Part two

Vitamin D
Chapter 5
Vitamin d and mortality in older men and women*

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ABSTRACT

Objective Vitamin D deficiency is common among the elderly and may contribute to cardiovascular disease. The aim of our study was to elucidate whether low serum levels of 25-hydroxyvitamin D [25(OH)D] are associated with an increased risk of all-cause and cardiovascular mortality.

Design and patients The Hoorn Study is a prospective population-based study among older men and women.

Measurements Fasting serum 25(OH)D was determined in 614 study participants at the follow-up visit in 2000–2001, the baseline for the present analysis. To account for sex differences and seasonal variations of 25(OH)D levels we formed sex-specific quartiles, which were calculated from the 25(OH)D values of each season.

Results After a mean follow-up period of 6.2 years, 51 study participants died including 20 deaths due to cardiovascular causes. Unadjusted Cox proportional hazard ratios (HRs; with 95% confidence intervals) for all-cause and cardiovascular mortality in the first when compared with the upper three 25(OH)D quartiles were 2.24 (1.28–3.92; P = 0.005) and 4.78 (1.95–11.69; P = 0.001), respectively. After adjustment for age, sex, diabetes mellitus, smoking status, arterial hypertension, high-density lipoprotein-cholesterol, glomerular filtration rate and waist-to-hip ratio, the HRs remained significant for all-cause [1.97 (1.08–3.58; P = 0.027)] and for cardiovascular mortality [5.38 (2.02–14.34; P = 0.001)].

Conclusions Low 25(OH)D levels are associated with all-cause mortality and even more pronounced with cardiovascular mortality, but it remains unclear whether vitamin D deficiency is a cause or a consequence of a poor health status. Therefore, intervention studies are warranted to evaluate whether vitamin D supplementation reduces mortality and cardiovascular diseases.

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Introduction

Older persons are prone to low vitamin D concentrations because the capacity of the skin to produce vitamin D decreases with aging and the time of sun exposure is often limited [1, 2]. This results in reduced dermal synthesis of vitamin D, which can hardly be compensated by dietary intake of vitamin D [1, 2]. The estimated worldwide prevalence of vitamin D deficiency among the elderly of almost 50% underlines that adverse health effects attributed to vitamin D deficiency may be important for public health [3]. It is well known that vitamin D deficiency contributes to a higher prevalence of fractures and falls and causes muscle weakness [1]. In addition, there is growing evidence that vitamin D deficiency may increase the risk of cancer, autoimmune diseases and infections [2, 4-7]. It was recently shown that low levels of 25-hydroxyvitamin D [25(OH)D] are also independently associated with cardiovascular events in patients with hypertension, suggesting a role of vitamin D for the maintenance of cardiovascular health [8]. This hypothesis is further supported by the ability of vitamin D to suppress the renin-angiotensin system [9]. Furthermore, accumulating evidence suggests that vitamin D deficiency may contribute to myocardial dysfunction, arterial hypertension and diabetes mellitus [10-15].

We have previously shown that low levels of 25(OH)D are an independent risk factor of total and cardiovascular mortality in a large cohort of patients referred to coronary angiography [16]. These results are in line with a recent meta-analysis, in which a significant reduction of all-cause mortality was reported for persons receiving vitamin D supplementation that were largely derived from trials among frail elderly people with vitamin D deficiency [17]. In the present work, we aimed to address the largely unknown association between 25(OH)D and cardiovascular as well as all-cause mortality in a population-based study. For this purpose, we measured 25(OH)D values, which are considered to be the best indicator of vitamin D status [1, 2, 4], in 614 study subjects from the population-based Hoorn Study which includes older men and women who were followed with respect to total and cardiovascular mortality [18-20].

