Vitamin D and Mortality: A Mendelian Randomization Study

Olivia Trummer,1 Stefan Pilz,1 Michael M. Hoffmann,2 Bernhard R. Winkelmann,3 Bernhard O. Boehm,4 Winfried März,5,6,7 Thomas R. Pieber,1 Barbara Obermayer-Pietsch,1,* and Wilfried Renner5

BACKGROUND: Decreased circulating 25-hydroxy-vitamin D (25-OH-vitamin D) concentrations have been associated with mortality rates, but it is unclear whether this association is causal. We performed a Mendelian randomization study and analyzed whether 3 common single-nucleotide polymorphisms (SNPs) associated with 25-OH-vitamin D concentrations are causal for mortality rates.

METHODS: Genotypes of SNPs in the group-specific component gene (GC, rs2282679), 7-dehydrocholesterol reductase gene (DHCR7, rs12785878), and cytochrome P450 IIR-1 gene (CYP2R1, rs10741657) were determined in a prospective cohort study of 3316 male and female participants [mean age 62.6 (10.6) years] scheduled for coronary angiography between 1997 and 2000. 25-OH-vitamin D concentrations were determined by RIA. The main outcome measures were all-cause deaths, cardiovascular deaths, and noncardiovascular deaths.

RESULTS: In a linear regression model adjusting for month of blood sampling, age, and sex, vitamin D concentrations were predicted by GC genotype (P < 0.001), CYP2R1 genotype (P = 0.068), and DHCR7 genotype (P < 0.001), with a coefficient of determination (r²) of 0.175. During a median follow-up time of 9.9 years, 955 persons (30.0%) died, including 619 deaths from cardiovascular causes. In a multivariate Cox regression adjusted for classical risk factors, GC, CYP2R1, and DHCR7 genotypes were not associated with all-cause mortality, cardiovascular mortality, or noncardiovascular mortality.

CONCLUSIONS: Genetic variants associated with 25-OH-vitamin D concentrations do not predict mortality. This suggests that low 25-OH-vitamin D concentrations are associated with, but unlikely to be causal for, higher mortality rates.

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25-Hydroxy-vitamin D (25-OH-vitamin D)8 is known for its crucial role in calcium and bone metabolism (1, 2). In the past several years, attention has turned to potential effects of 25-OH-vitamin D on cardiovascular disease (CVD) (3), hypertension (4), and metabolic syndrome (5, 6). We have previously shown that low 25-OH-vitamin D concentrations are associated with all-cause and cardiovascular mortality (7). These results are in line with other studies (8, 9). It remains unclear whether 25-OH-vitamin D is the cause or the consequence of these effects (3).

Vitamin D deficiency has been shown to be highly prevalent among general populations (10) and is more common among elderly individuals, probably due to the decreasing capacity of skin to produce vitamin D with aging (3).

Mendelian randomization refers to the random allocation of alleles at the time of gamete formation. A specific genotype carried by a person results from 2 such randomized transmissions, 1 from the paternally inherited allele and the other from the maternally inherited allele. A logical consequence of these randomizations is that genotypes are not expected to be associated with known (measurable or not) or unknown confounders for any outcome of interest, except those lying on the causal pathway between the genotype and the outcome. This allows the analysis of the genotype–risk factor association and the genotype–outcome association in an unconfounded manner. By combining

1 Division of Endocrinology and Metabolism, Department of Internal Medicine, Medical University of Graz, Graz, Austria; 2 Department of Clinical Chemistry, University Medical Center, Freiburg, Germany; 3 Cardiology Group Sachsenhausen, Frankfurt-Sachsenhausen, Germany; 4 Division of Endocrinology and Diabetes, Ulm University, Ulm, Germany; 5 Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz, Graz, Austria; 6 Mannheim Institute of Public Health, Social and Preventive Medicine, Medical Faculty Mannheim, University of Heidelberg, Heidelberg, Germany; 7 Synlab Academy, Synlab Services LLC, Mannheim, Germany.

* Address correspondence to this author at: Division of Endocrinology and Metabolism, Department of Internal Medicine, Medical University of Graz, Auenbruggerplatz 15, 8036 Graz, Austria. Fax +43-431-385-13428; e-mail barbara.obermayer@medunigraz.at.

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8 Nonstandard abbreviations: 25-OH-vitamin D, 25-hydroxy vitamin D; CVD, cardiovascular disease; SNP, single-nucleotide polymorphism; LURIC, Ludwigshafen Risk and Cardiovascular Health; CAD, coronary artery disease; r², coefficient of determination.
the results of these 2 analyses appropriately, one can estimate the risk factor–outcome association, which is itself not confounded. This is analogous to randomized controlled trials, in which the random allocation of treatment or preventive measures is expected to lead to an even distribution of known or unknown confounding factors across each group (11).

