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Plasma 25-Hydroxyvitamin D and Its Genetic Determinants in Relation to Incident Myocardial Infarction and Stroke in the European Prospective Investigation into Cancer and Nutrition (EPIC)-Germany Study

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Abstract

Circulating 25-hydroxyvitamin D (25(OH)D) has been associated with cardiovascular disease (CVD) risk in observational studies. Also, SNPs to explain variation in 25(OH)D have been identified by genome-wide association studies. Detection of direct associations between SNPs that significantly affect 25(OH)D and CVD risk would indicate a causal role of vitamin D, as reverse causation could be excluded and confounding could be better controlled. Thus, a combined analysis of candidate SNPs in relation to circulating 25(OH)D and CVD risk was carried out. A case-cohort study within the EPIC-Germany study was conducted comprising a randomly drawn subcohort of 2,132 subjects (57.9% women, mean age: 50.6 years) and incident cases of myocardial infarction (n=559) and stroke (n=471) that occurred during a mean follow-up duration of 7.6 years. 25(OH)D concentrations were measured by LC-MS/MS in baseline plasma samples. Additionally, eight candidate SNPs were assayed. Associations between 25(OH)D, SNPs and the risks of myocardial infarction and stroke were assessed by multivariable regression analyses. Mean 25(OH)D level was 47.2 nmol/L in the subcohort. Four SNPs were associated with 25(OH)D (p<0.05). In subjects with 25(OH)D levels <25 nmol/L, the risks of CVD as composite endpoint (Hazard Ratio: 1.53, 95% confidence interval: 1.12–2.09), myocardial infarction, and stroke were significantly increased compared to subjects with levels ≥50 nmol/L, while no significant linear associations were observed. A SNP score was not related to the risks of total CVD (Hazard Ratio: 1.0, 95% confidence interval: 0.71–1.42), myocardial infarction, or stroke. The same was true concerning single SNPs. Given the lack of association between SNPs and the risks of stroke and myocardial infarction, the present findings do not point to a major causal role of vitamin D in the development of these diseases. However, a detection of modest associations between genetic markers and CVD risk in larger consortia cannot be ruled out.

Introduction

Beyond its role in bone health, vitamin D has been proposed to lower the risks of a variety of non-skeletal health outcomes including cardiovascular diseases (CVD) [1,2]. Its active form, 1,25(OH)2vitamin D, is a secosteroid hormone whose synthesis starts by a UV–B induced conversion of 7-dehydrocholesterol to cholecalciferol (vitamin D3) in the human skin [3]. In addition, dietary cholecalciferol from animal food sources as well as dietary ergocalciferol (vitamin D2) from mushrooms contribute to vitamin D supply, even though to a lesser extent than cholecalciferol from cutaneous production [2,4]. In the circulation, vitamin D3 and D2 are transported to the liver bound to the vitamin D binding protein and converted to 25-hydroxvitamin D (25(OH)D) by the vitamin D 25-hydroxylase. 25(OH)D, the accepted clinical indicator of vitamin D status, is...
activated to 1,25(OH)2vitamin D by the vitamin D 1-alpha-hydroxylase in the kidneys and, in a paracrine fashion, in several extra-renal tissues. Biological actions of activated vitamin D in the target organs are mediated through the vitamin D receptor (VDR) [3].

The detection of the VDR and the 1-alpha-hydroxylase in cells throughout the cardiovascular system has motivated multiple studies on the role of vitamin D in the etiology of CVD [5,6]. Several pathways linking vitamin D status with atherogenesis and CVD risk have been identified in experimental studies. Vitamin D seems to down-regulate the renin-angiotensin aldosterone system (RAAS), inflammation, coagulation, proliferation of vascular smooth muscle cells and cardiomyocytes, vascular calcification, and parathyroid hormone levels [5-8]. At the same time, vitamin D may improve insulin secretion and insulin sensitivity, as well as endothelial function [6,8].

CVD risk may be lowered by these mechanisms and pooled results from observational studies actually also suggest that higher circulating 25(OH)D levels are associated with decreased risks of stroke [9] and myocardial infarction [10]. However, it has been challenged that vitamin D is causally linked with the development of CVD [11]. Instead, low circulating 25(OH)D levels might be a surrogate for a poor overall health status, rather reflecting the inability to get outdoors because of multimorbidity, low exercise resistance, or increased BMI [12].

Although large randomized controlled trials (RCT) to elucidate the effects of vitamin D supplement use on CVD risk are under way, results are not to be expected before 2017 [13]. Also, concerns have already been raised about compliance, statistical power, latitude of the study centers, target populations, or the applied vitamin D dosages in these trials [13-15]. As a complementary approach to RCTs, observational studies with combined analyses of circulating 25(OH)D and its genetic determinants in relation to CVD endpoints may help to assess, whether the link between vitamin D status and CVD risk is causal [14,16]. Several single nucleotide polymorphisms (SNPs) coding for the vitamin D binding protein and enzymes involved in vitamin D synthesis and metabolism have been found to be associated with vitamin D status in genome wide association studies (GWAS) [17,18]. Concurrent associations of such SNPs with both vitamin D status and CVD risk in observational studies would indicate a causal link between vitamin D status and CVD risk, as genetic variation is assigned randomly during meiosis and reverse causation could be ruled out [16,19].

To date, only one study has assessed the association between vitamin D related SNPs, vitamin D status and the risk of myocardial infarction (MI) [20], while no comparable study on stroke risk has been carried out. Therefore, we investigated the associations between genetic vitamin D status determinants, circulating 25(OH)D, and the risks of MI and stroke in the German branch of the European Prospective Investigation into Cancer and Nutrition (EPIC).

**Methods**

**Population and study design**

The European Investigation into Cancer and Nutrition (EPIC) is an ongoing prospective study of chronic diseases in 10 European countries. In Germany, it comprises two cohorts at centers in Heidelberg and Potsdam [21]. Between 1994 and 1998, 25,440 participants (13,612 women and 11,928 men) were recruited in Heidelberg and 27,548 participants (16,644 women and 10,904 men) in Potsdam. Subjects were mostly aged between 35 and 65 years at both centers. Baseline examinations included detailed self-administered questionnaires and interviews on health status, socio-economic status and lifestyle, anthropometric measurements, and a dietary assessment by food frequency questionnaire [21]. A blood sample from about 95% of the participants was drawn, processed and stored at -196° Celsius in liquid nitrogen [21]. Participants are being followed-up by active and passive procedures [22].

