Serum Bioavailable and Free 25-Hydroxyvitamin D Levels, but Not Its Total Level, Are Associated With the Risk of Mortality in Patients With Coronary Artery Disease

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Rationale: **Bioavailable and free 25-hydroxyvitamin D (25(OH)D) are emerging measurements of vitamin D. Whether serum bioavailable or free 25(OH)D level is associated with mortality in patients with coronary artery disease (CAD) is unknown.**

Objective: **Our aim is to determine the potential association between serum total, bioavailable, and free 25(OH)D levels and the risk of mortality among patients with CAD.**

Methods and Results: **We measured serum 25(OH) levels in 1387 patients with angiographically confirmed CAD from the Guangdong Coronary Artery Disease Cohort. Serum DBP (vitamin D-binding protein) levels were measured using a polyclonal immunoassay, and serum-free 25(OH)D levels were measured using a 2-step immunoassay. Bioavailable 25(OH)D levels were calculated using a previously validated formula. By the median follow-up time of 6.7 years, 205 patients had died, including 134 deaths from cardiovascular diseases. In multivariate analyses, low serum bioavailable 25(OH)D level was significantly associated with increased risks of mortality, independent of established cardiovascular risk factors, features and treatments of CAD, factors associated with vitamin D and mineral metabolism, and CRP (C-reactive protein). The multivariable-adjusted hazard ratios across quartiles of bioavailable 25(OH)D were 1.79, 1.35, 1.36, and 1.00 for all-cause mortality (*P* for trend=0.01) and 2.58, 1.85, 1.73, and 1.00 for cardiovascular mortality (*P* for trend=0.001), respectively. Serum-free 25(OH)D level was inversely associated with the risk of mortality, with the extreme-quartile hazard ratios of 1.64 for all-cause mortality (*P* for trend=0.024) and 1.97 for cardiovascular mortality (*P* for trend=0.013). In contrast, serum total 25(OH)D level was not significantly associated with all-cause mortality or cardiovascular mortality.**

Conclusions: **Lower serum bioavailable and free 25(OH)D levels rather than total 25(OH)D level are independently associated with an increased risk of all-cause mortality and cardiovascular mortality in a population-based CAD cohort. (*Circ Res*. 2018;123:996-1007. DOI: 10.1161/CIRCRESAHA.118.313558.)**

Key Words: coronary artery disease ■ mortality ■ prognosis ■ risk ■ vitamin D

Vitamin D deficiency is a prevalent health problem all over the world that has been associated with many chronic diseases, including coronary artery disease (CAD).[1] It has been suggested that vitamin D may play a protective role in the cardiovascular system,[2] and vitamin D deficiency is associated with cardiovascular events in the general population.[3,4] However, studies concerning vitamin D status and the prognosis of CAD are scarce, and the conclusions are inconsistent.[5,6] Furthermore, recent clinical trials that used total 25-hydroxyvitamin D (25(OH)D) for vitamin D status did not prove causality.[7–9] Whether vitamin D status is associated with mortality in patients with CAD is still unknown.

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Serum total 25(OH)D is the most commonly used marker for the assessment of vitamin D nutritional status.[1] Approximately 85% to 90% of circulating 25(OH)D are tightly bound to DBP (vitamin D-binding protein), which restricts access of vitamin D metabolites to most cells. While 10% to 15% of 25(OH)D exists in a loose albumin-bound state, and <0.01% of 25(OH)D is free in the circulation.[10,11] The bioavailable hormone hypothesis suggests that only the nonbound fraction of hormones is

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Novelty and Significance

What Is Known?

- Vitamin D is the precursor of a steroid hormone that may be associated with the prognosis of coronary artery disease (CAD).
- The bioavailable hormone hypothesis suggests that only free or bioavailable vitamin D metabolites in circulation are able to enter cells to perform biological actions.

What New Information Does This Article Contribute?

- Serum-free and bioavailable 25-hydroxyvitamin D (25(OH)D) levels, but not total 25(OH)D level, are independently associated with the risk of mortality in patients with CAD.

Rather than total 25(OH)D levels, the levels of free and bioavailable 25(OH)D may better assess the true vitamin D status. However, the relationship between free or bioavailable 25(OH)D and the prognosis of established CAD has not been examined. In an angiographically confirmed CAD cohort, we found that lower serum free and bioavailable 25(OH)D levels, rather than total 25(OH)D level, were associated with an increased risk of mortality, independent of established cardiovascular risk factors, features and treatments of CAD, CRP (C-reactive protein), and others associated with vitamin D and mineral metabolism. These findings suggest that free and bioavailable 25(OH)D may be useful predictors for the prognosis of patients with CAD.

Nonstandard Abbreviations and Acronyms

25(OH)D	25-hydroxyvitamin D
BMI	body mass index
CAD	coronary artery disease
DBP	vitamin D-binding protein
HR	hazard ratio

able to enter cells to perform biological actions.[12] As a precursor of the steroid hormone calcitriol, the bioavailable vitamin D is consistently defined as the vitamin D metabolites that are easily accessible to most cells. Accordingly, free 25(OH)D is a bioavailable form. The loose albumin-bound 25(OH)D is able to dissociate rapidly in the circulation and is also biologically available for tissues. Thus, bioavailable 25(OH)D is generally recognized as 25(OH)D that is not bound to DBP (free plus albumin-bound 25(OH)D). It has been suggested recently that the traditional vitamin D nutritional status which is assessed using total 25(OH)D may be inaccurate under many pathological conditions,[13–15] and non-DBP-bound 25(OH)D may be a better biomarker for vitamin D status, highlighting the need for studies with assessment of free or bioavailable 25(OH)D.

As far as we know, most of previous studies focused on vitamin D deficiency and cardiovascular diseases only with regards to total 25(OH)D[2]. Whether serum bioavailable or free 25(OH)D levels are related to the prognosis of established CAD has not been investigated before. Here, we hypothesized that the vitamin D bioavailable status, different from the traditional nutritional status, is related to the prognosis of CAD patients. Therefore, the present study is aimed at evaluating the association of total, bioavailable, and free 25(OH)D with mortality in a cohort of CAD patients diagnosed by coronary angiography.

Methods

The data, analytic methods, and study materials that support the findings of this study are available from corresponding author on reasonable request.

Study Design and Participants

The Guangdong Coronary Artery Disease Cohort is a prospective observational study designed to investigate the influence of genetic, social, and environmental factors on the progression of CAD. Briefly, patients aged 40 to 85 years were recruited from the cardiology departments of 3 major hospitals (the First Affiliated Hospital, Sun Yat-sen University; the Second Affiliated Hospital, Sun Yat-sen University; and General Hospital of Guangzhou Military Command of People's Liberation Army) in Guangzhou in South China (23° 16' north latitude) between October 2008 and December 2011. All participants underwent coronary angiography and were diagnosed with CAD according to WHO 1999/2000 guidelines.[16,17] The rationale and design of this study, selection criteria, methods, and definitions have been previously published.[18,19] Our study population comprised 1439 participants in whom we performed baseline measurements of serum levels of 25(OH)D and DBP. For the purpose of the current study, we have excluded 31 subjects as they were on moderate- to high- dose vitamin D supplementation (≥400 U/d) treatment. Of the remaining 1408 participants, 21 with missing baseline data of albumin were also excluded, resulting in a final sample size of 1387 (96% of the entire study population). The analysis was restricted to this group. The study protocol was approved by the Sun Yat-sen University Ethics Committee. All participants provided written informed consent.

