.6-29.0)	.006
Waist to hip ratio, median (IQR)	0.96 (0.91-1.01)	0.97 (0.92-1.02)	0.96 (0.92-1.01)	0.96 (0.91-1.00)	.61
Charlson Comorbidity Index category, %					
Group 0	17.8	28.4	28.3	31.6	<.001
Group 1	27.8	29.6	36.7	37.7	
Group 2	54.4	42.1	35.0	30.7	
Physical activity level, %					
Below average	36.9	26.7	22.3	17.7	<.001
Average	51.7	55.3	56.1	53.1	
Above average	11.4	18.0	21.6	29.2	
New York Heart Association functional class, %					
1	46.9	51.1	53.3	57.7	<.001
2	26.2	29.9	30.8	29.5	
3	22.4	15.7	13.7	11.0	
4	4.5	3.2	2.3	1.7	
Current smokers, %	23.6	18.8	19.7	17.1	.009
Systemic hypertension, %	74.6	73.6	73.4	68.4	.02
Diabetes mellitus, %	41.4	35.3	28.0	22.3	<.001
History of myocardial infarction, %	43.8	40.0	40.3	40.4	.36
Significant coronary artery disease, %	70.4	69.0	67.7	65.6	.20
Medication use, %					
β-Blocker	60.5	64.0	64.8	64.4	.24
Angiotensin-converting enzyme inhibitor	62.4	53.6	49.0	48.2	<.001
Statin	46.2	47.3	47.8	46.6	.91
Aspirin	72.6	70.4	70.8	72.6	.67
Hemoglobin level, median (IQR), g/dL	13.4 (12.3-14.5)	13.9 (13.1-15.0)	14.0 (13.1-15.0)	14.2 (13.4-15.0)	<.001
Glomerular filtration rate, median (IQR), mL·min·1.73 m^2	79 (66-92)	81 (70-92)	81 (71-93)	82 (71-92)	<.001
Cystatin C level, median (IQR), mg/L	0.96 (0.83-1.15)	0.93 (0.81-1.08)	0.89 (0.79-1.02)	0.88 (0.79-0.99)	<.001
Blood lipid level, median (IQR), mg/dL					
Cholesterol	186 (161-213)	192 (165-219)	191 (166-216)	192 (168-217)	.02
High-density lipoprotein	36 (30-44)	37 (31-44)	38 (32-45)	39 (33-47)	<.001
Low-density lipoprotein	112 (93-138)	115 (95-138)	116 (95-138)	115 (95-139)	.55
Triglyceride level, median (IQR), mg/dL	149 (109-202)	152 (112-202)	144 (106-199)	140 (109-196)	.08
N-terminal pro-BNP level, median (IQR), ng/mL	429 (166-1343)	302 (110-908)	244 (96-660)	196 (84-567)	<.001
Serum calcium level corrected, median (IQR), mg/dL	9.3 (9.0-9.6)	9.4 (9.0-9.6)	9.3 (8.9-9.6)	9.3 (9.0-9.6)	.37
Serum magnesium level, mEq/L	2.08 (1.92-2.24)	2.07 (1.92-2.21)	2.04 (1.92-2.21)	2.07 (1.92-2.21)	.04
Serum phosphate level, median (IQR), mg/dL	3.6 (3.2-4.0)	3.5 (3.1-3.9)	3.5 (3.1-3.9)	3.4 (3.1-3.8)	<.001
Parathyroid hormone level, median (IQR), pg/mL	34 (26-46)	30 (23-40)	28 (21-36)	25 (19-33)	<.001
1,25-Dihydroxyvitamin D level, median (IQR), pg/mL	26.9 (21.1-35.6)	32.0 (24.9-40.9)	35.6 (28.8-45.1)	39.1 (31.0-48.4)	<.001

Abbreviations: BNP, brain natriuretic peptide; IQR, interquartile range.

SI conversion factors: To convert 25-hydroxyvitamin D levels to nanomoles per liter, multiply by 2.496; 1,25-dihydroxyvitamin D levels to picomoles per liter, multiply by 2.6; hemoglobin levels to grams per liter, multiply by 10.0; cholesterol levels to millimoles per liter, multiply by 0.0259; triglyceride levels to millimoles per liter, multiply by 0.0113; calcium levels to millimoles per liter, multiply by 0.25; magnesium levels to millimoles per liter, multiply by 0.50; parathyroid hormone levels to nanograms per liter, multiply by 1.0.

