Research Article

Free 25-Vitamin D Is Correlated with Cardiovascular Events in Prevalent Hemodialysis Patients but Not with Markers of Renal Mineral Bone Disease

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Keywords

Free vitamin D · Cardiovascular disease · Hemodialysis patients · 25-Hydroxyvitamin D

Abstract

Free vitamin D is the biologically active form of vitamin D. Vitamin D deficiency is associated with cardiovascular disease, the most common cause of mortality in hemodialysis patients. The goal of our current study was to investigate the relation between blood concentrations of free 25-hydroxyvitamin D with cardiovascular events in end-stage chronic kidney disease patients on hemodialysis, because this is unknown so far. We measured free vitamin D levels in 117 stable consecutive prevalent patients in September as a surrogate of vitamin D exposure during the past 6 months, and recorded the number of cardiovascular events during the previous 6 months defined as hospitalization due to heart failure, episodes of acute coronary syndrome, and stroke. Fourteen events occurred during the observation period. In patients without any cardiovascular events the free vitamin D levels were significantly higher as compared to those with cardiovascular events (patients without events: 5.68 [4.37–9.27] pg/mL; patients with events: 4.74 [3.46–5.37] pg/mL, $p = 0.015$). This finding remained stable after multiple regression analysis considering confounding factors such as age, time on dialysis, preexisting diabe-

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tes, hypertension, and coronary heart disease. In conclusion, our study shows that free vitamin D serum concentrations are independently associated with major cardiovascular events in chronic kidney disease patients on dialysis.

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Published by S. Karger AG, Basel

Introduction

Hemodialysis (HD) is the most common form of renal replacement therapy for end-stage renal disease (ESRD). With the development of HD, the survival of ESRD patients has significantly improved in the past decades. However, patients undergoing HD still suffer from a huge burden of various cardiovascular events. Their morbidity and mortality is similar to patients with certain forms of solid cancer [1, 2] and stroke [3, 4]. Cardiovascular events are the major causes of mortality in HD patients [5–7]. Stroke in HD patients has a particularly poor prognosis [3, 8].

Vitamin D is a fat-soluble steroid hormone. It plays an essential role in calcium and phosphate homeostasis and bone metabolism. Vitamin D deficiency is common among patients undergoing dialysis, and is associated with increased morbidity and poor outcomes [9]. In the early 1980s, Scragg [10] raised the hypothesis that low 25(OH)D levels during wintertime increased cardiovascular diseases, mortality, and morbidity during the seasonal low sun exposure. In the following decades some studies [11–13] confirmed that low levels of 25(OH)D are associated with increased risk of cardiovascular diseases and mortality. One meta-analysis including 20 studies demonstrated that vitamin D supplementation decreased the risk of all-cause and cardiovascular mortality in ESRD patients requiring dialysis [14]. Therefore, accurately and effectively measuring the level of vitamin D is very important for HD patients.

In general, about 85–90% of vitamin D metabolites are bound to vitamin D binding protein and 10–15% are bound to serum albumin, less than 1% (0.4% for 1,25(OH)₂D and 0.03% for 25(OH)D) exist in a free form [15]. Chronic kidney diseases, in particular nephrotic syndrome, are associated with tubular damage, which result in urinary loss of vitamin D bound to vitamin D binding protein, which will alter the serum concentrations of total vitamin D [16, 17]. However, free vitamin D measurements are independent of the confounding factor of kidney diseases, and thus are potentially much better correlated with cardiovascular morbidity and mortality. Therefore, in our recent review, we recommended that measuring free, biologically active vitamin D might be advantageous [18]. So far, it has not been shown whether or not free vitamin D levels in patients on HD are linked to cardiovascular outcome.

Materials and Methods

Participants and Data Collection

A total of 117 prevalent HD patients treated at the department of nephrology of the First Affiliated Hospital of Jinan University were included and prospectively followed up. They were dialyzed three times a week for 4 h. All of them were stable and on dialysis for at least 2 months before entering the study. The key inclusion criteria were ESRD for more than 2 months and age >18 years. Patients with recent acute heart failure, acute coronary syndrome, infection, and stroke were excluded. None of the patients received treatment with active vitamin D, ergocalciferol, or cholecalciferol before initiating dialysis and during the study. The patients were followed for 6 months and checked for the composite endpoint.