Methods

Study population

The Hoorn Study is a population-based cohort study on type 2 diabetes and cardiovascular diseases [18]. Baseline measurements of the white population in Hoorn, a medium-sized town in the Netherlands, were performed between 1989 and 1992 and were described elsewhere [18]. The initial study cohort was a random sample from the population register of the municipal registry of Hoorn, and encompassed 2484 participants aged from 50 to 75 years. All subjects with type 2 diabetes at a follow-up visit between 1996 and 1998 (n = 176) and a random samples of the remaining study cohort (n = 898), including 193 persons with impaired and 705 persons with normal glucose metabolism, were invited for a follow-up examination between 2000 and 2001 [19, 20]. In this work, we present the data of the 648 subjects (60.3% of the invited individuals) who participated in that examination between 2000 and 2001.
The reasons for not participating in the follow-up examination were lack of interest (30%), morbidity (23%), advanced age (7%), unwillingness to travel (6%), participation considered too time-consuming (6%) and miscellaneous reasons (15%) [19, 20]. The Hoorn Study complied with the Declaration of Helsinki. Written informed consent was obtained from all study subjects and the Ethics Committee of the VU University Medical Centre approved the study.

**Measurements**

Blood samples were taken after an overnight fasting. Serum 25(OH)D was measured by means of a competitive binding protein assay (DiaSorin, Stillwater, MN, USA) [19]. This assay determines both 25-hydroxyvitamin D₂ and 25-hydroxyvitamin D₃ and the interassay coefficient of variation was 10–15%, with slightly lower coefficient of variation at higher 25(OH)D levels [19]. Serum parathyroid hormone (PTH) was determined with an immunoradiometric assay (Incstar Corp., Stillwater, MN, USA). Methods for routine laboratory measurements were described previously [20]. We performed a 75-g oral-glucose tolerance test in all study participants, except in those with capillary fasting whole blood glucose levels ≥8 mmol/l or in patients with known diabetes mellitus, who were already treated with oral antidiabetics and/or insulin. Diabetes mellitus and impaired glucose metabolism (persons with impaired glucose tolerance and/or impaired fasting glucose) were classified according to the 1999 WHO criteria [21]. Glomerular filtration rate (GFR) was calculated according to the abbreviated Modification of Diet in Renal Disease formula [22]. Systolic and diastolic blood pressures were determined with a random-zero sphygmomanometer (Hawksley-Gelman, Lancing, UK) at the right-upper arm after 5 min at rest and the average of two measurements was used. Arterial hypertension was defined as systolic blood pressure ≥140, diastolic blood pressure ≥90 or use of antihypertensive medication. Information about smoking status was evaluated by a self-administered questionnaire. Percentage of whole-body fat was determined by whole-body dual-energy X-ray absorptiometry scan using fan beam technology (QDR-2000, software version 7.2OD; Hologic, Brussels, Belgium) [19]. Physical activity, expressed in h/day, was assessed by a validated questionnaire and included sports, bicycling, gardening, walking, doing odd jobs and household work [23]. Prior cardiovascular disease was defined as previously described as Minnesota Code 1•1–1•3, 4•1–4•3, 5•1–5•3 or 7•1 on the electrocardiogram, coronary bypass operation or angioplasty, and/or peripheral arterial bypass or amputation [24].

Information on mortality was obtained from the municipal register of the city of Hoorn. Medical records of general practitioners and local hospitals were used to determine the causes of death. Causes of death were classified according to the ninth edition of the Classification of Diseases (ICD-9). Cardiovascular mortality was classified for deceased patients with ICD codes 390–459 (diseases of the circulatory system) or ICD code 798 (sudden death). The follow-up time for all-cause mortality is defined as the time between the baseline examination (2000–2001) and the date of death or the censoring date (1 July 2007). For cardiovascular mortality, the follow-up time is defined as the time between the baseline examination (2000–2001) and the date of death because of cardiovascular causes or the censoring date, which is either
the end of the observational period (1 July 2007) or the date of death because of noncardiovascular causes.

**Statistical analysis**

For each season of the year (summer: 21 June till 22 September, autumn: 23 September till 21 December, winter: 22 December till 19 March and spring: 20 March till 20 June), we formed sex-specific vitamin D quartiles that were calculated from the 25 (OH)D concentrations of the blood samples that were drawn within the respective season. We then combined the vitamin D quartiles of each season and of both genders into a single variable [25(OH)D quartiles]. This was done because of the recently described sex difference in 25(OH)D levels of our study [19], and because of the well-known seasonal variations of serum 25(OH)D levels that are mainly attributed to circannual differences in sun exposure of the skin [20, 25-27]. Thus, for the estimation of the long-term vitamin D status of an individual, it is mandatory to consider the season of blood draw because recent data indicate that the same person has significantly higher 25(OH)D levels when blood is drawn in summer when compared with winter [20, 25-27]. However, 25(OH)D levels tend to be consistent when measured 12 months apart in the same person [27]. It can, thus, be assumed that those individuals in a specific vitamin D quartile are likely to remain in that quartile throughout all seasons of a year, despite significant circannual variations in 'absolute' 25(OH)D concentrations [27].