Analysis of Coronary Angiography

Coronary angiography was performed by at least 2 interventional cardiologists who were unaware of the study protocol. According to the standard definitions of flow-limiting stenoses,[20,21] we classified the severity of CAD as mild (≥20%–<50% stenosis in at least 1 major epicardial coronary artery), moderate (≥50%–<70% stenosis), and obstructive CAD (≥50% stenosis in the left main coronary artery, or ≥70% in any other coronary artery, or both). Patients with obstructive CAD were further categorized by the number of diseased vessels, namely a single, double, or triple-vessel distribution. Taken together, we defined 5 categories of CAD extent in current study: mild CAD, moderate CAD, and 1-, 2-, and 3-vessel obstructive CAD.

Measurements of 25(OH)D and DBP

Fasting serum samples were collected before performing the planned coronary angiography. They were stored at −80°C. Serum 25(OH)D and DBP were measured from duplicates of frozen sample aliquots that had undergone 1 or 2 freeze cycles. Levels of serum total 25(OH)D (sum of 25(OH) D_2 and D_3) were evaluated by ultraperformance liquid chromatography-tandem mass spectrometry. The intra-assay and interassay coefficients of variations were ≤8.3%. DBP levels were measured using a commercial polyclonal antibody enzyme-linked immunosorbent assay (ELISA) kit (Immunodiagnostik AG, Bensheim, Germany). The coefficients of variations for the intra-assay and interassay samples were <10%. Serum DBP levels of Gc1F isoforms have been supposed to be underestimated by using the monoclonal ELISA method.[22] However, the polyclonal assay is suggested to be an appropriate method to assess DBP levels in diverse populations.[23]

Genotyping for DBP single nucleotide polymorphisms (rs4588 and rs7041) was determined using the multiplex SNaPshot assay with the ABI 3130 Genetic Analyzer (Applied Biosystem, CA).

Calculation of Bioavailable 25(OH)D

Serum bioavailable 25(OH)D was calculated using total 25(OH)D, DBP, albumin levels, and affinity constants for albumin and DBP isoforms[12,13] (Online Methods).

Measurements of Free 25(OH)D

Serum-free 25(OH)D was directly measured by a 2-step ELISA kit (Future Diagnostics, Wijchen, The Netherlands). The intra-assay and interassay coefficients of variations were ≤7.0%. The values of free 25(OH)D obtained using this method are well consistent with the values obtained with the liquid chromatography-tandem mass spectrometry method and centrifugal ultrafiltration.

Measurements of Covariates

A standardized questionnaire to provide general information about age, sex, leisure-time physical activity, smoking habits, medical history, and medicine use, including vitamin D supplements, was used in a face-to-face interview. Medical records were reviewed by medical staff to confirm the clinical characteristics, treatments of patients, and angiographic findings documentations. Anthropometric measurements were conducted by trained nurses using standard protocols and procedures. Body mass index (BMI), in kg/m^2, was calculated as weight divided by height in meters squared. Blood pressure (BP) was ascertained as the mean of the last 3 seated measurements except for some emergent patients (<2%). Smoking status was defined as >1 cigarette per day and lasting >6 months, and classified as never, past, or current. Diabetes mellitus was defined by elevated fasting glucose levels (≥126 mg/dL) or the use of antidiabetic medication (oral hypoglycemic agents or insulin). Patients were considered to have acute CAD if they were diagnosed with myocardial infarction (with or without ST-segment elevation) or unstable angina.[21] We calculated the estimated glomerular filtration rate with the use of the Modification of Diet in Renal Disease Study equation[24] and chronic kidney disease was defined as estimated glomerular filtration rate <60 mL/min per 1.73 m^2.

The biochemical variables, including albumin, total cholesterol, triglyceride, HDL (high-density lipoprotein) cholesterol, LDL (low-density lipoprotein) cholesterol, creatinine, calcium, phosphorus, and CRP (C-reactive protein) were immediately tested by standard methods using a Hitachi automatic analyzer 7600-020 (Hitachi, Tokyo, Japan) and using Roche reagents (Roche Diagnostics Corp, Germany). Parathyroid hormone was measured with a commercially available ELISA kit (Abnova, Taibei, Taiwan). All interassay and intra-assay coefficients of variations were ≤8.6%.

Outcomes

The primary outcomes were all-cause mortality and cardiovascular mortality. Annual follow-up information was collected and confirmed by a combination of medical records, telephone interviews with patients or family members, and death registration from Guangdong Provincial Centers for Disease Control and Prevention. The surveys were followed to the end of June 2017 or patient death, whichever occurred first. Death certificates were coded by nosologists according to the *International Classification of Diseases*. Cardiovascular mortality was defined by *International Classification of Diseases Tenth Revision* codes I00-I99.

Statistical Analyses

The baseline clinical characteristics were expressed as means (SD) or medians (interquartile ranges) for continuous variables and percentages (%) for categorical variables. The skewed variables were log transformed before analysis. Serum levels of total, bioavailable, and free 25(OH)D were initially assessed as categorical variables by categorizing into quartiles and then as continuous variable by per SD decrement. Using the Cox proportional hazards models, hazard ratios (HRs) of mortality with 95% CIs were calculated from 2 sets of models: age- and sex-adjusted models, as well as multivariable models adjusted for conventional risk factors for cardiovascular disease[25] (smoking status, presence or absence of diabetes mellitus, systolic BP, total cholesterol, HDL cholesterol, and BMI), features of CAD (presence or absence of acute CAD and coronary artery stenosis extent), treatments of CAD (presence or absence of coronary revascularization, use or nonuse of statins, angiotensin-converting enzyme inhibitors/angiotensin receptor blockers, and β-blockers), factors associated with vitamin D and mineral metabolism[1] (season of blood-drawing, leisure-time physical activity, estimated glomerular filtration rate, parathyroid hormone, and calcium), and biomarker of inflammation (CRP). To display continuous associations of vitamin D with mortality, we examined the Cox regression model using restricted cubic splines with 4 degrees of freedom. And cumulative survival curves were constructed using the Kaplan-Meier method and compared using the log-rank test.