After obtaining written informed consent, a structured medical history was taken. The following data were entered into our database: age, sex, body height and weight, time of dialysis, heart rate, hypertension and diabetes mellitus history, systolic and diastolic blood pressure. Information about hospitalization due to acute coronary syndrome, heart failure, and stroke events, as well as all-cause mortality, were obtained.

Systolic blood pressure and diastolic blood pressure were obtained as the mean of three blood pressure measurements before dialysis treatment. The diagnosis of stroke was made only when confirmed by computed tomography or cerebral magnetic resonance imaging [19–21]. The diagnosis of coronary artery disease was only made with a stenosis of more than 50% by coronary angiography [22, 23]. The diagnosis of acute heart failure was based on the symptoms and clinical findings, chest X-ray, and biomarkers (N-terminal pro-brain natriuretic peptide; NT-proBNP). Symptoms and clinical findings included dyspnea, tachypnea, orthopnea, elevated jugular venous pressure, and wet rales in the lung. Chest X-ray is used both for assessing pulmonary congestion and excluding pneumonia or other reasons for the symptoms. NT-proBNP was considered elevated if higher than 300 pg/mL [24].

Sample Collection

The blood samples were collected in September 2018. Blood samples were centrifuged at 3,000 rpm/min for 10 min. Obtained serum samples were then stored at -80°C until analysis.

Serum-free 25(OH)D (25(OH)D₃ and 25(OH)D₂) was measured directly using a commercial kit by Future Diagnostics Solutions B.V. (Wijchen, The Netherlands), distributed by DIASource Immunoassays (Louvain-la-Neuve, Belgium), based on a two-step immunoassay procedure [25]. This assay is CE-IVD marked, i.e., it fully complies with the requirements of the European Directive regulating the quality of the products present on the market for in vitro diagnostics. The performance data of the assay are provided in online supplementary Figure 1 (see www.karger.com/doi/10.1159/000499878 for all online suppl. material).

Laboratory parameters, such as hemoglobin, white blood cell count, neutrophil count, lymphocyte count, thrombocytes count, immunoreactive parathyroid hormone, calcium, phosphate, total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglyceride, serum albumin, serum creatinine, serum urea, uric, aspartate aminotransaminase, alanine aminotransaminase, alkaline phosphatase, C-reactive protein, fasting sugar, and glycosylated hemoglobin, were measured in the central clinical laboratory of the First Affiliated Hospital of Jinan University. These measurements are subject to routine quality controls according to the laws of the People's Republic of China. The urea clearance (KT/V) was calculated from the predialysis to postdialysis urea nitrogen ratio (R) and the ratio of the ultrafiltrate volume removed to the postdialysis weight (UF/W), using the equation: $KT/V = -\ln(R - 0.03) + (4 - 3.5 \times R) \times UF/W$ [26].

Statistical Analysis

The data were analyzed with SPSS version 22.0. Results of normally distributed parameters are expressed as the arithmetic mean $\pm$ SD. Two-group comparisons were performed using the two-sample *t* test; results of non-normally distributed parameters are expressed as the median (IQR), and two-group comparisons were performed using the Wilcoxon rank-sum test. Pearson's χ^2 test was used for testing qualitative data. Bivariate correlation analysis was performed to find the correlation between clinical/laboratory parameters and heart events. Logistic regression models were adjusted for age [27–29], dialysis time [30, 31], diabetes mellitus [31, 32], hypertension [31, 32], and coronary artery disease [30], which are all known risk factors for heart events (defined as episodes of acute coronary syndrome and heart failure events) and stroke. A *p* value <0.05 was considered significant.