[a] Analysis of variance with *P* for trend was used for continuous variables and χ^2 test for categorical variables.

[b] Calculated as weight in kilograms divided by height in meters squared.

36% higher and the 1,25-dihydroxyvitamin D levels 31% lower among patients in the lowest 25-hydroxyvitamin D quartile compared with those in the highest quartile. Differences in corrected serum calcium and phosphate levels were absent or small.

SURVIVAL STATISTICS

Kaplan-Meier curve analysis followed by log-rank test showed that risk for all-cause and cardiovascular mortality increases significantly ($P<.001$) across 25-hydroxyvitamin D quartiles (**Figure 2**). Hazard ratios (with 95% CIs) for all-cause mortality (**Table 2**) and for cardiovascular mortality (**Figure 3**) among 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D quartiles are given for 3 different statistical models that include traditional cardiovascular risk factors or other variables directly or indirectly correlated with vitamin D metabolism. After adjustment for the respective other serum vitamin D level, 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D associations with all-cause and cardiovascular mortality remained significant.

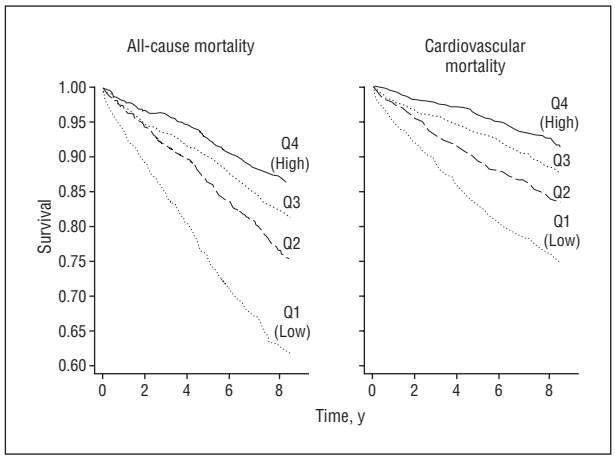

Figure 2. Kaplan-Meier plots of all-cause and cardiovascular mortality in the 25-hydroxyvitamin D quartiles (Q). Log-rank test indicated a significant difference across all 25-hydroxyvitamin D quartiles ($P<.001$). Multivariate-adjusted hazard ratios for patients in the lower two 25-hydroxyvitamin D quartiles (median, 7.6 and 13.3 ng/mL) were higher for all-cause mortality (hazard ratio, 2.08; 95% confidence interval, 1.60-2.70; and hazard ratio, 1.53; 95% confidence interval, 1.17-2.01; respectively) and for cardiovascular mortality (hazard ratio, 2.22; 95% confidence interval, 1.57-3.13; and hazard ratio, 1.82; 95% confidence interval, 1.29-2.58; respectively) compared with patients in the highest 25-hydroxyvitamin D quartile (median, 28.4 ng/mL). To convert 25-hydroxyvitamin D levels to nanomoles per liter, multiply by 2.496.

SUBGROUP ANALYSIS

We performed a multivariate-adjusted subgroup analysis (based on covariates of model 3, described in the legend to Figure 3) for all-cause mortality among patients in different Charlson Comorbidity Index, New York Heart Association functional class, and physical activity level categories. These results are shown in **Figure 4**.

CORRELATION OF 25-HYDROXYVITAMIN D LEVELS WITH 1,25-DIHYDROXYVITAMIN D LEVELS

Univariate correlation analysis between 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D levels was weak ($r=0.36$, $P<.001$) even after adjustment for cystatin C levels ($r=0.32$, $P<.001$). Patients in a given 25-hydroxyvitamin D category could have 1,25-dihydroxyvitamin D serum levels ranging from low to high (**Figure 5**A). Patients' concurrent assignment to both vitamin D quartiles suggests a synergistic increase in all-cause mortality within a respective 25-hydroxyvitamin D or 1,25-dihydroxyvitamin D quartile with declining levels of the respective other vitamin D level (Figure 5B).