Differences across 25(OH)D quartiles were calculated by $\chi^2$-test with $P$ for linear by linear test for categorical and with analysis of variance (anova) with $P$ for trend for continuous parameters. Differences in overall survival and survival without fatal cardiovascular events between the lowest (first) and the upper three quartiles were graphically displayed using Kaplan–Meier curves. Previous reports are showing that the association between low 25(OH)D and cardiovascular events is nonlinear with a steep increase of cardiovascular risk at very low 25(OH)D levels [8]. This prompted us to calculate Cox proportional hazard ratios (HRs) for all-cause and cardiovascular mortality for the lowest when compared with the upper three 25(OH)D quartiles. The upper three quartiles were used as the reference group [8]. In addition to unadjusted HRs, we present age- and sex-adjusted HRs in model 1. In model 2, we additionally adjusted for several cardiovascular risk factors including dichotomous categorical variables for diabetes mellitus, smokers (ex- and active smokers vs. never smokers) and arterial hypertension, and continuous variables for high-density lipoprotein-cholesterol, GFR and waist-to-hip ratio. Alternatively, we also included percentage of body fat instead of waist-to-hip ratio in the covariate list of model 2, because we have recently shown that percentage of body fat is strongly associated with a poor vitamin D status [28]. In addition to all covariates of model 2, we adjusted for PTH in model 3, for habitual physical activity in model 4, for albumin in model 5 and we calculated a multivariable adjusted model (backward LR selection method) that includes all the above-mentioned covariates. Adjustment for PTH was performed because previous study results indicated that secondary hyperparathyroidism is an important mediator of the deleterious effects of vitamin D deficiency [29, 30]. Habitual physical activity was included as a covariate because low physical activity is known to
be associated with increased mortality and vitamin D deficiency and might thus be an important confounder for the association between 25(OH)D and mortality [12, 13, 16, 19, 31]. However, the reduced physical activity associated with low 25(OH)D levels may also be regarded as a consequence of vitamin D deficiency by considering that low levels of 25(OH)D can cause muscle weakness. It is, therefore, debatable whether low physical activity is rather a mediator or confounder of harmful effects of vitamin D deficiency. Inclusion of serum albumin in the covariate list was done to adjust for a marker of malnutrition that is also predictive for mortality [32]. To reduce possible confounding by prior cardiovascular disease, we additionally included this variable (prior cardiovascular disease) to the covariate list of model 2 and calculated the respective HRs for all-cause and cardiovascular mortality. All statistical tests were two-sided and a $P$-value below $0.05$ was considered statistically significant. All our statistical analyses were performed with spss 15.0 statistical package (SPSS Inc., Chicago, IL, USA).

**Results**

Serum 25(OH)D levels were available in 614 study participants. Depending on the season of blood draw, the mean serum 25(OH)D levels ± SD in nmol/l were $51.4 \pm 18.3$ in winter, $52.1 \pm 19.2$ in spring, $59.7 \pm 20.5$ in summer and $56.0 \pm 20.3$ in autumn. 25(OH)D concentrations were higher in men ($56.5 \pm 18.8$ nmol/l) than in women ($50.8 \pm 19.8$ nmol/l). Baseline characteristics according to sex-specific 25(OH)D quartiles, which are based on the 25(OH)D values within each season, are presented in Table 1. Low levels of 25(OH)D were significantly associated with higher age, waist-to-hip ratio, percentage of body fat, PTH concentrations, and systolic and diastolic blood pressure, whereas high-density lipoprotein-cholesterol, serum albumin, GFR and physical activity were increased in groups with higher 25(OH)D levels (Table 1). Arterial hypertension and type 2 diabetes were associated with low serum 25(OH)D concentrations (Table 1).