A case-cohort design was chosen for the analyses on 25(OH)D in relation to CVD risk [23,24]. Random subcohorts were drawn in Heidelberg and Potsdam according to the protocol of the Europe-wide EPIC InterAct study on diabetes type 2 [25]. As opposed to the InterAct selection criteria, prevalent cases of diabetes mellitus type 2 were kept in the present study. After the exclusion of participants with prevalent MI or stroke (n=76), without complete follow-up information (n=66), missing 25(OH)D values (n=4), and missing covariate data (n=2), the common subcohort consisted of 2,132 participants (Figure 1). In addition, incident cases of primary MI and stroke from complete follow-up rounds up to the end of December 2006 were included into the case-cohort study. MIs and strokes were ascertained by study physicians who carried out a detailed and systematic medical verification of self-reports and death certificates by clinical records in cooperation with treating physicians and hospitals. Outcomes were classified as fatal in case of death within the first 28 days after diagnosis. The following conditions were coded according to the International Statistical Classification of Diseases, 10th revision, 2011 (ICD-10): MI (ICD-10 I21), ischemic stroke (ICD-10 I63), hemorrhagic stroke (ICD-10 I60 and I61) and unspecified stroke (ICD-10 I64). After exclusion of incident cases with undetectable 25(OH)D levels (n=1) or missing covariate data (n=1, n=2), 559 verified cases of MI and 471 verified cases of stroke that had occurred during average follow-up periods of 7.5 (±2.3) and 7.7 (±2.1) years, respectively, were included into the CVD case-cohort study (Figure 1). Out of these, 20 MI cases and 27 stroke cases were part of the random subcohort. 90 (16.1%) MI cases and 50 (10.6%) stroke cases were fatal. 363 (77.1%) stroke cases were classified as ischemic, 88 (18.7%) as hemorrhagic, and 20 (4.2%) cases as unspecified.

**Ethics statement**

The study was approved by ethics committees at both study sites (Potsdam: Ethics Committee of the Medical Association of the State of Brandenburg; Heidelberg: Ethics Committee of the
Heidelberg University Medical School) and all participants gave written informed consent.

Laboratory methods

Vitamin D status was determined in the laboratory of the Institute of Agricultural and Nutritional Sciences at the University of Halle-Wittenberg, Germany. 25(OH)D$_2$ and 25(OH)D$_3$ were assayed in plasma samples from baseline that had been stored at -196°C for about 15 years on average. A MassChrom® reagent kit (Chromsystems, Munich, Germany) was used on an LC/MS-MS system (API 2000™, Applied Biosystems, Darmstadt, Germany) to measure both metabolites. Samples of incident cases and subcohort members were assigned to 78 analytical batches. Each batch contained at least 30 samples from both study centers and included both case and control samples. Laboratory staff was blinded to the case status of the analyzed samples. The within-batch coefficients of variation for 25(OH)D$_2$ and 25(OH)D$_3$ were 4.9% at 43.5 nmol/L and 4.2% at 96.8 nmol/L (n=3), and 5.6% at 41.8 nmol/L and 3.5% at 95.8 nmol/L (n=3), respectively. Between-batch coefficients of variation for 25(OH)D$_2$ and 25(OH)D$_3$ were 5.8% at 43.5 nmol/L and 5.6% at 96.8 nmol/L, as well as 7.8% at 41.8 nmol/L and 6.6% at 95.8 nmol/L. The use of quality control samples from the Vitamin D External Quality Assessment Scheme (www.deqas.org) revealed that 80% of the results fell within ±15% of the ALTM (All Laboratory Trimmed Mean), as compared to ±25% being the goal. The lower limit of quantification (LLOQ) was 8.25 nmol/L for 25(OH)D$_2$ and 11.5 nmol/L for 25(OH)D$_3$, the lower limit of detection (LLOD) was 2.75 nmol/L for 25(OH)D$_2$ and 4.5 nmol/L for 25(OH)D$_3$. For measured values above the LOD, but below the LLOQ, values equal to half of the LLOQ were assigned for statistical analyses. Overall, 25 out of 3 115 detected 25(OH)D$_2$ values, and 127 out of 174 detected 25(OH)D$_3$ values were below the LLOQ.

Candidate SNPs were selected based on the results of two recent GWAS [17,18] and a systematic review [26]. Information regarding linkage disequilibrium (LD) and minor-allele frequencies (MAF) of candidate SNPs was obtained using HapMap data (database release 27, CEU and TSI populations). SNPs characterized by a MAF <0.15 or a high LD with other candidates (R$^2$≥0.8) were not considered. Overall 8 SNPs from 6 regions were selected: rs1155563 and rs2282679 in the GC (group specific component) locus that codes for the vitamin D binding protein; rs3829251 and rs12785878 in the DHCR7/NADSYN1 locus that codes for the 7-dehydrocholesterol reductase involved in converting 7-dehydrocholesterol, the substrate for cholecalciferol, to cholesterol; rs10741657 in the CYP2R1 locus (25-hydroxylase); rs10877012 in the CYP27B1 locus (1-alpha-hydroxylase); rs6013897 in the CYP24A1 locus (24-hydroxylase that deactivates 1,25(OH)$_2$ vitamin D); rs6599638 in the C10orf88 locus in the vicinity of the gene coding for the
acetyl-Coenzyme A dehydrogenase, an enzyme involved in cholesterol synthesis;

Buffy-coat samples retrieved from liquid nitrogen storage were used for DNA extraction. Genotyping was carried out at KBioscience (Hoddesdon, Herts, UK) using the KASP assay, a homogeneous fluorescent genotyping system based on competitive allele-specific PCR. For quality control purposes non-template controls (NTC) were included on each plate. Genotyping call rates exceeded 96% for each SNP.

Statistical evaluation

It was decided to use total 25(OH)D values for the statistical analyses, because 25(OH)D$_2$ was detected in 5.6% of the samples only. To standardize 25(OH)D levels for season of blood draw, residuals obtained from locally weighted polynomial regression of 25(OH)D on calendar week of blood draw were added to the overall case-cohort mean value [27,28]. Hazard ratios (HR) of MI and stroke were calculated with Cox proportional-hazards models modified by the Prentice method to account for the case-cohort design of the study [23,24]. 25(OH)D levels were grouped into quartiles setting the highest quartile as the reference. In addition, we used clinical categories of 25(OH)D levels as defined by the Robert Koch Institute (severe to moderate deficiency: <25 nmol/L, mild deficiency: 25-49.9 nmol/L, sufficiency: ≥50 nmol/L) as predictors in our models [29]. Age was used as the underlying timescale. The baseline hazard function was allowed to differ by age (1 year integers) and center. First, a crude model was built adjusting for sex and BMI (continuous). Further potential confounders identified by literature search, i.e. waist circumference (cm, continuous), smoking ('never’, ‘former’, ‘1-20 cigarettes per day’, ‘>20 cigarettes per day’), physical activity according to the Cambridge Physical Activity Index [30] (‘active’, ‘moderately active’, ‘moderately inactive’, ‘inactive’), and alcohol consumption (g/d, continuous), as well as educational attainment as proxy for the subjects’ socioeconomic status ('low': primary school or no school graduation; ‘medium’: secondary, technical or professional school graduation; ‘high’: longer education including university degree) were additionally included in the second model. Linear trends were tested treating quartile medians as quantitative exposure scores. Besides the analyses with season-standardized 25(OH)D values, regression models were recalculated using quartiles of raw 25(OH)D values with adjustment for month of blood draw or season-specific 25(OH)D quartiles.