Table 1. Characteristics of the study population at baseline ($n = 117$)

Variable	All patients ($n = 117$)	Patients with events ($n = 14$)	Patients with no events ($n = 103$)	$t/z/\chi^2$	p
Age, years	58.50±15.02	61.64±14.60	58.07±15.09	0.84	0.406
Male	66 (56.40)	7 (50.00)	59 (57.30)	0.05	0.820
BMI	20.40 (18.26–22.99)	20.61 (19.37–22.45)	20.35 (18.17–23.11)	–0.60	0.551
Time on dialysis, months	24.00 (10.00–39.00)	18.50 (6.00–35.25)	24.00 (10.00–44.00)	–0.62	0.534
Systolic blood pressure, mm Hg	147.91±21.99	151.50±29.78	147.41±20.84	0.65	0.516
Diastolic blood pressure, mm Hg	78.16±13.10	78.00±17.76	78.18±12.44	–0.05	0.963
Heart rate, beats/min	81.78±10.31	80.14±12.58	80.86±10.03	–0.24	0.808
Diabetes mellitus	53 (45.30)	9 (64.29)	44 (42.72)	–1.53	0.220
Hypertension	89 (76.07)	13 (92.86)	76 (73.79)	1.53	0.220
Urea clearance indication (KT/V)	1.48 (1.31–1.65)	1.44 (1.22–1.52)	1.49 (1.32–1.67)	–1.05	0.292
Free vitamin D, pg/mL	5.46 (4.33–7.63)	4.74 (3.46–5.37)	5.68 (4.37–9.27)	–2.43	0.015
Natural log transformation of free vitamin D	1.70 (1.47–2.03)	1.55 (1.24–1.68)	1.74 (1.48–2.23)	–2.43	0.015
Total cholesterol, mmol/L	4.18±1.15	4.21±1.26	4.17±1.14	0.12	0.902
Low-density lipoprotein cholesterol, mmol/L	2.28±0.86	2.32±0.96	2.27±0.85	0.18	0.860
High-density lipoprotein cholesterol, mmol/L	0.94 (0.79–1.06)	0.95 (0.75–1.18)	0.94 (0.79–1.05)	–0.02	0.983
Triglyceride, mmol/L	1.53 (1.04–2.69)	1.79 (1.24–2.52)	1.53 (0.99–2.78)	–0.42	0.675
Serum albumin, g/L	36.60 (34.40–39.15)	36.35 (34.53–38.63)	36.60 (34.40–39.20)	–0.05	0.960
Serum creatinine, umol/L	950.67±297.67	786.88±334.23	972.93±286.97	–2.23	0.028
Serum urea, mmol/L	26.08±8.07	20.80±10.33	26.53±7.67	–1.63	0.105
Uric acid, μmol/L	464.07±98.55	431.54±92.86	468.50±98.90	–1.32	0.189
Aspartate aminotransaminase, U/L	14.00 (11.00–19.00)	14.50 (12.00–26.00)	14.00 (11.00–19.00)	–0.97	0.333
Alanine aminotransaminase, U/L	11.00 (8.00–16.50)	9.50 (8.00–17.75)	11.00 (8.00–16.00)	–0.18	0.856
Alkaline phosphatase, U/L	89.00 (68.00–123.00)	109.00 (67.50–391.50)	88.00 (68.00–120.00)	–1.11	0.266
Immunoreactive parathyroid hormone, pg/mL	242.91 (140.30–505.02)	266.53 (127.95–759.14)	242.91 (142.52–508.95)	–0.49	0.626
Calcium, mmol/L	2.26±0.24	2.28±0.25	2.26±0.24	0.38	0.706
Phosphate, mmol/L	2.09±0.70	1.95±1.02	2.11±0.65	–0.57	0.576
C-reactive protein, mg/L	4.89 (1.84–9.19)	5.46 (1.98–7.39)	4.65 (1.80–10.78)	–0.23	0.819
Hemoglobin, g/L	101.14±17.50	98.79±15.75	101.46±17.77	–0.53	0.595
White blood cell count, $\times 10^9$ /L	6.14 (4.78–7.76)	5.35 (4.36–6.49)	6.26 (4.84–7.81)	–1.38	0.168
Neutrophil count, $\times 10^9$ /L	4.06 (3.01–5.37)	3.71 (2.83–4.51)	4.21 (3.01–5.42)	–0.93	0.353
Lymphocyte count, $\times 10^9$ /L	1.24 (0.96–1.53)	0.97 (0.89–1.14)	1.27 (1.02–1.57)	–2.27	0.023
Thrombocytes count, $\times 10^9$ /L	190.43±59.77	181.62±51.89	191.63±60.88	–0.59	0.559
Fasting blood glucose, mmol/L	7.81 (6.43–11.04)	8.56 (7.05–11.44)	7.67 (6.28–11.06)	–0.89	0.376