MORTALITY RISK IN PATIENTS WITHOUT SIGNIFICANT CAD

We split the results of multivariate-adjusted Cox proportional hazards regression models by different categories of angiographic CAD. A total of 2190 patients (67.3%) had significant CAD (≥50% stenosis). Six hundred ninety-three patients had virtually no CAD (<20% stenosis). Even in patients with CAD of less than 50% stenosis or less than 20% stenosis, there was a gradual increase in all-cause mortality for all lower 25-hydroxyvitamin D or 1,25-dihydroxyvitamin D quartiles (**Figure 6**).

CARDIOVASCULAR RISK MARKERS

We performed multivariate-adjusted comparisons among 25-hydroxyvitamin D quartiles for biological markers potentially linked to cardiovascular risk (**Table 3**). In addition, multiple linear regression analyses were performed for 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D levels, which were found to be correlated with the following log values (significant for all 25-hydroxyvitamin D correlations and for most 1,25-dihydroxyvitamin D correlations): glutathione, interleukin 6, phospholipids, C-reactive protein, intercellular adhesion molecule 1, and vascular cell adhesion molecule 1, with standard β coefficients of 0.10 to 0.20 ($P<.001$ for all) after adjustments for age, sex, body mass index, and physical activity level and of 0.05 to 0.10 after additional multivariate adjustments. The direction of these correlations pointed toward an increase in cardiovascular risk at low vitamin D levels. Variables available for all patients (C-reactive protein and phospholipid levels) were also added to multivariate Cox proportional hazards regression models and were found not to significantly affect the vitamin D mortality relationship.

COMMENT

This study demonstrates that 25-hydroxyvitamin D levels that are in the lower 50% of the vitamin D range of our study population have a hazard ratio (HR) of 1.53 to 2.08 for all-cause mortality after adjustment for traditional cardiovascular risk factors. In subgroup analysis, the relationship of low 25-hydroxyvitamin D levels to mortality was consistent regardless of comorbidity, physical activity level, or New York Heart Association functional class. We could also exclude that variables known to be vitamin D dependent such as serum PTH, calcium, or phosphate level were driving confounders in the association between mortality and serum vitamin D levels. Therefore, a low 25-hydroxyvitamin D level can be considered a strong risk indicator for all-cause mortality in women and in men.

For this analysis, a retrospective classification of all individual 25-hydroxyvitamin D levels into quartiles was performed on the basis of 202 to 358 measurements of 25-hydroxyvitamin D levels each month. A patient belonging to the lowest monthly quartile could have 25-hydroxyvitamin D values ranging from 1.9 to 15.3 ng/mL depending on the season of the year. Although in theory such an approach of quartile-based monthly cutoffs seems logical and appealing, we reached similar results when absolute serum 25-hydroxyvitamin D levels were considered.

However, the cutoff discriminating vitamin D insufficiency and sufficiency is still a matter of debate, and there is likely a "gray area" of overlap between these 2 entities. A cutoff value of 20 ng/mL for normal 25-hydroxyvitamin D levels may be considered a more conservative threshold value because vitamin D levels below that have clearly been shown to be associated with in-

Table 2. Hazard Ratios (HR) for All-Cause Mortality According to 25-Hydroxyvitamin D and 1,25-Dihydroxyvitamin D Quartiles