After a mean follow-up time of 6.2 years, 51 study participants (34 men and 17 women) had died, including 20 deaths (12 men and 8 women) because of cardiovascular causes. We recorded 21 deaths (13%8%) in the lowest, and 30 deaths (6.5%) in the upper three quartiles of 25(OH)D [10 deaths (6.5%) in the second, 13 (8.6%) in the third and 7 (4.5%) in the fourth quartile]. Cardiovascular deaths were classified for 12 deceased persons (7.9%) in the lowest, and 8 (1.7%) persons in the highest three 25(OH)D quartiles [3 deaths (1.9%) in the second, 2 (1.3%) in the third and 3 (1.9%) in the fourth quartile]. Kaplan–Meier curves that show unadjusted data for all-cause and cardiovascular mortality according to 25(OH)D quartiles (lowest vs. highest three quartiles) are graphed in Fig. 1a and b. Cox proportional HRs for all-cause and cardiovascular mortality, according to 25(OH)D quartiles, are shown in Table 2.
Differences between groups were assessed by χ²-test and analysis of variance (anova) with P-value.

For the lowest 25(OH)D quartile, the unadjusted risk of all-cause and cardiovascular mortality was significantly increased when compared with the highest three 25(OH)D quartiles. Age- and sex-adjusted HRs [with 95% confidence interval (CI)] in the first when compared with the upper three 25(OH)D quartiles were 1.70 (0.96–3.03; P = 0.069) for all-cause and 3.25 (1.30–8.15; P = 0.012) for cardiovascular mortality. After multivariate adjustments, low 25(OH)D levels were significantly predictive for all-cause and cardiovascular mortality (see Table 2). Inclusion of percentage of body fat instead of waist-to-hip ratio in the covariate list of model 3 in Table 2 resulted in an HR for all-cause mortality of 2.08 (1.12–3.86; P = 0.021) and an HR for cardiovascular mortality of 7.11 (2.28–22.14; P = 0.001). After adjustment for prior cardiovas-
cular disease in addition to all covariates in model 2 in Table 2, the HRs for all-cause and cardiovascular mortality were $1 \cdot 91 (1 \cdot 02-3 \cdot 58; P = 0 \cdot 45)$ and $5 \cdot 90 (1 \cdot 93-18 \cdot 07; P = 0 \cdot 002)$, respectively. In a multivariable adjusted model, including all covariates of Table 2, the HRs for all-cause and cardiovascular mortality remained significant with $1 \cdot 84 (1 \cdot 02-3 \cdot 32; P = 0 \cdot 043)$ and $4 \cdot 61 (1 \cdot 76-12 \cdot 01; P = 0 \cdot 002)$, respectively.

Figure 1. (a) Kaplan–Meier curve for all-cause mortality in the first and the upper three 25(OH)D quartiles. (b) Kaplan–Meier curve for cardiovascular mortality in the first and the upper three 25(OH)D quartiles.
Table 2. Cox proportional hazard ratios (with 95% CI) for all-cause and cardiovascular mortality in the first when compared with the upper three sex-specific 25(OH)D quartiles