In addition, we performed an analysis on subpopulations stratified by age, sex, BMI, obstructive CAD, chronic kidney disease, and hypertension. To investigate whether the addition of bioavailable or free 25(OH)D to the established cardiovascular risk factors could provide a better prediction on mortality risk, we compared the predictive ability of a model that included the conventional cardiovascular risk factors[25,26] with a corresponding model to which bioavailable or free 25(OH)D was added. The added predictive ability was assessed by C statistics.[27] All statistical analyses were performed with SPSS 19.0 (IBM SPSS Inc, Chicago, IL) and STATA 11.2 (StataCorp, College Station, TX). A 2-sided P value <0.05 was considered statistically significant.

Results

Baseline Characteristics

Baseline characteristics of the 1387 patients are presented in Table 1. At the baseline, the mean age was 63.2 years, and 65.1% of the participants were men, 31.9% were current smokers, 23.7% had diabetes mellitus, and 90.1% had hypertension. Median (interquartile range) serum total, bioavailable, and free 25(OH)D levels were 20.5 (16.6, 25.1) ng/mL, 3.17 (2.11, 4.87) ng/mL, and 4.52 (3.54, 5.95) pg/mL respectively.

The correlation between total, bioavailable, and free 25(OH)D and CAD patient characteristics is shown in Online Table I. Serum total 25(OH)D was associated with sex, presence of diabetes mellitus, season, leisure-time physical activity, calcium, parathyroid hormone, CAD extent, and CRP in the multivariate model. Meanwhile, serum bioavailable and free 25(OH)D were associated with sex, BMI, parathyroid hormone, and season in the same model. There was a strong correlation between serum bioavailable and free 25(OH)D (r=0.811; P<0.001; Online Figure I).

Serum Bioavailable 25(OH)D and Mortality

After a median follow-up of 6.7 years, there were 205 deaths recorded. Of them, 134 patients died of cardiovascular diseases. Figure 1A and 1B shows a significant inverse association between serum bioavailable 25(OH)D as quartiles and the risks of both all-cause mortality (Kaplan-Meier, log-rank P=0.03) and cardiovascular mortality (log-rank P=0.003). In age- and sex-models, HRs across quartiles of serum bioavailable 25(OH)D were 1.98, 1.63, 1.52, and 1.00 for all-cause mortality (P for trend=0.001) and 2.89, 2.26, 1.89, and 1.00 for cardiovascular mortality (P for trend <0.001), respectively. This association remained significant even further adjustment for conventional cardiovascular risk factors, features and treatments of CAD, factors associated with vitamin D and mineral metabolism, and CRP. When serum bioavailable 25(OH)D was examined as a continuous variable, the multivariate-adjusted HRs for each 1 SD decrease in serum bioavailable

Table 1. Baseline Characteristics of Patients by Quartiles of Bioavailable 25-Hydroxyvitamin D

Characteristics	Bioavailable 25-hydroxyvitamin D				*P* Value
	Quartile 1	Quartile 2	Quartile 3	Quartile 4	
Cut points, ng/mL	≤2.11	2.12–3.17	3.18–4.87	≥4.88	
Participants, N	346	347	347	347	
Vitamin D metabolism factors					
25-hydroxyvitamin D, median (IQR), ng/mL*	16.8 (12.6–21.0)	20.4 (17.3–24.5)	21.3 (17.1–26.4)	23.2 (19.8–27.4)	<0.001
Free 25-hydroxyvitamin D, median (IQR), pg/mL*	3.17 (2.68–3.74)	4.19 (3.73–4.66)	5.14 (4.30–5.89)	7.41 (5.89–8.37)	<0.001
Vitamin D-binding protein, median (IQR), μg/mL*	284.7 (218.1–354.9)	209.7 (162.1–256.8)	139.0 (102.8–181.9)	76.9 (57.0–98.4)	<0.001
Vitamin D-binding protein isoform, N (%)					
Gc-1S/GC-1S	49 (14.1)	25 (7.2)	34 (9.8)	31 (8.9)	<0.001
Gc-1S/GC-1F	85 (24.6)	89 (25.6)	85 (24.5)	62 (17.9)	<0.001
Gc-1S/GC-2	59 (17.1)	72 (20.7)	72 (20.7)	61 (17.6)	<0.001
Gc-1F/GC-1F	73 (21.1)	61 (17.6)	47 (13.5)	69 (19.9)	<0.001
Gc-1F/GC-2	44 (12.7)	73 (21.0)	83 (23.9)	85 (24.5)	<0.001
Gc-2/GC-2	36 (10.4)	27 (7.8)	26 (7.5)	39 (11.2)	<0.001
Albumin, median (IQR), g/L*	39.7 (37.5–41.7)	40.1 (37.8–42.0)	40.3 (38.1–42.9)	40.4 (38.3–42.5)	0.001
Season of blood-drawing, N (%)					
Spring	110 (31.8)	80 (23.1)	70 (20.2)	59 (17.0)	<0.001
Summer	112 (32.4)	122 (35.2)	134 (38.6)	112 (32.3)	<0.001
Autumn	93 (26.9)	102 (29.4)	107 (30.8)	126 (36.3)	<0.001
Winter	31 (9.0)	43 (12.4)	36 (10.4)	50 (14.4)	<0.001
Clinical characteristics					
Age, mean (SD), y	63.3 (12.3)	63.9 (11.1)	62.8 (11.2)	63.0 (11.7)	0.576
Men, N (%)	211 (61.0)	229 (66.0)	222 (64.0)	241 (69.5)	0.122
Body mass index,† mean (SD), kg/m^2	23.8 (3.7)	23.6 (3.2)	24.3 (3.7)	24.1 (3.2)	0.027
Smoking status, N (%)					
Never	212 (61.3)	203 (58.5)	204 (58.8)	194 (55.9)	0.746
Former	29 (8.4)	30 (8.6)	33 (9.5)	40 (11.5)	0.746
Current	105 (30.3)	114 (32.9)	110 (31.7)	113 (32.6)	0.746
Leisure-time physical activity, N (%)					
None	145 (41.9)	120 (34.6)	121 (34.9)	140 (40.3)	0.305
<30 min/d	82 (23.7)	97 (28.0)	91 (26.2)	79 (22.8)	0.305
≥30 min/d	119 (34.4)	130 (37.5)	135 (38.9)	128 (36.9)	0.305
Presence of hypertension, N (%)	310 (89.6)	316 (91.1)	307 (88.5)	316 (91.1)	0.606
Systolic blood pressure, mean (SD), mm Hg	133.0 (22.9)	134.5 (22.4)	133.6 (22.8)	133.2 (21.3)	0.823
Diastolic blood pressure, mean (SD), mm Hg	76.1 (12.8)	77.5 (13.1)	76.3 (13.1)	77.0 (11.8)	0.461
Presence of diabetes mellitus, N (%)	77 (22.3)	87 (25.1)	81 (23.3)	83 (23.9)	0.851
Total cholesterol, mean (SD), mg/dL	184.6 (44.4)	179.2 (42.5)	179.2 (40.5)	179.5 (42.9)	0.24
High-density lipoprotein cholesterol, mean (SD), mg/dL	41.7 (10.8)	47.3 (10.8)	42.1 (10.8)	42.8 (10.8)	0.231
Low-density lipoprotein cholesterol, mean (SD), mg/dL	115.8 (37.5)	112.4 (35.5)	112.7 (37.5)	115.4 (39.0)	0.443
Coronary factors					
Presence of acute CAD, N (%)	218 (63.0)	223 (64.3)	208 (59.9)	206 (59.4)	0.481
CAD extent, N (%)					
Mild	34 (9.8)	41 (11.8)	74 (21.3)	65 (18.7)	<0.001