Data are presented as the mean $\pm$ SD for normally distributed parameters, the median (IQR) for non-normally distributed parameters, or n (%). Age, systolic blood pressure, diastolic blood pressure, heart rate, total cholesterol, low-density lipoprotein cholesterol, serum creatinine, serum urea, uric acid, calcium, phosphate, hemoglobin, and thrombocytes count are normally distributed parameters. Group comparisons of normally distributed parameters were performed using the two-sample t test. Group comparisons of non-normally distributed parameters were performed using the Wilcoxon rank-sum test.

t is the value of the two-sample t test. z is the value of the Wilcoxon rank-sum test. χ^2 is the value of Pearson's χ^2 test.

Results

Characteristics of Study Population

The descriptive data of the study population, which represented a regular HD cohort in regard to key characteristics, such as age, BMI, dialysis time, systolic/diastolic blood pressure, presence of diabetes mellitus, and hypertension, are provided in Table 1. The levels of free vitamin D in September (Fig. 1a) showed no normal distribution. Therefore, we calculated the natural log transformation of free vitamin D in September (Fig. 1b) using the equation: $X' = \ln(X/\text{free vitamin D})$. Patients with heart events plus stroke had significantly lower levels of free vitamin D ($p = 0.015$; Table 1; Fig. 2) and natural log transformation of free vitamin D ($p = 0.015$; Table 1) than the no event control group.

Correlation of Clinical and Laboratory Parameters with Heart Events and Stroke

Cardiovascular events were defined as episodes of acute coronary syndrome and acute decompensated heart failure. Free vitamin D and natural log transformation of free vitamin D were negatively correlated with cardiovascular events ($p < 0.05$; Table 2). For heart events plus stroke, free vitamin D, natural log transformation of free vitamin D, and serum urea were negatively correlated with heart events ($p < 0.05$; Table 3).

Fig. 1. **a** Distribution of free vitamin D. **b** Natural log transformation of free vitamin D.

Fig. 2. Wilcoxon rank-sum test of free vitamin D between heart events plus stroke and no events. Data are given as the median (IQR): events group, 4.74 (3.46–5.37) pg/mL; control group, 5.68 (4.37–9.27) pg/mL, $p = 0.015$.

Correlation of Free Vitamin D/Natural log Transformation of Free Vitamin D with Clinical and Laboratory Parameters

No clinical or laboratory parameters were correlated with free vitamin D (all $p > 0.05$; Table 4, 5).

Logistic Regression

Following the bivariate correlation analysis, logistic regression models were calculated to see whether natural log transformation of free vitamin D was independently associated with heart events and stroke. Logistic regression models were adjusted for age [27–29], dialysis time [30, 31], diabetes mellitus [31, 32], hypertension [31, 32], and coronary artery disease [30], which are all known risk factors for acute cardiovascular events and stroke. Natural log transformation of free vitamin D in September had a negative association with heart events plus stroke (OR = 0.195, $p = 0.041$; Table 6).

Table 2. Correlation of clinical and laboratory parameters with cardiovascular events (episodes of acute coronary syndrome and heart failure events; $n = 117$)

Variable	Spearman's correlation	<i>p</i> value
BMI	0.04	0.668
Free vitamin D	−0.21	0.023
Natural log transformation of free vitamin D	−0.21	0.023
Time on dialysis	−0.08	0.425
Total cholesterol	0.01	0.915
Triglyceride	−0.00	0.986
Serum albumin	−0.01	0.943
Serum creatinine	−0.13	0.151
Serum urea	−0.15	0.103
Uric	−0.15	0.111
Fasting sugar	0.08	0.369
Glycosylated hemoglobin	−0.06	0.726
Aspartate transaminase	0.10	0.299
Alanine transaminase	−0.01	0.926
Alkaline phosphatase	0.10	0.291
Immunoreactive parathyroid hormone	0.06	0.519
Calcium	0.05	0.560
Phosphate	−0.06	0.521
C-reactive protein	−0.03	0.762