To check for non-linearity, Cox models with first and second degree fractional polynomials of 25(OH)D levels were applied allowing for seven transformations of continuous 25(OH)D values (by $x^4$, $x^3$, $x^{0.5}$, $x^{-0.5}$, $x^{-1}$, and log(x)) [31]. 25(OH)D values below the lower limit of quantification and in the highest percentile of the distribution were omitted from fractional polynomial analyses. The fractional polynomial with the best fit according to deviance testing was selected [31]. Log likelihood ratio tests were conducted to detect interactions between 25(OH)D levels with adjustment variables. Sensitivity analyses were carried out excluding the cases that occurred within the first two years after baseline. Correlation coefficients and Lewontin’s D’ were calculated as measures for the Linkage disequilibrium (LD) between SNP pairs. Chi-squared tests were conducted to assess if genotypes were in Hardy-Weinberg equilibrium (HWE). Generalized Linear Models were used to assess the association between SNPs and 25(OH)D in the subcohort adjusting for age, sex and study center. P values were multiplied by 8 to account for multiple testing. Partial $R^2$ values were calculated as estimates for the effect sizes of the SNPs. HRs of MI and stroke across alleles were calculated by Cox proportional-hazards models adjusted for sex and center using age as the underlying timescale. Co-dominant, dominant, log-additive, and recessive models were used for analyses on single SNPs. Further, risk allele scores were calculated based on SNPs that were significantly associated with 25(OH)D levels. First, a simple score was created summing the number of alleles associated with lower 25(OH)D levels. Alternatively, risk alleles were weighted by beta coefficients obtained from the regression of SNPs on 25(OH)D levels using an additive model. Statistical tests were two-sided and p-values of <0.05 were considered statistically significant. SAS 9.2 (SAS Institute, NC, USA) was used for all analyses.

Results

Characteristics of the study participants

The seasonal variation in 25(OH)D levels in the subcohort is shown in Figure 2. Mean season-adjusted 25(OH)D levels were higher among non-cases in the subcohort (47.3 nmol/L, ±18.3) and in the total subcohort (47.2 nmol/L, ±18.3) when compared to the mean levels in MI cases (44.6 nmol/L, ±18.9) and stroke cases (44.6 nmol/L, ±18.3). Further baseline characteristics of the subcohort and the incident cases are presented in Table 1. Incident MI and stroke cases were older and more likely to be men compared to the subcohort members. They had higher risk profiles concerning classical CVD risk factors including overweight and obesity, smoking, physical activity, hypertension, and diabetes. Also, CVD cases had higher waist circumference values and lower formal education levels. Finally, average alcohol intake levels in stroke cases were higher than in subcohort members.

In the subcohort, there were more women in the lowest 25(OH)D quartile compared to the overall percentage of women. Age at baseline was similar across 25(OH)D quartiles. The proportions of overweight and obese subjects, current smokers, physically inactive subjects, and subjects with prevalent hypertension or diabetes were lower at higher 25(OH)D levels, while average alcohol intakes increased across 25(OH)D quartiles. There was no clear relation between education levels and 25(OH)D levels. Mean plasma 25(OH)D levels were similar among participants from Heidelberg (47.4 nmol/L, ±19.6) and Potsdam (47.0 nmol/L, ±17.2) (p=0.63).
Associations between plasma 25(OH)D and incident myocardial infarction and stroke

Prospective associations between plasma 25(OH)D levels and the risks of MI and stroke are shown in Table 2. In the basic model adjusted for sex and BMI, 25(OH)D was significantly inversely associated with the risk of MI (HR between quartile 1 and quartile 4 [95% confidence interval]: 1.43 [1.07-1.92], $p_{\text{linear trend}}<0.01$). After additional adjustment for waist circumference, alcohol intake, physical activity, smoking, and education levels, the association attenuated and was no longer significant (HR [95% CI]: 1.17 [0.86-1.58], $p_{\text{linear trend}}=0.19$). Concerning incident stroke, the basic model revealed a significant association with 25(OH)D levels (HR [95% CI]: 1.37 [1.02-1.84], $p_{\text{linear trend}}=0.05$). This association also attenuated by multiple adjustment (HR [95% CI]: 1.25 [0.92-1.70], $p_{\text{linear trend}}=0.19$). Similarly, 25(OH)D levels were significantly inversely related to CVD risk in the basic model (HR [95% CI]: 1.41 [1.11-1.79], $p_{\text{linear trend}}<0.01$), but not in the multivariable model (HR [95% CI]: 1.19 [0.93-1.52], $p_{\text{linear trend}}=0.12$) when using MI and stroke as a composite endpoint (Table 2).

With regard to MI and total CVD, there was no indication for a nonlinear relation with 25(OH)D using fractional polynomials. By contrast, there was a significant ($p<0.01$) nonlinear association between 25(OH)D and incident stroke characterized by a j-shaped curve after transforming 25(OH)D levels to a second degree polynomial (Figure 3). A categorization by clinical cut-points revealed an increased risk of stroke in participants with deficient 25(OH)D levels (<25 nmol/L) as compared to participants with levels above 50 nmol/L (HR [95% CI]: 1.54 [1.05-2.27], $p=0.03$, Table 3). The same was true concerning MI risk (HR [95% CI]: 1.56 [1.08-2.25], $p=0.02$) and CVD risk (HR [95% CI]: 1.53 [1.12-2.09], $p<0.01$). The risks of both MI and stroke in subjects with 25(OH)D levels between 25 and 49.9 nmol/L did not differ significantly from those of subjects with levels of ≥50 nmol/L.

Additional adjustment for self-reported prevalent hypertension, dyslipidemia, diabetes, drug use (anti-hypertensive drugs, NSAIDs, glucocorticosteroids,
Table 1. Characteristics of the subcohort presented by quartiles of season-standardized 25(OH)D.