(*Continued*)

Table 1. Continued

Characteristics	Bioavailable 25-hydroxyvitamin D				*P* Value
	Quartile 1	Quartile 2	Quartile 3	Quartile 4	
Moderate	122 (35.3)	98 (28.3)	74 (21.3)	91 (26.2)	<0.001
1-vessel obstructive	30 (8.7)	25 (7.2)	26 (7.5)	29 (8.4)	<0.001
2-vessel obstructive	70 (20.2)	67 (19.3)	65 (18.7)	79 (22.8)	<0.001
3-vessel obstructive	90 (26.0)	116 (33.4)	108 (31.1)	83 (23.9)	<0.001
Presence coronary revascularization, N (%)	188 (54.3)	198 (57.1)	202 (58.2)	192 (55.3)	0.736
Use of medicine					
Statins, N (%)	265 (76.6)	272 (78.4)	279 (80.4)	271 (78.1)	0.68
β-blockers, N (%)	193 (55.8)	197 (56.8)	203 (58.5)	186 (53.6)	0.622
ACEIs/ARBs, N (%)	191 (55.2)	201 (57.9)	184 (53.0)	203 (58.5)	0.436
Mineral metabolism					
eGFR‡ of <60 mL/min per 1.73 m^2, N (%)	55 (15.9)	58 (16.7)	50 (14.4)	49 (14.1)	0.749
Parathyroid hormone, median (IQR), pg/mL*	78.6 (51.1–123.3)	72.1 (50.5–111.7)	67.6 (46.9–100.5)	63.0 (44.2–95.3)	<0.001
Calcium, median (IQR), mg/dL*	8.88 (8.52–9.20)	9.00 (8.64–9.32)	9.00 (8.68–9.36)	8.96 (8.68–9.28)	0.123
Inflammatory markers					
C-reactive protein, median (IQR), mg/L*	4.12 (1.13–14.21)	4.10 (1.24–13.75)	3.80 (0.90–13.63)	2.95 (0.79–11.35)	0.103

SI conversion factors: to convert 25-hydroxyvitamin D to nmol/L, multiply by 2.496; cholesterol to mmol/L, multiply by 0.0259; calcium to mmol/L, multiply by 0.25; phosphorus to mmol/L, multiply by 0.323; C-reactive protein to nmol/L, multiply by 9.524. ACEI indicates angiotensin-converting enzyme inhibitors; ARB, angiotensin receptor blockers; CAD, coronary artery disease; IQR, interquartile range; and eGFR, estimated glomerular filtration rate.

*The skewed variables were log transformed before analysis.

†Body mass index was the weight in kilograms divided by the square of the height in meters.

‡The eGFR was calculated with the use of the Modification of Diet in Renal Disease Study equation.

25(OH)D were 1.24 (95% CI, 1.06–1.47) for all-cause mortality and 1.38 (95% CI, 1.11–1.71) for cardiovascular morality, respectively (Table 2). The restricted cubic splines further displayed a dose-response association between bioavailable 25(OH)D and mortality (Figure 2A and 2B).

Serum-Free 25(OH)D and Mortality

Similar to bioavailable 25(OH)D, low serum-free 25(OH)D levels also had an association with increased risks of all-cause mortality (log-rank *P*=0.03; Figure 1C) and cardiovascular mortality (log-rank *P*=0.016; Figure 1D). The multivariable-adjusted HRs across quartiles of free 25(OH)D were 1.64, 1.35, 1.23, and 1.00 for all-cause mortality (*P* for trend=0.024), and 1.97, 1.59, 1.40, and 1.00 for cardiovascular mortality (*P* for trend=0.013), and the multivariate-adjusted HRs for each 1 SD decrease in serum-free 25(OH)D were 1.23 (95% CI, 1.05–1.45) for all-cause mortality, and 1.31 (95% CI, 1.07–1.61) for cardiovascular morality, respectively (Table 2; Figure 2C and 2D).

Serum Total 25(OH)D and Mortality

There was no significant association between serum total 25(OH)D and the risk of mortality in the multivariable models (Table 2). When serum total 25(OH)D was examined as a continuous variable (each 1 SD decrease), the direction or magnitude of estimates did not change to any relevant extent. To better define the individual contribution of the confounder to the association between 25(OH)D and mortality, we further assessed the percent change in the magnitude of the association before and after adjustment for each confounder, and found that CRP, extent of CAD, and season of blood-drawing were largely responsible for the attenuated associations (Online Figure II).

DBP and Mortality

No significant difference was observed in terms of mortality according to the different allelic variants of DBP (Online Table II). In addition, there was no association of serum DBP levels with mortality using the Cox proportional hazards models that incorporated DBP as quartiles or a continuous variable (Online Table III).

Discrimination

The incorporation of a combination of bioavailable 25(OH)D into a model with conventional cardiovascular risk factors improved the prediction capability of cardiovascular death (C statistic with serum bioavailable 25(OH)D versus without serum bioavailable 25(OH)D, 0.733 versus 0.715, *P*=0.042; C statistic increment, 0.018; 95% CI, 0.001–0.034; Online Table IV).

Subgroup Analyses

As shown in Table 3, the association between bioavailable and free 25(OH)D levels and cardiovascular mortality was evident in patients with uncontrolled hypertension (BP ≥140/90 mm Hg or higher) but not in those with BP <140/90 mm Hg (*P* for interaction <0.05). There was no significant association between bioavailable or free 25(OH)D levels and mortality based on age, sex, BMI, estimated glomerular filtration rate, or extent of CAD (Table 3).