Table 3. Correlation of clinical and laboratory parameters with heart events and stroke (episodes of acute coronary syndrome and heart failure events and stroke; $n = 117$)

Variable	Spearman's correlation	<i>p</i> value
BMI	0.06	0.544
Free vitamin D	−0.22	0.015
Natural log transformation of free vitamin D	−0.22	0.015
Time on dialysis	−0.06	0.550
Total cholesterol	0.04	0.668
Triglyceride	0.04	0.660
Serum albumin	−0.00	0.972
Serum creatinine	−0.17	0.068
Serum urea	−0.18	0.047
Uric	−0.12	0.202
Fasting sugar	0.08	0.390
Glycosylated hemoglobin	−0.06	0.726
Aspartate transaminase	0.09	0.360
Alanine transaminase	−0.02	0.839
Alkaline phosphatase	0.11	0.249
Immunoreactive parathyroid hormone	0.05	0.591
Calcium	0.05	0.622
Phosphate	−0.09	0.322
C-reactive protein	−0.02	0.845

Discussion

Our study demonstrates for the first time in a dialysis cohort that the free vitamin D level is correlated with the occurrence of cardiovascular events (acute coronary syndrome, heart failure) and stroke, and that this correlation is independent of known confounders for cardio-

Table 4. Correlation of free vitamin D with clinical and laboratory parameters ($n = 117$)

Variable	Spearman's correlation	<i>p</i> value
BMI	−0.07	0.450
Time on dialysis	−0.08	0.377
Total cholesterol	−0.06	0.508
Triglyceride	−0.11	0.248
Serum albumin	0.04	0.571
Serum creatinine	0.09	0.326
Serum urea	0.11	0.233
Uric acid	0.11	0.261
Fasting sugar	−0.07	0.457
Glycosylated hemoglobin	−0.01	0.978
Aspartate transaminase	−0.10	0.282
Alanine transaminase	−0.02	0.847
Alkaline phosphatase	−0.15	0.113
Immunoreactive parathyroid hormone	−0.06	0.559
Calcium	−0.03	0.784
Phosphate	−0.02	0.822
C-reactive protein	0.00	0.972

Table 5. Correlation of natural log transformed free vitamin D with clinical and laboratory parameters ($n = 117$)

Variable	Spearman's correlation	<i>p</i> value
BMI	−0.07	0.450
Time on dialysis	−0.08	0.377
Total cholesterol	−0.06	0.508
Triglyceride	−0.11	0.248
Serum albumin	0.04	0.571
Serum creatinine	0.09	0.326
Serum urea	0.11	0.233
Uric	0.11	0.261
Fasting sugar	−0.07	0.457
Glycosylated hemoglobin	−0.01	0.978
Aspartate transaminase	−0.10	0.282
Alanine transaminase	−0.02	0.847
Alkaline phosphatase	−0.15	0.113
Immunoreactive parathyroid hormone	−0.06	0.559
Calcium	−0.03	0.784
Phosphate	−0.02	0.822
C-reactive protein	0.00	0.972

vascular events. In our study, we measured free vitamin D in September and retrospectively checked for events in the previous 6 months.

Previous studies showed that total vitamin D is correlated with morbidity and mortality in much larger cohorts. Wolf et al. [30] could show that a higher vitamin D level is associated with a higher survival rate. They could show that Blacks and Hispanics with higher vitamin D levels had a statistically higher survival rate compared to white non-Hispanics who had lower vitamin D levels (16% and 16% vs. 23%). This difference might be due to a higher prescription rate of vitamin D. The same authors could show that total 25- and 1,25-vitamin D was not correlated with markers of mineral metabolism, but with mortality [33]. Our study is very

Table 6. Adjusted logistic regression models analyzing risk factors for heart events plus stroke (episodes of acute coronary syndrome and heart failure events plus stroke)