<table>
<thead>
<tr>
<th></th>
<th>All-cause mortality</th>
<th>Cardiovascular mortality</th>
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</thead>
<tbody>
<tr>
<td>Unadjusted</td>
<td>Upper three quartiles</td>
<td>1.00 reference</td>
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<tr>
<td>First quartile</td>
<td>2.24 (1.28–3.92; P = 0.005)</td>
<td>4.78 (1.95–11.69; P = 0.001)</td>
</tr>
<tr>
<td>Model 1</td>
<td>Upper three quartiles</td>
<td>1.00 reference</td>
</tr>
<tr>
<td>First quartile</td>
<td>1.70 (0.96–3.03; P = 0.069)</td>
<td>3.25 (1.30–8.15; P = 0.012)</td>
</tr>
<tr>
<td>Model 2</td>
<td>Upper three quartiles</td>
<td>1.00 reference</td>
</tr>
<tr>
<td>First quartile</td>
<td>1.97 (1.08–3.58; P = 0.027)</td>
<td>5.38 (2.02–14.34; P = 0.001)</td>
</tr>
<tr>
<td>Model 3</td>
<td>Upper three quartiles</td>
<td>1.00 reference</td>
</tr>
<tr>
<td>First quartile</td>
<td>1.90 (1.04–3.48; P = 0.037)</td>
<td>4.70 (1.75–12.62; P = 0.002)</td>
</tr>
<tr>
<td>Model 4</td>
<td>Upper three quartiles</td>
<td>1.00 reference</td>
</tr>
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<td>First quartile</td>
<td>1.93 (1.06–3.51; P = 0.032)</td>
<td>5.02 (1.88–13.42; P = 0.001)</td>
</tr>
<tr>
<td>Model 5</td>
<td>Upper three quartiles</td>
<td>1.00 reference</td>
</tr>
<tr>
<td>First quartile</td>
<td>1.88 (1.02–3.44; P = 0.042)</td>
<td>5.33 (1.97–14.45; P = 0.001)</td>
</tr>
</tbody>
</table>

Model 1: Adjusted for age and sex; model 2: additionally adjusted for diabetes mellitus, ex- and active smokers, arterial hypertension, high-density lipoprotein-cholesterol, glomerular filtration rate and waist-to-hip ratio; model 3: adjusted for all covariates from model 2 plus parathyroid hormone; model 4: adjusted for all covariates from model 2 plus physical activity; model 5: adjusted for all covariates from model 2 plus albumin.

Discussion

In this study, we found that low serum levels of 25(OH)D are prospectively associated with all-cause and cardiovascular mortality in a selected sample of a population-based study cohort of older men and women.

Apart from the maintenance of muscular and skeletal health, vitamin D may also protect against cancer, infections, autoimmune and vascular diseases, suggesting that vitamin D deficiency might contribute to a reduced life expectancy [1, 2, 4-15]. Towards this, it has already been shown that low 25(OH)D levels are associated with an increased risk of all-cause mortality in patients with renal failure and in patients scheduled for coronary angiography [16, 33]. After adjustment for age and sex, low 25(OH)D levels were also predictive for mortality in very frail older people residing in hostels and nursing homes, but this association was no longer significant after multivariate adjustments including comorbidities, nutritional status and renal function [29]. The association between serum 25(OH)D levels and all-cause mortality was also addressed by the Longitudinal Aging Study Amsterdam (LASA), which included 1260 community-dwelling persons aged 65 years and older at baseline [34]. In that study, low vitamin D status was a significant predictor of mortality after adjustments for possible confounders such as age, sex, creatinine, cognitive status, depressive symp-
toms, comorbidities and lifestyle variables, but significance was lost after additional adjustments for frailty indicators (mobility performance, low serum albumin and low serum total cholesterol concentrations) [34]. Importantly, the investigators of the LASA concluded that the frailty indicators, which they included as covariates in their mortality analyses might hypothetically rather be mediators than confounders of harmful effects of vitamin D deficiency, and it is therefore conceivable that they ‘overadjusted’ their analyses [34]. Results from the Third National Health and Nutrition Examination Survey (NHANES-III), which were published during the revision of our present work, showed for the general US population that after multivariable adjustments for possible confounders the risk of all-cause mortality was significantly reduced by 26% in the highest vs. the lowest 25(OH)D quartile [35]. Our results concerning the association between 25(OH)D and mortality are well in line with and extend the findings of the LASA and NHANES-III and further support the notion that vitamin D deficiency is a risk factor for mortality.