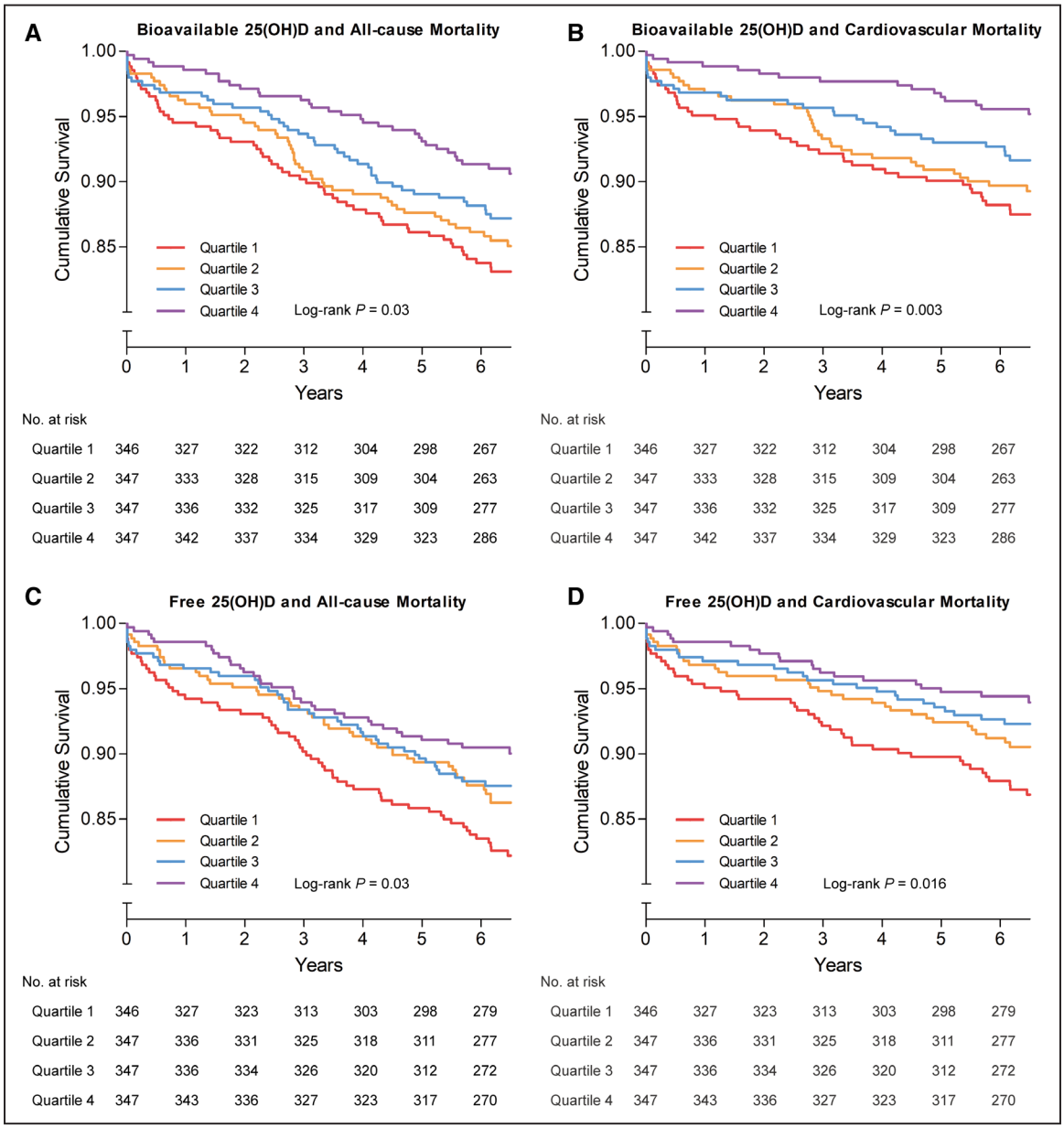

Figure 1. Kaplan-Meier plots for all-cause mortality (A) and cardiovascular mortality (B) according to bioavailable 25-hydroxyvitamin D (25(OH) D) quartiles and all-cause mortality (C) and cardiovascular mortality (D) according to free 25(OH)D quartiles among patients with coronary artery disease.

Discussion

We present in this study that lower serum bioavailable and free 25(OH)D levels are associated with increased risks of all-cause mortality and cardiovascular mortality among patients with CAD, independent of established cardiovascular risk factors, features and treatments of CAD, factors associated with vitamin D and mineral metabolism, and CRP. In contrast, serum total 25(OH)D levels are not associated with the risk of mortality once the confounders were accounted for various cardiovascular protective actions of vitamin D have been demonstrated.[28,29] However, 2 published cohort studies, the LURIC study (Ludwigshafen Risk and Cardiovascular Health)[6] and the KAROLA study (Langzeiterfolge der KARdiOLogischen Anschlussheilbehandlung),[5] have addressed the prognostic value of total 25(OH)D for mortality among suspected CAD patients. Both studies were conducted in Germany while the authors have drawn opposite conclusions. The LURIC study suggested an independent association of low 25(OH)D levels with mortality in a population scheduled for coronary angiography. The KAROLA study and our current study, however, did not support such association. It needs to be pointed out that total 25(OH)D levels presented in the LURIC study were much lower than that in our current study. The difference could be attributed to several potential reasons, including the lower latitude of Guangzhou and the racial differences. Of note, despite geographical and racial heterogeneities, there was no data on the angiographic findings in previous observational studies. Thus, effects of CAD extent on mortality, which may be a substantially confounding factor from CAD features, were not available before. In general,

Table 2. HRs for Mortality According to Total, Bioavailable, and Free 25-Hydroxyvitamin D in Patients With CAD

	Total 25-Hydroxyvitamin D					
	Quartile 1	Quartile 2	Quartile 3	Quartile 4	*P* for Trend	25-Hydroxyvitamin D as a Continuous Variable (1 SD Decrease)
All-cause mortality						
No. of deaths/person-years	73/2043	47/2194	42/2294	43/2354		
Age- and sex- adjusted HR (95% CI)	1.80 (1.22–2.64)	1.13 (0.75–1.72)	1.04 (0.68–1.59)	1	0.002	1.29 (1.11–1.50)
Multivariable-adjusted HR (95% CI)*	1.36 (0.88–2.12)	0.91 (0.58–1.44)	0.99 (0.63–1.56)	1	0.159	1.16 (0.98–1.37)
Cardiovascular mortality						
No. of deaths/person-years	51/2043	28/2194	27/2294	28/2354		
Age- and sex- adjusted HR (95% CI)	1.95 (1.22–3.13)	1.06 (0.62–1.80)	1.04 (0.61–1.76)	1	0.004	1.34 (1.11–1.62)
Multivariable-adjusted HR (95% CI)	1.49 (0.87–2.56)	0.84 (0.47–1.51)	0.98 (0.56–1.74)	1	0.145	1.20 (0.98–1.48)
	Bioavailable 25-hydroxyvitamin D					
	Quartile 1	Quartile 2	Quartile 3	Quartile 4	*P* for Trend	Bioavailable 25-hydroxyvitamin D as a Continuous Variable (1 SD Decrease)
All-cause mortality						
No. of deaths/person-years	63/2141	56/2183	48/2250	38/2310		
Age- and sex- adjusted HR (95% CI)	1.98 (1.32–2.97)	1.63 (1.08–2.46)	1.52 (0.99–2.33)	1	0.001	1.32 (1.13–1.54)
Multivariable-adjusted HR (95% CI)	1.79 (1.17–2.72)	1.35 (0.88–2.06)	1.36 (0.88–2.12)	1	0.01	1.25 (1.06–1.47)
Cardiovascular mortality						
No. of deaths/person-years	46/2141	39/2183	30/2250	19/2310		
Age- and sex- adjusted HR (95% CI)	2.89 (1.69–4.95)	2.26 (1.30–3.91)	1.89 (1.06–3.37)	1	< 0.001	1.48 (1.21–1.82)
Multivariable-adjusted HR (95% CI)	2.58 (1.47–4.52)	1.85 (1.05–3.25)	1.73 (0.96–3.13)	1	0.001	1.39 (1.12–1.72)
	Free 25-hydroxyvitamin D					
	Quartile 1	Quartile 2	Quartile 3	Quartile 4	*P* for Trend	Free 25-hydroxyvitamin D as a Continuous Variable (1 SD Decrease)
All-cause mortality						
No. of deaths/person-years	67/2186	53/2242	47/2254	38/2202		
Age- and sex- adjusted HR (95% CI)	1.78 (1.20–2.66)	1.60 (1.05–2.43)	1.37 (0.89–2.10)	1	0.003	1.30 (1.12–1.51)
Multivariable-adjusted HR (95% CI)	1.64 (1.08–2.50)	1.35 (0.87–2.07)	1.23 (0.92–1.65)	1	0.024	1.23 (1.05–1.45)
Cardiovascular mortality						
No. of deaths/person-years	47/2186	36/2242	29/2254	22/2202		
Age- and sex- adjusted HR (95% CI)	2.19 (1.32–3.64)	1.88 (1.11–3.21)	1.45 (0.83–2.53)	1	0.002	1.39 (1.15–1.69)
Multivariable-adjusted HR (95% CI)	1.97 (1.15–3.37)	1.59 (0.91–2.76)	1.40 (0.79–2.49)	1	0.013	1.31 (1.07–1.61)