Independent variable	<i>p</i> value	OR	95% confidence interval for B
Age	0.912	1.00	0.95–1.04
Time on dialysis	0.848	1.00	0.98–1.01
Diabetes mellitus	0.481	0.62	0.16–2.34
Hypertension	0.212	0.25	0.03–2.22
Coronary artery disease	0.574	0.66	0.15–2.84
Natural log transformation free vitamin D	0.041	0.20	0.04–0.93

similar in some respects, but we measured the biologically active and relevant vitamin D, not the total vitamin D, and rather than testing for mortality we analyzed cardiovascular endpoints, as defined above. Drechsler et al. [34] used the cohort from the NECOSAD trial in the Netherlands to test the hypothesis that low 25-vitamin D levels are associated with cardiovascular and non-cardiovascular mortality, and they obtained a hazard ratio (HR) of 2.7 for short-term (6 months) and of 1.7 or long-term (3 years) cardiovascular mortality. There was no correlation for non-cardiovascular mortality. The same hypothesis was tested in the study cohort of the German Diabetes and Dialysis Study [35]. Here the authors studied 1,108 diabetic HD patients for a median follow-up of 4 years. The authors found a 3-fold increase of sudden cardiac death (HR 2.99), and a HR of 1.78 for cardiovascular events and of 1.74 for all-cause mortality. There were borderline but non-significant correlations for stroke and fatal infection, while there was no correlation for deaths due to heart failure. The impact of low 25-vitamin D levels was also tested by Pilz et al. [36] in non-dialysis patients with chronic kidney disease with an eGFR of less than 60 mL/min. They followed up 444 patients for a median of 9.4 years. During this time 227 patients died, including 159 of cardiovascular causes. They could show a clear correlation between low levels and mortality. The HR for cardiovascular mortality was 5.61 (1.89–16.6), while the HR for all-cause mortality was 3.79 (1.71–8.43). In this study there was no correlation found with intact parathyroid hormone (iPTH).

A recent study [37] with 94 incident HD patients tried to correlate markers of mineral metabolism (Ca, phosphate, and iPTH) and clinical outcomes with the so-called bioavailable vitamin D. This bioavailable vitamin D is the total of free vitamin D and vitamin D bound to albumin. The study found a correlation of the bioavailable vitamin D with calcium and iPTH, but not with cardiovascular events.

Our findings add to this body of data, showing correlations for total 25-vitamin D and for bioavailable vitamin D, and for the first time that the biologically active free vitamin D is also correlated to cardiovascular events. We chose to measure free vitamin D because only this form of vitamin D can pass the cell and nuclear membrane passively and activates the nuclear vitamin D receptor to exert its effects on the cardiovascular system. The activation of the nuclear receptor is known to control genes which are responsible for endothelial dysfunction and vascular remodeling [38]. These are the underlying molecular pathways leading to cardiovascular events.

The limitations of our study are in some respects the same as in all the above-mentioned total vitamin D observational studies – we cannot show a causal relation between the level of free vitamin D and the clinical events because the nature of the study does not allow to control for other known and as yet unknown confounding factors. Moreover, the assumption that the free vitamin D levels in September reflect the vitamin D exposure in the previous months was not tested. In addition, our study was conducted in southern China in the Han Chinese popu-

lation. It needs to be shown that our findings can be repeated in other ethnic groups and other geographic regions with a different sun exposure. Therefore, we are already planning to increase the size of the study group, and to measure free vitamin D levels serially in summer and in winter. We also acknowledge that the sample size is limited, but as mentioned it will be increased in the same dialysis center, but should also be independently confirmed in another patient population with a different background and different exposure to sun. In this first study we only tested the correlation of free vitamin D with clinical endpoints. As mentioned above, the association of total vitamin D was already shown. The measurement of all vitamin D forms (total, bioavailable, and free) in one study and a comparison of all these forms regarding the association with clinical endpoints should be addressed in further studies.

Statement of Ethics

This study was approved by the Ethics Committee of the Jinan University, Guangzhou, China.

Disclosure Statement

The authors have no conflicts of interest to declare.

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