| Subcohort* (quartiles of season-standardized plasma 25(OH)D) | Q1       | Q2       | Q3       | Q4       | All     | MI cases | cases |
|-------------------------------------------------------------|----------|----------|----------|----------|---------|----------|-------|
| N                                                          | 533      | 533      | 533      | 533      | 2132    | 559      | 471   |
| Women                                                       | 62.5     | 58.5     | 55.4     | 55.2     | 57.9    | 22.4     | 41.1  |
| Age at baseline (years)                                     | (±8.5)   | (±8.4)   | (±8.6)   | (±7.3)   |         |          |       |
| 25(OH)D (nmol/L)                                            | 27.3     | 40.4     | 50.8     | 70.3     | 47.2    | 44.6     | 44.6  |
| Season of blood draw                                        | Jan–Mar  | 25.9     | 29.8     | 26.1     | 21.0    | 25.7     | 23.2  | 28.5  |
|                                                          | Apr–Jun  | 21.6     | 26.8     | 23.5     | 24.6    | 24.1     | 21.5  | 22.6  |
|                                                          | Jul–Sept | 26.8     | 22.2     | 27.0     | 27.8    | 25.9     | 28.3  | 24.9  |
|                                                          | Oct–Sept | 25.7     | 21.2     | 23.4     | 26.6    | 24.3     | 27.0  | 24.0  |
| BMI categories                                              | Normal weight | 43.0   | 37.3     | 46.3     | 52.7    | 44.8    | 22.2  | 32.7  |
|                                                          | Overweight | 34.5     | 42.4     | 40.2     | 38.5    | 38.9    | 51.3  | 47.0  |
|                                                          | Obese     | 22.5     | 20.3     | 13.5     | 8.8     | 16.3    | 26.5  | 21.3  |
| Waist circumference (cm)                                    | 82.9     | 82.6     | 79.2     | 77.1     | 80.6    | 86.5     | 84.5  |
|                                                          | (±13.1)  | (±12.2)  | (±9.9)   | (±9.1)   | (±11.5) | (±12.5)  |       |
|                                                          | 96.5     | 96.9     | 94.8     | 93.1     | 95.2    | 98.9     | 97.5  |
|                                                          | (±11.2)  | (±10.7)  | (±9.9)   | (±8.8)   | (±10.2) | (±9.7)   |       |
| Alcohol intake (g/day)                                      | 14.8     | 14.6     | 15.3     | 19.0     | 15.9    | 16.8     | 20.1  |
|                                                          | (±22.6)  | (±16.8)  | (±17.1)  | (±21.0)  | (±19.6) | (±19.7)  |       |
| Smoking status                                              | Never     | 47.8     | 46.9     | 46.9     | 45.4    | 46.8    | 29.2  | 37.3  |
|                                                          | Former    | 25.2     | 31.9     | 35.7     | 38.1    | 32.7    | 30.6  | 34.2  |
|                                                          | Current   | 27.0     | 21.2     | 17.4     | 16.5    | 20.5    | 40.2  | 28.5  |
| Physical activity index                                     | Inactive  | 21.4     | 20.3     | 13.7     | 12.8    | 17.0    | 22.9  | 25.5  |
|                                                          | Moderately Inactive | 38.3    | 35.5     | 38.6     | 36.2    | 37.2    | 36.9  | 35.7  |
|                                                          | Moderately Active | 25.7     | 25.5     | 25.0     | 28.3    | 26.1    | 23.6  | 20.2  |
|                                                          | Active    | 14.6     | 18.7     | 22.7     | 22.7    | 19.7    | 16.6  | 18.6  |
| Education level                                             | Low       | 24.0     | 21.8     | 21.6     | 20.8    | 22.1    | 34.0  | 33.5  |
|                                                          | Medium    | 44.5     | 44.1     | 40.9     | 44.7    | 43.5    | 36.3  | 39.3  |
|                                                          | High      | 31.5     | 34.1     | 37.5     | 34.5    | 34.4    | 29.7  | 27.2  |
|                                                          | Prevalent hypertension† | 32.8    | 35.5     | 32.1     | 27.4    | 31.9    | 48.4  | 52.1  |
|                                                          | Prevalent diabetes† | 5.6      | 4.9      | 3.0      | 2.4     | 4.0     | 14.5  | 13.9  |

Table 1 (continued).

* Includes 20 incident cases of MI and 27 incident cases of stroke;
† Season-standardized;
‡ Self-reported;
¶ P-values are percentages. Means (standard deviations) are shown for age, 25(OH)D, waist circumference, and alcohol intake;

** Associations between SNPs and plasma 25(OH)D **

Four SNPs (rs1155563 and rs2282679 in the GC locus, as well as rs12785878 and rs3829251 in the DHCR7/NADSYN1 locus) were significantly associated with season-adjusted 25(OH)D levels in the subcohort (Table 4). The variance in 25(OH)D explained by these four SNPs ranged from 0.7% (rs3829251) to 3.0% (rs2282679). As the two SNP pairs in the GC locus (rs1155563 and rs2282679) and the DHCR7/NADSYN1 locus (rs12785878 and rs3829251) were in high LD (r²>0.8), only rs2282679 and rs12785878 were used to build SNP scores. A simple SNP score based on the sum of risk alleles from both SNPs explained 4.4% of the variance in plasma 25(OH)D levels, while a SNP score based on risk alleles weighted by beta coefficients from linear regression models explained 4.2% adjusting for age, sex and center (data not shown). SNP associations with 25(OH)D levels were similar when analyzing data of the entire case-cohort or when using raw 25(OH)D levels adjusting for month of blood draw or season-specific quartiles (data not shown).

** Associations between SNPs and incident cardiovascular diseases **

Associations between genetic 25(OH)D determinants and incident MI and stroke are shown in Table 4. None of the SNPs was significantly related to the risks of MI or stroke in the co-dominant model, irrespective of the variance in 25(OH)D explained. Further, the selected SNPs were not significantly associated with incident CVD, i.e. stroke and MI as a composite endpoint. There were no significant linear trends for associations between the SNPs and incident MI, stroke, or CVD when using SNPs as continuous variables in additive models, even without correcting p-values for multiple testing. With respect to the SNP score, Cox regression revealed no

Vitamin D Status and Cardiovascular Disease Risk
Table 2. Hazard ratios for cardiovascular diseases according to quartiles of 25(OH)D.