CAD indicates coronary artery disease; and HR, hazard ratio.

*Adjusted for age, sex, body mass index, smoking status, presence or absence of diabetes mellitus, systolic pressure, total cholesterol, high-density lipoprotein cholesterol, extent of CAD, presence or absence of acute CAD, presence or absence of coronary revascularization, use or nonuse of statins, angiotensin-converting enzyme inhibitors/angiotensin receptor blockers, and β-blockers, season of blood-drawing, leisure-time physical activity, estimated glomerular filtration rate, calcium, parathyroid hormone, and C-reactive protein.

obstructive CAD patients are considered to have a more severe disease status than patients with a nonobstructive manifestation. Patients who had significant CAD at admission were more likely to lack the exposure to sunlight and may be susceptible to vitamin D deficiency. We have consistently found that total 25(OH) D levels were significantly associated with coronary artery stenosis extent at the baseline, confirming that low total 25(OH)D levels may be a manifestation of CAD severity, and worse health status may explain the association between total 25(OH)D and mortality in patients with CAD.

In contrast, we have observed an independent association between serum bioavailable and free 25(OH)D levels and mortality, indicating that bioavailable and free 25(OH)D rather than total 25(OH)D are an independent predictor of adverse outcomes among CAD patients. On the basis of data for cardiovascular mortality, bioavailable 25(OH)D was likely to be a more specific vitamin D biomarker to estimate the risk of cardiovascular mortality than total 25(OH)D according to the discrimination analysis. Although to date there is little evidence that poor vitamin D bioavailable status is capable of providing future insight about the risk of mortality among CAD patients

beyond total 25(OH)D, it has been suggested that bioavailable 25(OH)D might be better correlated with measures of mineral metabolism than total 25(OH)D in end-stage renal disease patients.[30] In another population-based study, low levels of bioavailable 25(OH)D as opposed to total 25(OH)D were found to be associated with new onset end-stage renal disease.[14] The relationship of bioavailable 25(OH)D and kidney disease may be a special case. However, renal insufficiency also predicts cardiovascular events among patients with CAD independently.[31,32] Furthermore, a recent community-based study demonstrated an association between bioavailable 25(OH)D and cardiovascular events in white populations.[33] These emerging evidence and our results highlight that assessment of vitamin D bioavailable status may be useful for the prediction of cardiovascular events.

Previous studies have demonstrated an interaction between vitamin D deficiency and hypertension,[3] and we consistently found a stronger association between bioavailable 25(OH)D and mortality among patients with uncontrolled hypertension. It is well known that vitamin D is a negative endocrine regulator of the renin-angiotensin system,[34] and hypertension promotes left ventricular hypertrophy, resulting in increased myocardial oxygen demand and decreased subendocardial perfusion.[21] It is likely that vitamin D deficiency, with its adverse effects of suppression of the rennin-angiotensin-aldosterone system, increased BP, together with the direct effect on cardiovascular system itself, promotes progress of CAD.

There remains scarce data on the role of vitamin D supplementation on cardiovascular outcomes. Nor did limited randomized controlled trials provide us with conclusive evidence.[2,7,8] The recent EVITA study (Effect of Vitamin D on All-Cause Mortality in Heart Failure Patients), which had an inclusion criteria of baseline serum 25(OH)D levels <30 ng/mL, demonstrated that vitamin D supplementation did not reduce mortality in patients with heart failure.[7] Another clinical trial with the interventional aim to increase the serum 25(OH)D levels to 32 to 40 ng/mL demonstrated that vitamin D supplementation did not prevent cardiovascular diseases in general population.[8] It is important to note that these randomized controlled trials only used total 25(OH)D to evaluate vitamin D status of subjects. However, as mentioned previously, the association between total 25(OH)D and cardiovascular events can be modified by residual confounding such as poor health status. Thus, these trials cannot assess the comprehensive vitamin D status of individuals in absence of bioavailable 25(OH)D measurements, leading to selection and information biases.

An additional consideration for vitamin D status beyond the traditional biomarker is DBP. In addition to its critical determination on the bioavailability of vitamin D, DBP per se is a multi-functional globulin with potential implication in the development and prognosis of cardiovascular diseases.[33,35] A