Variable	Quartile			
	1st	2nd	3rd	4th
25-Hydroxyvitamin D				
25-Hydroxyvitamin D level, median (interquartile range), ng/mL	7.6 (5.8-10.1)	13.3 (10.4-16.8)	18.9 (14.6-22.8)	28.4 (23.6-33.5)
Study participants at risk, No.	831	798	809	802
Deaths, No. (%)	307 (36.9)	185 (23.1)	142 (17.6)	103 (12.8)
Statistical model, HR (95% CI)[a]				
1	3.33 (2.66-4.16)	1.88 (1.48-2.39)	1.39 (1.08-1.79)	1 [Reference]
2	2.64 (2.09-3.34)	1.60 (1.25-2.05)	1.22 (0.94-1.58)	1 [Reference]
3	2.08 (1.60-2.70)	1.53 (1.17-2.01)	1.24 (0.94-1.65)	1 [Reference]
3 Plus				
Serum calcium level corrected	2.14 (1.65-2.78)	1.55 (1.18-2.02)	1.25 (0.95-1.66)	1 [Reference]
Serum phosphate level	2.05 (1.58-2.66)	1.51 (1.15-1.97)	1.24 (0.93-1.64)	1 [Reference]
Parathyroid hormone level	2.07 (1.60-2.69)	1.51 (1.16-1.98)	1.22 (0.92-1.62)	1 [Reference]
1,25-Dihydroxyvitamin D level	1.91 (1.45-2.51)	1.46 (1.11-1.91)	1.21 (0.91-1.61)	1 [Reference]
25-Hydroxyvitamin D level	NA	NA	NA	NA
1,25-Dihydroxyvitamin D				
1,25-Dihydroxyvitamin D, median (interquartile range), pg/mL	20.9 (17.1-23.9)	29.7 (27.5-32.3)	37.7 (35.0-40.9)	50.8 (45.9-58.1)
Study participants at risk, No.	820	818	803	799
Deaths, No. (%)	269 (32.8)	181 (22.1)	152 (18.9)	135 (16.9)
Statistical model, HR (95% CI)[a]				
1	2.20 (1.79-2.71)	1.37 (1.09-1.71)	1.15 (0.91-1.45)	1 [Reference]
2	1.92 (1.55-2.37)	1.27 (1.01-1.59)	1.13 (0.90-1.43)	1 [Reference]
3	1.61 (1.25-2.07)	1.26 (0.97-1.64)	1.16 (0.89-1.51)	1 [Reference]
3 Plus				
Serum calcium level corrected	1.64 (1.28-2.10)	1.27 (0.98-1.65)	1.15 (0.88-1.50)	1 [Reference]
Serum phosphate level	1.56 (1.22-2.01)	1.24 (0.96-1.62)	1.15 (0.88-1.51)	1 [Reference]
Parathyroid hormone level	1.60 (1.24-2.06)	1.26 (0.97-1.64)	1.16 (0.88-1.51)	1 [Reference]
1,25-Dihydroxyvitamin D level	NA	NA	NA	NA
25-Hydroxyvitamin D level	1.32 (1.02-1.71)	1.12 (0.86-1.46)	1.11 (0.85-1.46)	1 [Reference]

Abbreviations: CI, confidence interval; NA, not applicable.

SI conversion factors: To convert 25-hydroxyvitamin D levels to nanomoles per liter, multiply by 2.496; 1,25-dihydroxyvitamin D levels to picomoles per liter, multiply by 2.6.

[a] Model 1, unadjusted; model 2 adjusted for age, sex, body mass index, and physical exercise level; model 3 also adjusted for active smokers, diabetes mellitus, systolic and diastolic blood pressure, albumin level, cystatin C level, triglyceride level, N-terminal pro-brain natriuretic peptide level, low-density lipoprotein and high-density lipoprotein cholesterol levels, and the use of bronchodilators, aspirin, statins, β-blockers, and angiotensin-converting enzyme inhibitors.

creases in serum PTH levels,[25] decreases in physical performance,[26] and diminished lower extremity function.[27] Using such a vitamin D classification, we calculated multivariate-adjusted HRs for all-cause mortality of 2.13 (95% CI, 1.70-2.68) for patients with vitamin D deficiency (≤10 ng/mL) and 1.33 (95% CI, 1.07-1.65) for patients with vitamin D insufficiency (>10-20 ng/mL), values that are close to the HRs obtained using the quartile-based approach. If vitamin D deficiency is defined as 20 ng/mL or less and vitamin D insufficiency as greater than 20 to 30 ng/mL or less and is compared with values greater than 30 ng/mL, the adjusted HRs for all-cause mortality were 2.34 (95% CI, 1.48-3.39) for patients with vitamin D deficiency and 1.54 (95% CI, 0.99-2.41) for patients with vitamin D insufficiency. Using yet another approach, we expressed 25-hydroxyvitamin D levels as percentiles within the respective month. Here, the adjusted HRs for all-cause mortality among patients having hydroxyvitamin D values in the lowest 25-hydroxyvitamin D quartile became even higher compared with those among patients having 25-hydroxyvitamin D values above the 10th percentile (adjusted HR, 4.91; 95% CI, 3.33-7.20; and adjusted HR, 3.04; 95% CI, 1.95-4.75). Therefore, it cannot be excluded that risk gradients become even steeper when outcomes are compared with 25-hydroxyvitamin D levels well above those seen in our study population.