| 25(OH)D quartile | Cases | Model 1 | Model 2 |
|------------------|-------|---------|---------|
| (Median nmol/L)  | N (%) | HR      | 95% CI  | P trend† | HR      | 95% CI  | P trend† |
|                  |       |         |         |         |         |         |         |
| Myocardial Infarction |       |         |         |         |         |         |         |
| Q4 (66.5)        | 118 (21.1) | 1.0 (Referent) | 1.0 (Referent) | 0.93 (0.71-1.26) | 0.92 (0.70-1.21) | <0.01 | 0.93 (0.70-1.21) | 0.92 (0.70-1.21) | <0.01 |
| Q3 (50.5)        | 117 (20.9) | 0.95 (0.70-1.26) | 0.85 (0.62-1.17) | 0.95 (0.70-1.26) | 0.85 (0.62-1.17) | 0.26 | 0.85 (0.62-1.17) | 0.85 (0.62-1.17) | 0.26 |
| Q2 (40.4)        | 158 (28.3) | 1.24 (0.93-1.66) | 1.07 (0.78-1.46) | 1.24 (0.93-1.66) | 1.07 (0.78-1.46) | 0.07 | 1.07 (0.78-1.46) | 1.07 (0.78-1.46) | 0.07 |
| Q1 (28.9)        | 166 (29.7) | 1.43 (1.07-1.92) | 1.17 (0.86-1.58) | 1.17 (0.86-1.58) | 1.17 (0.86-1.58) | 0.19 | 1.17 (0.86-1.58) | 1.17 (0.86-1.58) | 0.19 |
| Stroke           |       |         |         |         |         |         |         |
| Q4 (66.6)        | 111 (23.6) | 1.0 (Referent) | 1.0 (Referent) | 0.90 (0.57-1.42) | 0.90 (0.57-1.42) | <0.01 | 0.90 (0.57-1.42) | 0.90 (0.57-1.42) | <0.01 |
| Q3 (50.5)        | 101 (21.4) | 0.86 (0.63-1.17) | 0.83 (0.60-1.13) | 0.86 (0.63-1.17) | 0.83 (0.60-1.13) | 0.26 | 0.83 (0.60-1.13) | 0.83 (0.60-1.13) | 0.26 |
| Q2 (40.4)        | 102 (21.7) | 0.83 (0.61-1.12) | 0.83 (0.61-1.12) | 0.83 (0.61-1.12) | 0.83 (0.61-1.12) | 0.26 | 0.83 (0.61-1.12) | 0.83 (0.61-1.12) | 0.26 |
| Q1 (28.9)        | 157 (33.3) | 1.37 (1.02-1.84) | 0.05 | 1.25 (0.92-1.70) | 0.19 |         |         |
| CVD as composite endpoint |       |         |         |         |         |         |         |
| Q4 (66.6)        | 229 (22.2) | 1.0 (Referent) | 1.0 (Referent) | 0.90 (0.57-1.42) | 0.90 (0.57-1.42) | <0.01 | 0.90 (0.57-1.42) | 0.90 (0.57-1.42) | <0.01 |
| Q3 (50.5)        | 218 (21.2) | 0.89 (0.70-1.14) | 0.84 (0.65-1.09) | 0.89 (0.70-1.14) | 0.84 (0.65-1.09) | 0.26 | 0.84 (0.65-1.09) | 0.84 (0.65-1.09) | 0.26 |
| Q2 (40.4)        | 260 (25.2) | 1.06 (0.83-1.35) | 0.96 (0.75-1.24) | 1.06 (0.83-1.35) | 0.96 (0.75-1.24) | 0.26 | 0.96 (0.75-1.24) | 0.96 (0.75-1.24) | 0.26 |
| Q1 (28.9)        | 323 (31.4) | 1.41 (1.11-1.79) | <0.01 | 1.19 (0.93-1.52) | 0.12 |         |         |

Hazard Ratios calculated by Cox regression analyses using Prentice weights to account for the case-cohort design;
Model 1 adjusted for BMI and sex, stratified by center and age at baseline;
Model 2 additionally adjusted for waist circumference, alcohol intake, education level, physical activity, and smoking
* Quartile medians from the subcohort;
† P for trend calculated modeling quartile medians as continuous variable;

Discussion

In the present study, we found no significant linear associations between 25(OH)D levels and the risks of stroke and MI, although we did observe increased risks of both diseases at 25(OH)D levels below 25 nmol/L. Four out of eight candidate SNPs were significantly associated with 25(OH)D levels. However, neither single SNPs nor allele scores were related to the risks of MI or stroke. Thus, our results provide no support for a major causal role of vitamin D in the development of MI and stroke.

With regard to the lack of linear associations between vitamin D status and the risks of MI and stroke in our study two aspects are worth noting. First, plasma 25(OH)D levels in our population were relatively low compared to other populations worldwide [32]. Only few subjects in our study had 25(OH)D values above 75 nmol/L (5.8% in the subcohort) that are considered optimal regarding CVD prevention by some researchers [33]. Second, the majority of incident cases in our study were nonfatal. Notably, a large prospective study from Denmark has recently shown that 25(OH)D levels were associated with incident fatal MI, but not nonfatal MI [10]. The authors discussed that their finding supported the notion that 25(OH)D may be related to fatal MI rather than nonfatal MI [34]. A similar tendency was observed in the MIDSPAN study [35]. Hence, the hypothesis that 25(OH)D may be related to fatal MI rather than nonfatal MI deserves further study, even though the Intermountain study and the Cardiovascular Health Study 25(OH)D did show associations between 25(OH)D and the risks of incident nonfatal MI [36,37]. Nonetheless, the observation that inverse associations detected in our raw models attenuated after adjusting for classical CVD risk factors may imply that low 25(OH)D is a marker of an adverse CVD risk profile as well.

Interestingly, associations between 25(OH)D and incident MI have been assessed only recently in the MONICA/KORA-
Figure 3. Nonlinear association between plasma 25(OH)D and incident stroke. The curve represents the following second degree fractional polynomial function of 25(OH)D that is significantly (p<0.01) associated with incident stroke after multivariable adjustment: $HR_{stroke} = \exp\{((x)^1\times(-0.19457) + (x)^1\times(0.03294)\times\log(x)) - ((66.5)^1\times(-0.19457) + (66.5)^1\times(0.03294)\times\log(x))\}$; Additionally, hazard ratios of stroke by 25(OH)D quartiles from the fully adjusted model are presented. The reference for the polynomial function was set at 66.5 nmol/L, the median value in the highest 25(OH)D quartile. The short vertical ticks above the horizontal axis represent the distribution of season-standardized 25(OH)D values in incident stroke cases and the subcohort. doi: 10.1371/journal.pone.0069080.g003

Table 3. Hazard ratios for cardiovascular diseases according to clinical vitamin D status categories.

| Clinical category | 25(OH)D (nmol/L) | N cases/subco. | Model 1 | Model 2 |
|-------------------|------------------|----------------|---------|---------|
|                   |                  |                | HR      | 95% CI  | P       |
|                   |                  |                | HR      | 95% CI  | P       |
| Myocardial Infarction |                  |                |         |         |         |
| Sufficiency       | ≥50              | 183/802        | 1.0     | (Referent) |         |
| Mild deficiency   | 25-49.9          | 315/1162       | 1.24    | (0.99-1.55) | 0.06 |
| Moderate to severe deficiency | <25        | 61/168         | 1.81    | (1.25-2.64) | <0.01 |
| Stroke            |                  |                |         |         |         |
| Sufficiency       | ≥50              | 171/802        | 1.0     | (Referent) |         |
| Mild deficiency   | 25-49.9          | 240/1162       | 0.99    | (0.79-1.24) | 0.93     |
| Moderate to severe deficiency | <25        | 60/168         | 1.81    | (1.25-2.62) | <0.01 |
| CVD (composite)   |                  |                |         |         |         |
| Sufficiency       | ≥50              | 354/802        | 1.0     | (Referent) |         |
| Mild deficiency   | 25-49.9          | 555/1162       | 1.11    | (0.93-1.33) | 0.25 |
| Moderate to severe deficiency | <25        | 121/168        | 1.80    | (1.33-2.45) | <0.01 |

Hazard ratios calculated by Cox regression analyses using Prentice weights to account for the case-cohort design;
Model 1 adjusted for BMI and sex, stratified by center and age at baseline;
Model 2 additionally adjusted for waist circumference, alcohol intake, education level, physical activity, and smoking.
Table 4. Associations between candidate SNPs, season-standardized 25(OH)D levels, and the risks cardiovascular diseases.