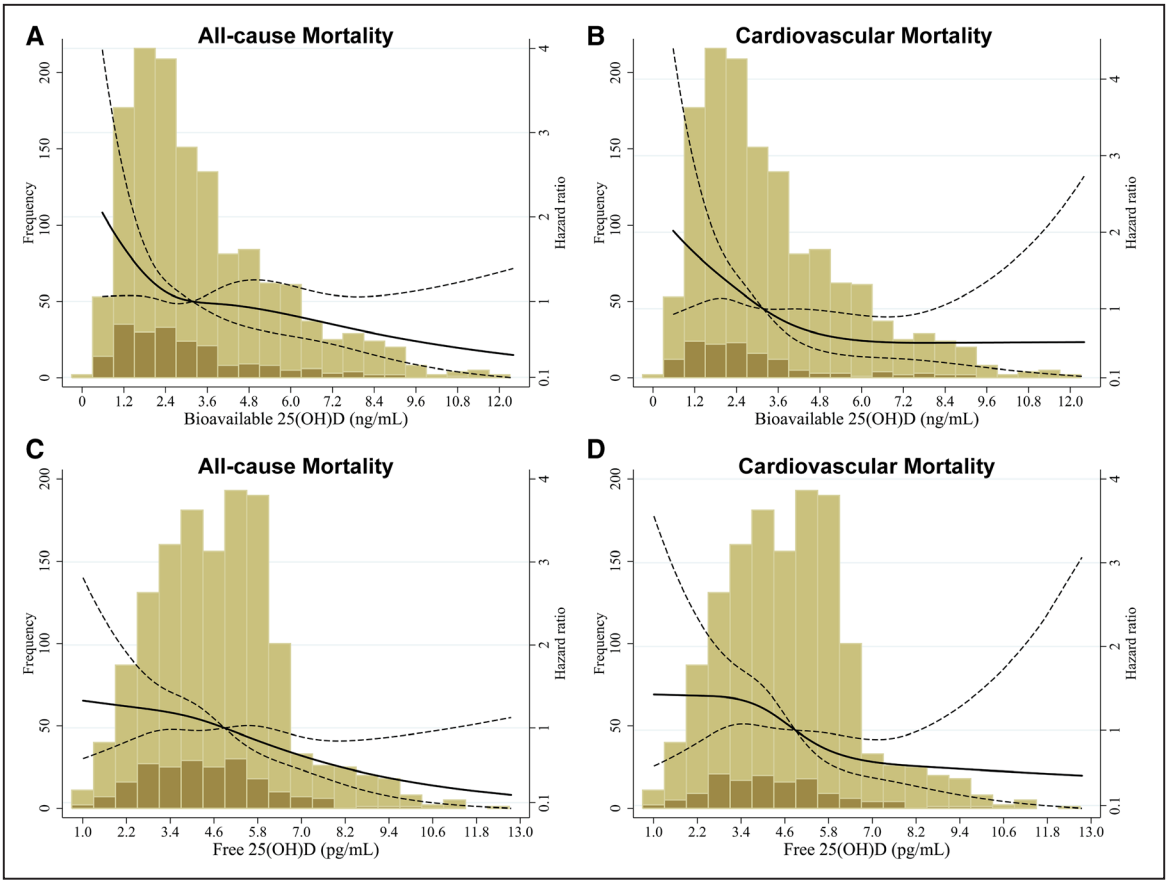

Figure 2. Multivariable-adjusted spline functions demonstrate the relation between bioavailable 25-hydroxyvitamin D (25(OH)D) and all-cause mortality (A) and cardiovascular mortality (B) and the relation between free 25(OH)D and all-cause mortality (C) and cardiovascular mortality (D). The histogram of the distribution of bioavailable or free 25(OH)D levels is also shown. Deaths were shown in dark brown column, and survivals were shown in light brown column.

Table 3. Hazard Ratios* for Mortality According to Serum Bioavailable and Free 25-Hydroxyvitamin D in CAD Patients of Various Subpopulations

Subpopulation	No. of Death/No. of Participants	Free 25-Hydroxyvitamin D				*P* for Trend	*P* for Interaction
		Quartile 1	Quartile 2	Quartile 3	Quartile 4		
All-cause mortality							
Age groups, y							0.745
≤65	42/725	1.43 (0.56–3.65)	1.32 (0.51–3.43)	1.18 (0.51–3.09)	1	0.433	
>65	163/662	2.04 (1.27–3.28)	1.38 (0.84–2.25)	1.58 (0.96–2.59)	1	0.009	
Sex groups							0.848
Men	141/903	1.92 (1.14–3.25)	1.65 (0.98–2.77)	1.34 (0.77–2.34)	1	0.009	
Women	64/484	1.98 (0.90–4.36)	0.82 (0.36–1.86)	1.37 (0.59–3.16)	1	0.107	
BMI, kg/m^2							0.09
<24	127/736	2.72 (1.53–4.82)	1.47 (0.82–2.67)	1.78 (0.97–3.28)	1	0.003	
≥24	78/651	0.94 (0.47–1.89)	1.19 (0.61–2.30)	1.11 (0.54–2.25)	1	0.966	
Extent of CAD							0.554
Nonobstructive	84/599	1.50 (0.83–2.71)	0.73 (0.37–1.44)	0.86 (0.41–1.81)	1	0.389	
Obstructive	121/788	1.93 (1.06–3.51)	1.64 (0.91–2.96)	1.99 (1.11–3.57)	1	0.046	
eGFR, mL/min per 1.73 m^2							0.847
<60	57/212	2.23 (0.89–5.59)	1.68 (0.71–3.99)	1.87 (0.75–4.62)	1	0.099	
≥60	148/1175	1.80 (1.10–2.96)	1.31 (0.79–2.17)	1.34 (0.80–2.26)	1	0.035	
Blood pressure, mm Hg							0.032
≥140/90	93/548	3.05 (1.53–6.09)	2.01 (0.99–4.07)	1.85 (0.88–3.86)	1	0.002	
<140/90	112/839	1.36 (0.76–2.44)	1.13 (0.62–2.04)	1.39 (0.78–2.48)	1	0.391	
Cardiovascular mortality							
Age groups, y							0.954
≤65	30/725	2.65 (0.77–8.52)	2.20 (0.63–7.74)	1.20 (0.32–4.59)	1	0.096	
>65	104/662	2.78 (1.47–5.26)	1.95 (1.02–3.73)	1.88 (0.95–3.72)	1	0.002	
Sex groups							0.684
Men	95/903	2.98 (1.48–6.00)	2.57 (1.28–5.15)	1.73 (0.82–3.66)	1	0.001	
Women	39/484	2.72 (0.97–7.60)	1.05 (0.35–3.08)	1.03 (0.33–3.26)	1	0.029	
BMI, kg/m^2							0.205
<24	84/736	5.18 (2.35–11.41)	2.52 (1.12–5.64)	2.85 (1.22–6.65)	1	<0.001	
≥24	50/651	1.25 (0.51–3.05)	1.83 (0.78–4.26)	1.20 (0.46–3.15)	1	0.393	
Extent of CAD							0.38
Nonobstructive	53/599	2.61 (1.16–5.86)	0.90 (0.35–2.34)	1.09 (0.40–2.99)	1	0.052	
Obstructive	81/788	2.29 (1.05–5.00)	2.47 (1.17–5.22)	2.08 (0.96–4.52)	1	0.019	
eGFR, mL/min per 1.73 m^2							0.842
<60	39/212	3.35 (1.04–10.82)	1.55 (0.48–5.05)	2.48 (0.77–8.03)	1	0.088	
≥60	95/1175	2.40 (1.22–4.69)	2.01 (1.03–3.90)	1.55 (0.76–3.15)	1	0.008	
Blood pressure, mm Hg							0.016
≥140/90	61/548	5.12 (1.91–13.70)	3.80 (1.40–10.34)	2.59 (0.89–7.52)	1	0.001	
<140/90	73/839	1.78 (0.84–3.75)	1.38 (0.65–2.92)	1.61 (0.76–3.43)	1	0.168	
Subpopulation	No. of death/No. of participants	Free 25-hydroxyvitamin D				*P* for Trend	*P* for Interaction
		Quartile 1	Quartile 2	Quartile 3	Quartile 4		
All-cause mortality							
Age groups, y							0.32
≤65	42/725	1.00 (0.38–2.68)	1.05 (0.39–2.86)	1.25 (0.49–3.23)	1	0.96	