At first glance, the percentage of patients with low 25-hydroxyvitamin D values seems unexpectedly high in the present study. Roughly two-thirds had serum levels below 20 ng/mL. However, our mean value of 17.3 ng/mL compares well with values reported from other large trials performed in middle European countries such as France, Italy, and Germany.[28-30]

Another important aspect supporting our conclusions is that, similar to low 25-hydroxyvitamin D levels, low 1,25-dihydroxyvitamin D levels were associated with increased HRs for mortality, despite a weak correlation between the 2 (r=0.36). This leads to the conclusion that 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D levels may yield similar but independent biologic effects. This is also in line with our finding that, when both variables are considered concomitantly, a synergistic effect on mortality risk seems evident (Figure 5B).

What are the major determinants of circulating 1,25-dihydroxyvitamin D levels? By multiple regression analysis with relevant variables included and with serum 1,25-dihydroxyvitamin D level set as the dependent variable, 25-hydroxyvitamin D levels (partial

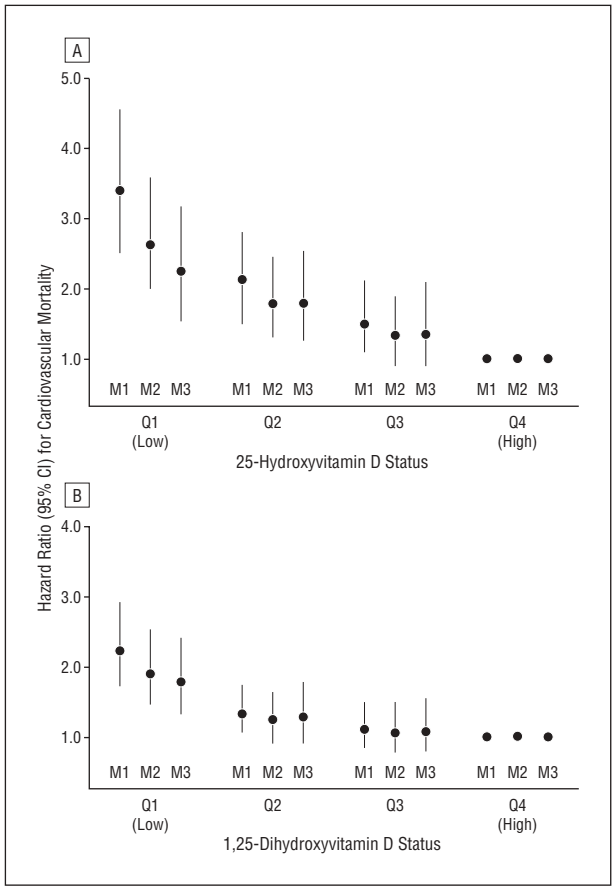

Figure 3. Cox proportional hazards regression model ratios (including 95% confidence intervals [CI]) for cardiovascular mortality are shown for 25-hydroxyvitamin D (A) and 1,25-dihydroxyvitamin D (B) quartiles (Q) for the following 3 different statistical models (M): (1) M1 (unadjusted), (2) M2 (adjusted for age, sex, body mass index, and physical activity level), and (3) M3 (variables of M2 plus active smokers, diabetes mellitus, albumin level, cystatin C level, triglyceride level, N-terminal pro-BNP level, systolic and diastolic blood pressure, low-density lipoprotein and high-density lipoprotein cholesterol levels, and the use of statins, aspirin, β-blockers, bronchodilators, and angiotensin-converting enzyme inhibitors).