| SNP, locus† | Allele N subcohort Mean 25(OH)D * | Risk of myocardial infarction† | Risk of stroke† | Risk of CVD (composite)† |
|-------------|-----------------------------------|-------------------------------|----------------|----------------------|
| rs1155653   | T/T 1059 49.7                      | 261 Ref.                      | 243 Ref.       | 524 Ref.             |
| GC          | G/C 832 45.1                       | 224 1.07 (0.86-1.33)          | 185 0.99 (0.79-1.24) | 409 1.05 (0.88-1.25) |
| rs2282679   | A/A 1058 50.0                      | 288 Ref.                      | 249 Ref.       | 537 Ref.             |
| GC          | A/C 827 45.0                       | 216 0.97 (0.77-1.21)          | 183 0.91 (0.73-1.14) | 399 0.94 (0.79-1.13) |
| rs3829251   | G/G 1415 48.2                      | 380 Ref.                      | 314 Ref.       | 694 Ref.             |
| DHCR7/      | G/A 594 45.1                       | 150 1.04 (0.83-1.31)          | 140 1.15 (0.91-1.45) | 290 1.09 (0.90-1.31) |
| NADSYN1     | A/A 60 44.0                        | <0.01 0.7 1.17 (0.63-2.17)    | 0.11 1.0 (0.50-1.98) | 0.34 26 1.09 0.65-1.63 0.38 |
| rs12785878  | T/T 1081 49.0                      | 290 Ref.                      | 241 Ref.       | 531 Ref.             |
| DHCR7/      | G/G 806 46.2                       | 210 1.04 (0.84-1.29)          | 181 1.05 (0.84-1.31) | 391 1.06 (0.89-1.27) |
| NADSYN1     | G/G 172 40.3                       | <0.01 0.7 1.12 (0.76-1.65)    | 0.55 42 1.23 (0.83-1.81) | 0.35 84 1.20 0.87-1.65 0.38 |
| rs5993638   | G/G 555 43.6                       | 135 Ref.                      | 127 Ref.       | 262 Ref.             |
| C10orf88    | G/A 1020 47.6                      | 268 1.10 (0.86-1.41)          | 231 1.02 (0.79-1.31) | 499 1.07 (0.87-1.31) |
| rs10741657  | G/G 768 46.2                       | 208 Ref.                      | 183 Ref.       | 391 Ref.             |
| CYP2R1      | G/G 966 47.0                       | 251 1.07 (0.86-1.34)          | 212 1.03 (0.82-1.30) | 463 1.06 (0.88-1.28) |
| rs10877012  | G/G 919 48.1                       | 243 Ref.                      | 213 Ref.       | 456 Ref.             |
| CYP27B1     | G/G 910 46.7                       | 251 1.10 (0.89-1.37)          | 203 1.00 (0.80-1.25) | 454 1.11 (0.89-1.40) |
| rs015897    | T/T 238 45.3                       | 0.16 0.3 0.97 (0.55-1.13)     | 0.47 0.86 (0.59-1.23) | 0.53 96 1.07 (0.86-1.35) 0.38 |
| CYP24A1     | T/T 1315 47.7                      | 337 Ref.                      | 276 Ref.       | 613 Ref.             |
| A/A 67      | G/G 46.5                           | 0.99 0.1 1.50 (0.90-2.52)     | 0.18 22 1.54 (0.90-2.63) | 0.07 49 1.48 (0.96-2.30) 0.07 |
| SNP Score‡ | Risk alleles                       | 0 546 52.4                    | 159 Ref.       | 127 Ref. 286 Ref.    |
| 1 885       | 47.3                               | 210 0.88 (0.69-1.13)          | 190 1.00 (0.77-1.29) | 400 0.94 (0.76-1.13) |
| 2 462       | 43.9                               | 123 0.93 (0.70-1.25)          | 113 1.05 (0.78-1.42) | 236 1.01 (0.79-1.29) |
| >3 161      | 36.9                               | <0.01 0.4 41 1.07 (0.71-1.62) | 1.0 30 0.97 (0.57-1.42) | 0.95 71 1.00 (0.71-1.42) 0.90 |

* Percentages of missing values for single SNPs: rs1155563 3.5%, rs2282679 3.4%, rs3829251 2.8%, rs6013897 2.9%, rs6596638 3.0%, rs10741657 3.4%, rs10877012 3.0%, and rs12785878 3.3%.

** P for linear trend calculated by ANCOVA within Generalized Linear Models modeling the number of risk alleles as continuous score, adjusted for age, sex and study center; Initial p values were multiplied by 8 to account for the number of tested SNPs.

†† Calculated by Cox regression analyses stratified by age and center, and adjusted for sex; P for linear trend calculated modeling the number of risk alleles as continuous score; P values from Cox regressions were not corrected for multiple testing because all associations were non-significant.

‡‡ Consisting of rs2282679 and rs12785878; rs1155563 and rs2282679 in the GC locus, as well as rs3829251 and rs12785878 in the DHCR7/NADSYN1 loci were in strong linkage disequilibrium (R²>0.8).

No deviation from Hardy-Weinberg-Equilibrium was detected for any of the SNPs.

The SNP selection in the present study appears to be in line with recent suggestions for genetic markers in Mendelian Randomization studies on vitamin D [16,20,39,40]. As opposed to us, rs4588 in the GC locus instead of rs2282679 was used in the 1958 British birth cohort study [16]. However, both SNPs were in strong LD so that similar results using rs4588 instead of rs2282679 in our study can be assumed. Likewise, rs2296850 was used instead of rs2282679 in the Tromsø Study. Yet, both SNPs were in high LD and between allele-differences in 25(OH)D were similar [20]. In the DHCR7/NADSYN1 locus, rs3794060 was used in the Tromsø Study and reported to be in perfect LD with rs12785878, the SNP that
we chose [20]. In a recent GWAS based on 5 cohorts, rs2282679, rs12785878, rs3829251, and rs6013897 were identified as the best predictors of circulating 25(OH)D [39]. Interestingly, it appeared that the explanatory power with respect to circulating 25(OH)D was better when using a score out of these SNPs as compared to polygenic scores consisting of several thousand SNPs [39]. Also, the variance explained by the two strongest SNPs was similar to the variance explained by the listed four candidate SNPs [39]. These observations are in line with our finding that adding further SNPs to an allele score consisting of rs2282679 and rs12785878 did not increase the explained variance in 25(OH)D.

Although we selected the most relevant SNPs, the variance in plasma 25(OH)D explained by our SNP score did not exceed 4.4%. This modest proportion of explained variance in 25(OH)D levels may have contributed to our failure to detect significant associations between SNPs and the risks of MI and stroke. Nevertheless, the average difference in 25(OH)D between extreme categories of our SNP score was 13.5 nmol/L. Considering the increased risks of stroke and MI at 25(OH)D levels below 25 nmol/L in our study, it does not seem implausible that even a smaller fraction of genetically determined 25(OH)D may affect CVD risk. That said, the limited sample size of our study did not allow for separate analyses of associations between SNPs and CVD risk in subjects with insufficient 25(OH)D levels.