(*Continued*)

Table 3. Continued

Subpopulation	No. of Death/No. of Participants	Free 25-Hydroxyvitamin D				*P* for Trend	*P* for Interaction
		Quartile 1	Quartile 2	Quartile 3	Quartile 4		
>65	163/662	1.81 (1.11–2.94)	1.75 (1.08–2.83)	1.23 (0.74–2.06)	1	0.007	
Sex groups							0.911
Men	141/903	2.05 (1.23–3.44)	1.43 (0.82–2.49)	2.08 (1.22–3.55)	1	0.018	
Women	64/484	0.93 (0.40–2.19)	1.44 (0.65–3.16)	0.54 (0.22–1.31)	1	0.701	
BMI, kg/m^2							0.108
<24	127/736	2.78 (1.50–5.14)	2.40 (1.32–4.39)	1.87 (0.99–3.53)	1	0.001	
≥24	78/651	0.54 (0.28–1.06)	0.61 (0.30–1.25)	0.70 (0.36–1.35)	1	0.65	
Extent of CAD							0.268
Nonobstructive	84/599	2.22 (1.10–4.51)	1.94 (0.91–4.15)	1.34 (0.61–2.97)	1	0.016	
Obstructive	121/788	1.15 (0.67–1.98)	1.25 (0.73–2.16)	1.16 (0.67–2.03)	1	0.538	
eGFR, mL/min per 1.73 m^2							0.754
<60	57/212	0.98 (0.41–2.30)	1.73 (0.77–3.90)	1.22 (0.50–2.97)	1	0.806	
≥60	148/1175	1.89 (1.15–3.12)	1.50 (0.89–2.52)	1.41 (0.83–2.40)	1	0.014	
Blood pressure, mm Hg							0.19
≥140/90	93/548	2.27 (1.15–4.46)	1.89 (0.95–3.74)	1.62 (0.78–3.35)	1	0.015	
<140/90	112/839	1.55 (0.87–2.75)	1.46 (0.80–2.66)	1.04 (0.57–1.92)	1	0.099	
Cardiovascular mortality							
Age groups, y							0.582
≤65	30/725	1.13 (0.36–3.52)	1.08 (0.33–3.55)	0.51 (0.14–1.86)	1	0.626	
>65	104/662	2.30 (1.23–4.33)	1.89 (1.00–3.59)	1.51 (0.77–2.93)	1	0.007	
Sex groups							0.645
Men	95/903	2.36 (1.24–4.48)	1.68 (0.85–3.33)	1.93 (0.97–3.85)	1	0.014	
Women	39/484	1.00 (0.35–2.89)	1.15 (0.41–3.18)	0.33 (0.10–1.11)	1	0.688	
BMI, kg/m^2							0.068
<24	84/736	4.51 (2.01–10.09)	3.13 (1.41–6.93)	1.95 (0.83–4.58)	1	<0.001	
≥24	50/651	0.54 (0.23–1.26)	0.61 (0.25–1.47)	0.83 (0.37–1.84)	1	0.125	
Extent of CAD							0.372
Nonobstructive	53/599	3.16 (1.18–8.50)	2.52 (0.88–7.21)	1.79 (0.62–5.23)	1	0.015	
Obstructive	81/788	1.40 (0.72–2.73)	1.39 (0.70–2.73)	1.09 (0.53–2.21)	1	0.26	
eGFR, mL/min per 1.73 m^2							0.871
<60	39/212	1.34 (0.44–4.05)	2.45 (0.86–6.97)	1.45 (0.45–4.65)	1	0.418	
≥60	95/1175	2.28 (1.21–4.31)	1.65 (0.84–3.24)	1.38 (0.70–2.75)	1	0.012	
Blood pressure, mm Hg							0.004
≥140/90	61/548	6.53 (2.28–18.77)	4.59 (1.57–13.42)	3.40 (1.08–10.76)	1	<0.001	
<140/90	73/839	1.15 (0.57–2.30)	1.03 (0.50–2.11)	0.87 (0.43–1.77)	1	0.697	

BMI indicates body mass index; CAD, coronary artery disease; and eGFR estimated glomerular filtration rate.

*Adjusted for age, sex, body mass index, smoking status, presence or absence of diabetes mellitus, systolic pressure, total cholesterol, high-density lipoprotein cholesterol, extent of CAD, presence or absence of acute CAD, presence or absence of coronary revascularization, use or nonuse of statins, angiotensin-converting enzyme inhibitors/angiotensin receptor blockers, and β-blockers, season of blood-drawing, leisure-time physical activity, estimated glomerular filtration rate, calcium, parathyroid hormone, and C-reactive protein.

recent observational study showed that high DBP level was associated with an increased risk of cardiovascular events in a community-based population.[33] However, another case-control study (382 cases) suggested an inverse association between serum DBP levels and risk of coronary heart disease.[35] Whether DBP levels positively or negatively affect the adverse outcomes in cardiovascular diseases is still under the debate, but it is vital to notice that DBP is involved in

the inflammatory processes via the mechanism of immunity modulation.[36] We did not find that DBP levels were related to mortality in this angiographically confirmed CAD cohort. Nevertheless, because of the difference in the systemic inflammatory status between patients with CAD and the general populations, we cannot rule out the possibility that DBP is correlated with cardiovascular outcomes among other populations. Further studies are warranted to determine such relationship.

It is necessary to point out that some unmeasured confounders may be prevalent in our cohort and associated with mortality. Although we have made adjustment for known conventional risk factors of cardiovascular disease and other potentially confounding factors before performing the mediation analysis, we were unable to rule out the possibility that residual confounding still remain. In addition, only baseline serum vitamin D metabolites levels were measured for the cohort. Aside from significant seasonal variation, biological variation of vitamin D status within an individual over time is expected, such as through improved lifestyle. Last, our cohort included only Han ethnic group of Chinese, so present results should be taken with caution when generalized to other ethnic populations. Nevertheless, this study also has several strengths, including a large sample size of CAD patients who have undergone a detailed clinical and metabolic investigation, including coronary angiography. Furthermore, to the best of our knowledge, this is the first study in the exploration of the relationship between biomarkers of vitamin D status beyond total 25(OH)D and mortality in a prospective CAD cohort.

In conclusion, the present study demonstrates that bioavailable and free 25(OH)D are potential predictors of adverse outcomes among CAD patients, and further studies in diverse populations are needed to verify whether our findings have broader implications.

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Disclosures

None.

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