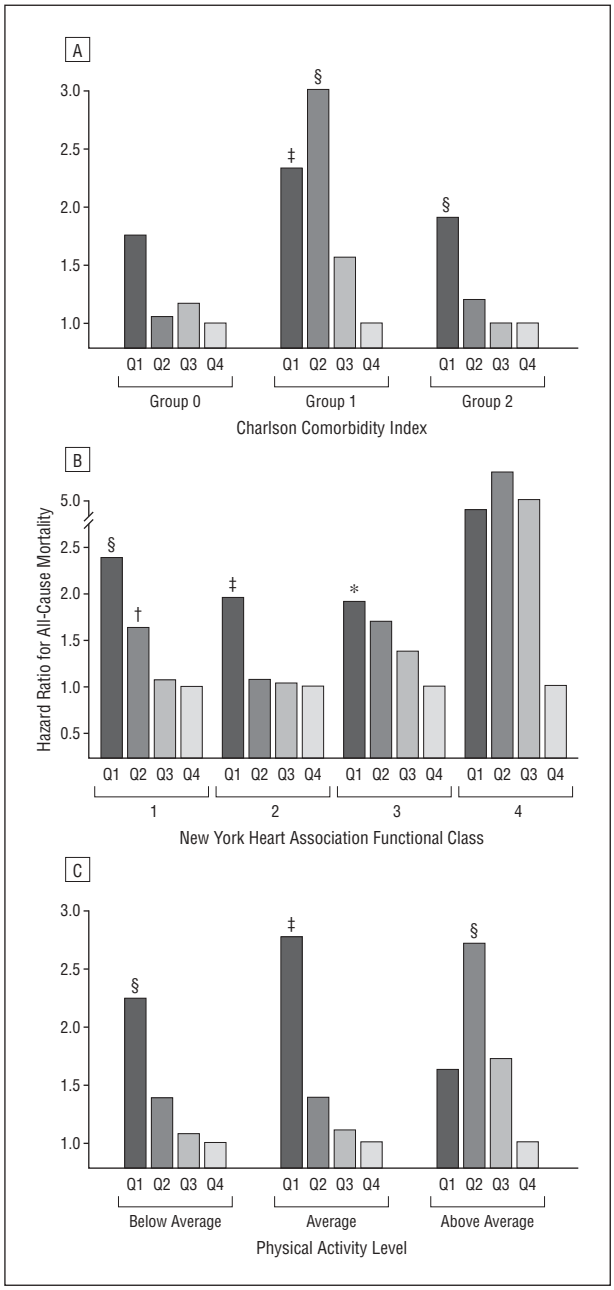

Figure 4. Multivariate-adjusted (covariates as in model 3 of Figure 3) hazard ratios for all-cause mortality in patients categorized by Charlson Comorbidity Index (A), New York Heart Association functional class (B), and physical activity level (C). Q1 indicates patients in the lowest 25-hydroxyvitamin D quartile; Q4, patients in the highest 25-hydroxyvitamin D quartile. *$P<.05$. †$P<.01$. ‡$P<.005$. §$P<.001$ compared with the reference group.

R^2=9.6%) and renal function (partial R^2=7.3% for log cystatin C) constituted the 2 most important independent predictors in the model, yielding an overall R^2 of 24%. We were unable to find an independent association with age, despite a study[31] demonstrating such an effect on 1,25-dihydroxyvitamin D production after infusion of PTH fragment 1-34. Consequently, availability of 25-hydroxyvitamin D substrate and renal function account for a small explainable proportion of 1,25-dihydroxyvitamin D serum levels. The question remains whether extrarenal production of 1,25-dihydroxyvitamin D contributes, at least in part, to the circulating pool of 1,25-dihydroxyvitamin D in individuals with normal renal function.

Another interesting aspect of the present study is that the association of vitamin D levels with mortality can be compared between patients with and without CAD. Low 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D levels were associated with significant increases in all-cause mortality but were consistently higher in patients without significant CAD. We conclude from these data that low 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D levels seem to be important mediators of mortality even when there is little or no indication of overt vascular disease.

A limitation of our study is that we are unable to decide whether the association between low 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D levels and mortality is causal or not. However, there are a few indications pointing to a possible link. Elevated C-reactive protein and interleukin 6 levels in patients with lower 25-hydroxyvitamin D levels suggests that 25-hydroxyvitamin D has anti-inflammatory properties. Similar relationships have been reported by others.[32,33] To-