The association between vitamin D deficiency and a higher risk of fatal cardiovascular events was highly significant (Table 2) in our study. There was also a statistically nonsignificant 20% reduction of cardiovascular mortality in the highest vs. the lowest 25(OH)D quartile in NHANES-III [35]. This associations between cardiovascular mortality (diseases) and 25(OH)D levels was less significant in NHANES-III than in previous studies and in our present work, which might be attributed to differences in study populations and the way of adjustments for seasonal 25(OH)D variations [8, 16, 35]. Considering that other studies showed a steep increase of cardiovascular risk at very low 25(OH)D concentrations [8, 16], it might be speculated that the relatively high 25(OH)D levels of the study participants of NHANES-III might have limited the chance to detect a more significant association of 25(OH)D levels and fatal cardiovascular events in that study. Concerning possible explanations for the link between vitamin D deficiency and cardiovascular diseases, it has been shown that low 25(OH)D levels might contribute to arterial hypertension and diabetes mellitus, which is consistent with the high prevalence of hypertensive and diabetic patients in the first 25(OH)D quartile of our study (Table 1) [10-15]. Interestingly, vitamin D deficiency remained a significant predictor for fatal cardiovascular events in our study even after adjustments for common cardiovascular risk factors, suggesting that possible harmful effects of low 25(OH)D levels on the cardiovascular system are independent of an involvement of vitamin D deficiency in the pathogenesis of diabetes mellitus and arterial hypertension [12-15]. There exists growing evidence that low serum 25(OH)D levels contribute to heart failure and it was shown that vitamin D treatment was associated with improved diastolic function and a regression of myocardial hypertrophy in haemodialysis patients [10, 11, 36, 37]. Carotid intima-media thickness was also found to be inversely and independently correlated with serum 25(OH)D levels and recent data from NHANES-III showed that low serum 25(OH)D concentrations are associated with a higher prevalence of peripheral arterial disease [38, 39]. Furthermore, results from the Framingham Offspring Study showed that patients with 25(OH)D levels <15 ng/ml (37•5 nmol/l) were at increased risk of incident cardiovascular events, even after adjustments for conventional cardiovascular risk factors [8]. We cannot prove causality for the relationship between 25(OH)D and fatal cardiovascular events, but our results and those of the Framingham Off-
spring Study point to the urgent need for interventional trials to further evaluate whether vitamin D supplementation protects against cardiovascular diseases [1-3, 40]. In this context, we have to acknowledge that in the Women’s Health Initiative (WHI), a daily intake of 400-IU vitamin D and 1000-mg calcium carbonate did not significantly reduce the risk of cardiovascular events [41], but the results of the WHI do not necessarily argue against a possible reduction of incident cardiovascular disease by vitamin D supplementation because the 400 IU of vitamin D used in the WHI are generally considered to be too low to adequately treat and prevent vitamin D deficiency [8, 42-44]. Furthermore, we have to note that a recent meta-analysis in patients with chronic kidney disease failed to demonstrate a significant beneficial effect of vitamin D (compound) treatment on biochemical markers such as PTH [45]. This finding contrasts the current recommendations for vitamin D treatment of patients with chronic kidney disease [45]. However, relevant data from randomised controlled trials on outcomes such as cardiovascular events or mortality, for which observational studies strongly suggest favourable effects of vitamin D treatment in chronic kidney disease, are still missing [45-48].

Our results are limited by the relatively low number of fatal events with subsequent high CIs of our HRs (Table 2), thus warranting further studies with larger study cohorts to confirm and extend our findings. Despite adjustments for various potential confounders, we cannot rule out that low serum 25(OH)D levels are only a non-specific marker for a high risk of mortality, which is confounded by other unconsidered or unmeasured factors. It is, however, important for us to point out that our analyses were adjusted for physical activity, which reduces the chance that limited mobility in persons with poor health is a main confounder for our results. Another limitation is that our study participants had only a single determination of 25(OH)D levels and not serial measurements that would provide a more reliable estimate of the long-term vitamin D status. Furthermore, our assumption made for the seasonal adjustments that a person within a certain vitamin D quartile, during e.g. summer, would also be within this quartile throughout the whole year is not necessarily validated. It should also be considered that the present study cohort is a selected sample of the initial population-based cohort of the Hoorn Study, thereby possibly limiting the generalizability of our results.

In summary, we have shown that low serum levels of 25-hydroxyvitamin D are prospectively associated with all-cause and cardiovascular mortality in older men and women. Our results provide a rationale for future studies to test whether vitamin D supplementation reduces mortality and/or cardiovascular diseases in persons with vitamin D deficiency. These studies are urgently needed to answer the question whether vitamin D deficiency is a cause or a consequence of a poor health status.

**Acknowledgements**

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