Thus far, two observational studies have reported on genetic determinants of circulating 25(OH)D in relation to CVD risk. The Tromsø Study included some 2000 incident MI cases [20], and, like our study, did not show associations between SNPs and MI risk, the HR of MI being 0.93 (95% CI: 0.82–1.06) for subjects in the highest quartile of a genotype score based on rs2298850 (GC), rs10741657 (CYP2R1), rs3794060 (DHCR7/NADSYN1), and rs6013897 (CYP24A1). Also, no association between genetic vitamin D status determinants (rs2282679, rs12785878, and rs10741657) and CVD mortality was observed in a population of 3316 subjects scheduled for coronary angiography comprising 619 later cases of fatal CVD [41]. Still, we cannot rule out the detection of modest associations between SNPs related to vitamin D status and MI or stroke in larger consortia, as a post hoc calculation revealed that we only had the power of 0.62, 0.46 and 0.42 to detect HRs of 1.3 for incident overall CVD, MI, and stroke, respectively, between extreme categories of our SNP score. Pooling data from observational studies in larger Mendelian Randomization projects may help overcome such limitations of statistical power.

Besides the sample size, a further limitation of our study was that we could assess 25(OH)D levels only at a single occasion. However, despite the seasonal variation in 25(OH)D, several large studies have shown acceptable long-term correlations between repeated 25(OH)D measurements that are comparable to those of other biomarkers of CVD [42-45]. Moreover, we did not have the opportunity to measure levels of the vitamin D binding protein or 1,25(OH)₂D. Vitamin D binding protein levels would have been of particular interest, as the strongest genetic determinants of 25(OH)D in GWAS and in our study were from the GC gene. Of note, recent prospective studies on cancer suggest that circulating levels of the vitamin D binding protein may crucially affect associations between 25(OH)D and disease risks by determining the free fraction of 25(OH)D [46-49]. Finally, we could not adjust for further potential confounders, such as blood lipids, blood pressure, or CRP.

In summary, we did not detect clear linear inverse relationships between 25(OH)D and CVD risk. The attenuation of the associations between 25(OH)D and CVD risks by adjustment for classical CVD risk factors in our study support the notion of 25(OH)D being a global health status marker rather than an independent causal factor in CVD etiology. The lack of associations between genetic vitamin D status determinants and CVD risk suggests that associations between low vitamin D levels and CVD risk may be due to uncontrolled confounding or reverse causation. However, a detection of genetic effects related to vitamin D status on CVD risk remains possible, and our results on genetic determinants of 25(OH)D require a cautious interpretation due to limited statistical power. The results of large consortia applying the Mendelian Randomization concept as well as upcoming RCTs will facilitate an evidence-based appraisal of the role of vitamin D with respect to CVD risk.

Author Contributions

Conceived and designed the experiments: BB CW TK BT RK HB. Performed the experiments: TK BB. Analyzed the data: TK BB. Wrote the manuscript: TK. Acquired the data: RK HB CW BT BB VK. Acquired biochemical data: FH GIS. Interpreted the data: TK RK CW BB. Critical revision of the article for important intellectual content: TK RK CW BB JD VK BT JD FH GIS.

References

1. Rosen CJ, Adams JS, Bikle DD, Black DM, Demay MB et al. (2012) The nonskeletal effects of vitamin D: an Endocrine Society scientific statement. Endocr Rev 33: 456-492. doi:10.1210/er.2012-1000. PubMed: 22596255.
2. Holick MF (2007) Vitamin D deficiency. N Engl J Med 357: 266–281. doi:10.1056/NEJMra070553. PubMed: 17634462.
3. Norman AW, Bouillon R (2010) Vitamin D nutritional policy needs a vision for the future. Exp Biol Med (Maywood) 235: 1034–1045. doi:10.1258/ebm.2010.010014.
4. Heaney RP, Davies KM, Chen TC, Holick MF, Barger-Lux MJ (2003) Human serum 25-hydroxycholecalciferol response to extended oral dosing with cholecalciferol. Am J Clin Nutr 77: 204-210. PubMed: 12499343.
5. Van der Schueren BJ, Verstuyf A, Mathieu C (2012) Straight from D-Heart: vitamin D status and cardiovascular disease. Curr Opin Lipidol 23: 17-23. doi:10.1097/MOL.0b013e32834d7357. PubMed: 22123672.
6. Motiwala SR, Wang TJ (2011) Vitamin D and cardiovascular disease. Curr Opin Nephrol Hypertens 20: 345-353. doi:10.1097/MNH.0b013e32834d7357. PubMed: 22123672.
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calcification and osteogenic key factor expression in mice. PLOS ONE 7: e35316. doi: 10.1371/journal.pone.0035316. PubMed: 22536373.

8. Brandenburg VM, Vervloet MG, Maris N (2012) The role of vitamin D in cardiovascular disease: from present evidence to future perspectives. Atherosclerosis 225: 253-263. doi: 10.1016/j.atherosclerosis.2012.08.005. PubMed: 22021424.

9. Sun Q, Pan A, Hu FB, Manson JE, Rxeorde KM (2012) 25-Hydroxyvitamin D levels and the risk of stroke: a prospective study and meta-analysis. Stroke 43: 1470-1477. doi: 10.1161/STROKEAHA.112.639867. PubMed: 22936341.

10. Brundum-Jacobsen B, Benn M, Jensen GB, Nordestgaard BG (2012) 25-hydroxyvitamin D levels and risk of ischemic heart disease, myocardial infarction, and early death: population-based study and meta-analyses of 18 and 17 studies. Arterioscler Thromb Vasc Biol 32: 2794-2802. doi: 10.1161/ATVBAHA.112.248039. PubMed: 22936341.

11. Institute of Medicine (US) Committee to Review Dietary Reference Intakes for Vitamin D and Calcium. Dietary Reference Intakes for Calcium and Vitamin D. Washington: The National Academies Press. 9780309163941 9780309163941.

12. McCreery C, Williams D (2011) New insights about vitamin D and cardiovascular disease: a narrative review. Ann Intern Med 155: 820-826. doi: 10.7326/0003-4819-155-12-20112200-00004. PubMed: 21648898.

13. Kupferschmidt K (2012) Uncertain verdict as vitamin D goes on trial. Science 337: 1476-1478. doi: 10.1126/science.337.6100.1476. PubMed: 22997323.

14. Bergmann A, Birgmann J, Gummert JF, Plz S (2012) Future directions in vitamin D and cardiovascular research. Nutr Metab Cardiovasc Dis 22: 541-546. doi: 10.1016/j.numecd.2012.02.004. PubMed: 22635667.

15. Plz S, Rutters F, Dekker JM (2012) Disease prevention: vitamin D trials. Science 338: 883. doi: 10.1126/science.338.6100.883-b. PubMed: 23161977.

16. Berry DJ, Vimaleswaran KS, Whittaker JC, Hingorni AD, Hypponen E (2012) Evaluation of genetic markers as instruments for Mendelian randomization studies on vitamin D. PLOS ONE 7: e37465. doi: 10.1371/journal.pone.0037465. PubMed: 22629401.