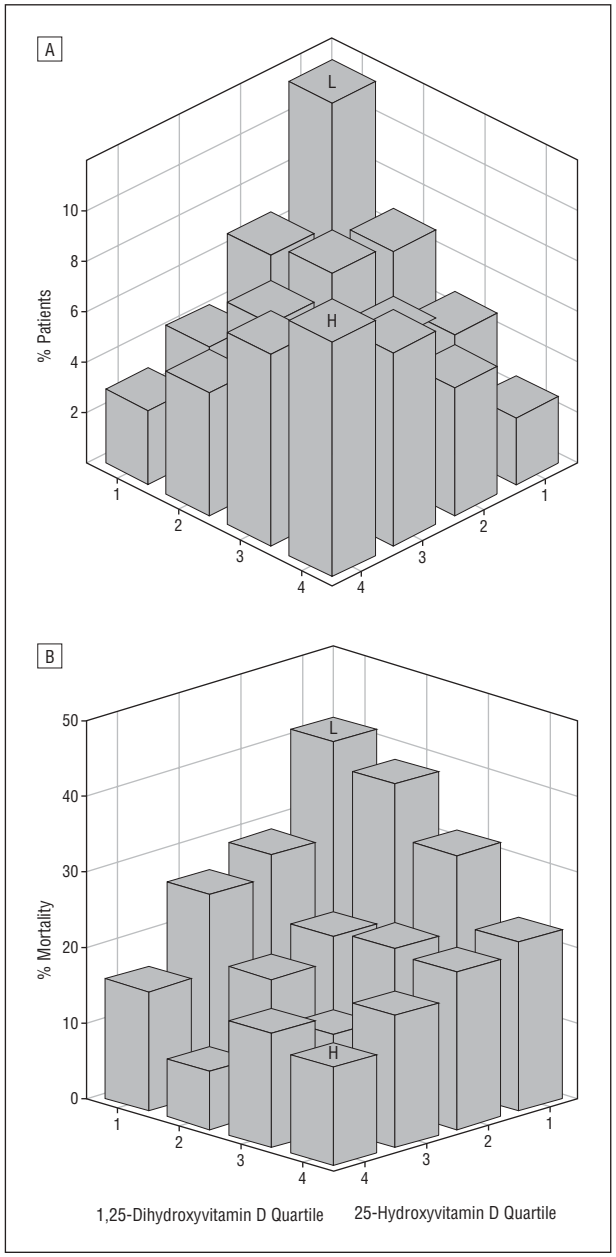

Figure 5. Univariate correlation analysis. A, Distribution profile for both serum vitamin D levels among patients assigned to different 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D quartiles. Although patients with concomitantly high (column H) or concomitantly low (column L) levels of 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D were frequent, the overall correlation between the 2 endogenous D vitamins was weak ($r=0.32$, $P<.001$). B, Effects on mortality rates when 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D levels were considered concomitantly. Patients of column H (both high) showed the lowest and those of column L (both low) the highest mortality rates during the study period.

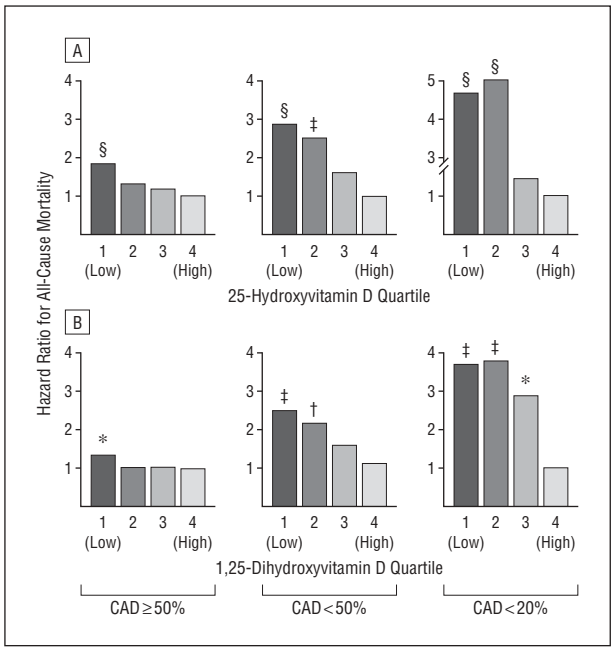

Figure 6. The 25-hydroxyvitamin D (A) and 1,25-dihydroxyvitamin D (B) patient quartiles and corresponding effects on multivariate-adjusted hazard ratios (covariates as in model 3 of Figure 3) for all-cause mortality according to patients' coronary artery disease (CAD) status as determined by coronary angiography at baseline. CAD ≥ 50% indicates patients with significant CAD; patients without significant CAD are presented as 2 categories (<50% or by the most stringent criteria of <20% narrowing of ≥1 of 15 coronary artery segments evaluated). * $P<.05$. † $P<.01$. ‡ $P<.005$. § $P<.001$ compared with the reference group.

gether with the effects related to oxidative stress and increased cell adhesion that we found, low levels of vitamin D may detrimentally affect vascular biologic function in multiple ways. Other mechanisms whereby low vitamin D levels may be associated with mortality include effects on matrix metalloproteinases,[32,34] which were shown to affect plaque production and stability, increased susceptibility to arterial calcification,[35,36] or an increase in renin messenger RNA expression.[37]

Further limitations of our study are related to 25-hydroxyvitamin D level determination and possibly race/ethnicity and changes of serum 25-hydroxyvitamin D levels over time. For measurements of 25-hydroxyvitamin D serum levels, we used a commercial radioimmunoassay that was validated against an LC-MS/MS as a reference method in a patient subgroup. The correlation between the 2 methods was strong ($r=0.87$). We have further confidence in our vitamin D measurements because the annual variation in 25-hydroxyvitamin D level and the rise in serum PTH levels in patients with vitamin D insufficiency were similar, as expected,[25] and because there was a graded biological effect with decreasing 25-hydroxyvitamin D levels. Serum 25-hydroxyvitamin D levels in a given individual seem to be constant over time. Among 100 healthy older individuals participating in a fall prevention study performed at 2 centers, we found a high correlation between two 25-hydroxyvitamin D levels that were 12 months apart ($r=0.758$, $P<.001$; H.D., unpublished data, 2004), suggesting low intraindividual variation over time. The results of this study may further apply only to white race/ethnicity; despite low 25-hydroxyvitamin D levels in African Americans, little or no correlation to cardiovascular disease was reported.[11]

In conclusion, this prospective cohort study demonstrates for the first time, to our knowledge, that low 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D levels are associated with increased risk in all-cause and cardiovascular mortality compared with patients with higher serum vitamin D levels. Both vitamins seem to have synergistic biologic action that is largely independent of each

Table 3. Associations of 25-Hydroxyvitamin D Quartiles With Cardiovascular Risk Markers[a]

Variable	Quartile				*P* Value for Trend[b]
	1st	2nd	3rd	4th	
Markers of inflammation, mean (95% CI)					
C-reactive protein level, mg/dL	3.94 (3.61-4.30)	3.52 (3.24-3.84)	3.08 (2.83-3.35)	3.39 (3.11-3.70)	.005
Interleukin 6 level, ng/L	5.27 (4.56-6.01)	4.15 (3.65-4.72)	4.07 (3.63-4.57)	3.55 (3.16-3.99)	<.001
Markers of cell adhesion, mean (95% CI)					
Intercellular adhesion molecule 1 level, mg/L	254 (248-259)	245 (240-259)	242 (236-247)	241 (235-247)	.003
Vascular cell adhesion molecule 1 level, mg/L	776 (759-793)	762 (744-780)	757 (738-774)	748 (729-769)	.05
Markers of oxidative stress, mean (95% CI)					
Glutathione level, µmol/L	3.54 (3.42-3.67)	3.89 (3.76-4.04)	4.19 (4.05-4.33)	4.08 (3.94-4.23)	<.001
Phospholipid level, mg/dL	212 (209-214)	216 (214-218)	216 (214-219)	218 (216-220)	.001

Abbreviation: CI, confidence interval.
SI conversion factor: To convert C-reactive protein levels to nanomoles per liter, multiply by 9.524.
[a] All data were $\log_{10}$ transformed for analysis and are shown as retransformed adjusted values.
[b] Analysis of covariance with adjustments for age, sex, active smokers, acute infection, diabetes mellitus, systemic hypertension, body mass index, cystatin C level, physical exercise level, N-terminal pro-BNP level, and low-density lipoprotein cholesterol level.

other. Apart from the proved effects that vitamin D has on bone metabolism and neuromuscular function, appropriate serum levels (that may also be higher than in the present investigation) are associated with a decrease in mortality. Although not proved, it seems possible that at least part of this effect may be due to lowering of a risk profile promoting atherosclerosis and preventing cardiovascular end points. Based on the findings of this study, a serum 25-hydroxyvitamin D level of 20 ng/mL or higher may be advised for maintaining general health.

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