17. Ahn J, Yu K, Stolzenberg-Solomon R, Simon KC, McCulloch ML et al. (2010) Genome-wide association study of circulating vitamin D levels. Hum Mol Genet 19: 2739-2745. doi: 10.1093/hmg/ddq155. PubMed: 20418485.

18. Wang TJ, Zhang F, Richards JB, Kestenbaum B, van Meurs JB et al. (2010) Common genetic determinants of vitamin D insufficiency: a genome-wide association study. Lancet 376: 180-188. doi: 10.1016/S0140-6736(10)60588-0. PubMed: 20541252.

19. Sheehan NA, Didelez V, Burton PR, Tobin MD (2008) Mendelian randomisation and causal inference in observational epidemiology. PLOS Med 5: e177. doi: 10.1371/journal.pmed.0050177. PubMed: 18752343.

20. Jorde R, Langenberg C, Sharp S, Forouhi NG, Franks PW, Schulze MB et al. (2012) Vitamin D, parathyroid hormone, and cardiovascular events and incident events in a general healthcare population. Am J Epidemiol 234: 225-234. doi: 10.1093/aje/kw209. PubMed: 21875231.

21. Kulathinal S, Karvanen J, Saarela O, Kuulasmaa K (2007) Case-cohort design in practice - experiences from the MORGAM Project. Epidemiol Prev 31: 373-380. doi: 10.1007/s11657-007-0093-0. PubMed: 17858097.

22. Brøndum-Jacobsen P, Benn M, Jensen GB, Nordestgaard BG (2012) 25-hydroxyvitamin D concentrations and risk of myocardial infarction, diabetes, and subsequent risk of cardiovascular disease: a narrative review. Ann Intern Med 155: 105-115. doi: 10.7326/0003-4819-155-2-20112200-00004. PubMed: 22936341.

23. Welsh P, Doolin O, McConnachie A, Boulton E, McNeil G et al. (2012) Circulating 25OHD, dietary vitamin D, PTH, and calcium associations with incident cardiovascular disease and mortality: the MIDSPAN Family Study. J Clin Endocrinol Metab 97: 4578-4587. doi: 10.1210/jc.2011-2272. PubMed: 22936342.

24. Anderson JL, May HT, Horne BD, Bair TL, Hall NL et al. (2012) Relation of vitamin D deficiency to cardiovascular risk factors, disease status, and incident events in a general healthcare population. Am J Cardiol 108: 963-968. doi: 10.1016/j.amjcard.2010.05.027. PubMed: 20854956.

25. Kestenbaum B, Katz R, de Boer I, Hoofnagle A, Sarnak MJ et al. (2011) Vitamin D, parathyroid hormone, and cardiovascular events among older adults. J Am Coll Cardiol 58: 1433-1441. doi: 10.1016/j.jacc.2011.03.069. PubMed: 21939825.

26. Karakas M, Thorand B, Zierer A, Huth C, Meisinger C et al. (2013) Low Levels of Serum 25-Hydroxyvitamin D Are Associated with Increased Risk of Myocardial Infarction, Especially in Women: Results from the MONICA/KORA Augsburg Case-Cohort Study. J Intern Med 273: 272-280. doi: 10.1111/j.1365-2702.2012.2368.8. PubMed: 23150960.

27. Hiraki LT, Major JM, Chen C, Cornelis MC, Hunter DJ et al. (2013) Exploring the genetic architecture of circulating 25-hydroxyvitamin D. Genetics Epidemiol 37: 92-98. doi: 10.1002/gepi.21694. PubMed: 23135809.

28. Vimaleswaran KS, Berry DJ, Lu C, Tikkanen E, Plz S et al. (2013) Causal Relationship between Obesity and Vitamin D Status: Bi-Directional Mendelian Randomization Analysis of Multiple Cohorts. PLOS Med 10: e1001383. PubMed: 23393431.

29. Trummer O, Plz S, Hoffmann MM, Winkelmann BR, Boehm BO et al. (2013) Vitamin D and mortality: a Mendelian randomization study. Clin Chem 59: 793-797. doi: 10.1373/clinchem.2012.193185. PubMed: 23319826.

30. Kotsovoulos J, Tworoger SS, Campos H, Chung FL, Cleveger CV et al. (2010) Reproducibility of plasma and urine biomarkers among premenopausal and postmenopausal women from the Nurses’ Health Studies. Cancer Epidemiol Biomarkers Prev 19: 938-946. doi: 10.1158/1055-9965.EPI-09-1318. PubMed: 20332276.

31. Platz EA, Leitzmann MF, Hollis BW, Willett WC, Giovannucci E et al. (2004) Plasma 1,25-dihydroxy- and 25-hydroxyvitamin D and subsequent risk of prostate cancer. Cancer Causes Control 15: 255-265. doi: 10.1023/B:CAICO.0000002425.24880.s. PubMed: 15090720.

32. Sonderman JS, Munro HM, Biot WJ, Signorello LB, Welsh P, Doolin O, McConnachie A, Boulton E, McNeil G et al. (2012) Causal Association between Obesity and Vitamin D Status: Bi-Directional Mendelian Randomization Analysis of Multiple Cohorts. J Intern Med 273: 1-12. doi: 10.1111/j.1365-2702.2012.2368.8. PubMed: 23150960.

33. Sonderman JS, Munro HM, Biot WJ, Signorello LB, Welsh P, Doolin O, McConnachie A, Boulton E, McNeil G et al. (2012) Causal Association between Obesity and Vitamin D Status: Bi-Directional Mendelian Randomization Analysis of Multiple Cohorts. J Intern Med 273: 1-12. doi: 10.1111/j.1365-2702.2012.2368.8. PubMed: 23150960.
46. Weinstein SJ, Stolzenberg-Solomon RZ, Kopp W, Rager H, Virtamo J et al. (2012) Impact of circulating vitamin D binding protein levels on the association between 25-hydroxyvitamin D and pancreatic cancer risk: a nested case-control study. Cancer Res 72: 1190-1198. doi: 10.1158/1538-7445.AM2012-1190. PubMed: 22232734.

47. Mondul AM, Weinstein SJ, Virtamo J, Albanes D (2012) Influence of vitamin D binding protein on the association between circulating vitamin D and risk of bladder cancer. Br J Cancer 107: 1589-1594. doi:10.1038/bjc.2012.417. PubMed: 22990651.

48. Weinstein SJ, Mondul AM, Kopp W, Rager H, Virtamo J et al. (2013) Circulating 25-hydroxyvitamin D, vitamin D-binding protein and risk of prostate cancer. Int J Cancer 132: 2940-2947. doi:10.1002/ijc.27969. PubMed: 23180681.

49. Chun RF (2012) New perspectives on the vitamin D binding protein. Cell Biochem Funct 30: 445-456. doi:10.1002/cbf.2835. PubMed: 22528606.