Results of twin studies have suggested a heritable component of circulating 25-OH-vitamin D concentrations, with heritability rates ranging from 29% to 77% (12, 13, 14). Genomewide association studies identified 3 common loci of genetic determinants for vitamin D insufficiency: group-specific component (GC, rs2282679), 7-dehydrocholesterol reductase (DHCR7, rs12785878), and cytochrome P450 11R-1 (CYP2R1, rs10741657). These loci are located within or near genes involved in vitamin D transport, cholesterol synthesis, and hydroxylation (15, 16).

In the present study we analyzed the roles of three 25-OH-vitamin-D–associated single-nucleotide polymorphisms (SNPs) in all-cause cardiovascular and noncardiovascular mortality.

Materials and Methods

STUDY POPULATION
The Ludwigshafen Risk and Cardiovascular Health (LURIC) study is a prospective cohort trial designed to evaluate the effects of genetic polymorphisms and plasma biomarkers on cardiovascular health. Participants are consecutive white patients hospitalized for coronary angiography between June 1997 and May 2001. The study was approved by the ethics review committee at the “Landesärztekammer Rheinland-Pfalz” (Mainz, Germany). Written informed consent was obtained from each of the participants.

PROCEDURES
A detailed description of the LURIC study design and baseline characteristics has been published previously (17). Briefly, the study population comprised 3316 participants. According to the classification of the American Heart Association, coronary artery disease (CAD) was defined as the presence of a visible luminal narrowing (≤20% stenosis) in at least 1 of 15 coronary segments (18). Individuals with stenosis ≤20% were considered as not having CAD. Cardiovascular risk factors such as type 2 diabetes, hypertension, and smoking were assessed. Type 2 diabetes mellitus was diagnosed according to the criteria of the American Diabetes As-

ociation. Further, individuals with a history of type 2 diabetes or those receiving oral antidiabetics or insulin were considered diabetic (19). Hypertension was defined as a systolic and/or diastolic blood pressure exceeding 140 and/or 90 mmHg or a history of hypertension documented in medical records. Data on smoking habits were retrieved using questionnaires. To detect “hidden” smokers, plasma cotinine concentrations were determined using a commercial RIA (cotinine RIA; DPC). Individuals suffering from acute illnesses other than acute coronary syndromes, chronic noncardiac diseases, and a history of malignancy within the past 5 years were not eligible.

A fasting blood sample was obtained in the morning before coronary angiography. Selected variables were measured after samples were frozen and stored at −80 °C. 25-OH-vitamin D concentrations were deter-

mined using an RIA (DiaSorin GmbH) with intraassay and interassay CVs of 8.6% and 9.2%, respectively.

Information on mortality rates was obtained from local registries. Death certificates were used to classify the deceased individuals into those who died from cardiovascular vs noncardiovascular causes. This classification was done independently by 2 experienced clinicians who were blinded to any data on the study participants except the information that was required to classify the causes of death.

GENOTYPING
Genomic DNA was prepared from EDTA anticoagu-
lated peripheral blood by using a common salting-out procedure. Genotypes were determined by fluorogenic 5′-exonuclease assays (TaqMan™). Primer and probe sets were designed and manufactured by Applied Biosystems (Life Tech). Assay IDs were C__26407519_10 (GC polymorphism), C__32063037_10 (DHCR7 polymorphism), and C__2958430_10 (CYP2R1 polymorphism). Endpoint fluorescence was measured in a POLARstar plate reader (BMG Labtech). Fluorescence data were exported into Excel format and analyzed as scatter plots. As a QC measure, genotyping was repeated in 184 samples and no discrepancies were observed.

STATISTICS
Statistical analysis was done using PASW 18.0.0 software (IBM). Continuous variables were compared between groups by univariate ANOVA. A linear regression model was performed to identify the predictors of 25-OH-vitamin D concentrations. Cox regression in-

cluding GC, DHCR7, and CYP2R1 genotypes, age, sex, CAD, smoking, and type 2 diabetes mellitus was used to estimate the effect of the polymorphisms on mortality (all cause, cardiovascular, and noncardiovascular). For regression analyses, an allelic model based upon
additive gene-dose effects was used and genotypes were coded as 0 (wild type, homozygous for common allele), 1 (heterozygous), or 2 (homozygous for minor allele). Seasonal variations of vitamin D concentrations were modeled using categorical variables for the month of blood taking. The criterion for statistical significance was $P < 0.05$.

Results

The investigated cohort consisted of 3316 persons, including 2310 men (69.7%) and 1006 women (30.3%). Demographic data for the study population are given in Table 1. GC, DHCR7, and CYP2R1 genotypes were successfully determined in 3130 (94.4%), 3109 (93.8%), and 2980 (89.9%) participants (Table 2). For the GC and the DHCR7 polymorphism, minor alleles (GC G-allele, DHCR7 G-allele) were associated with lower 25-OH-vitamin D concentrations. For the CYP2R1 polymorphism, the minor allele (CYP2R1 A-allele) was associated with higher 25-OH-vitamin D concentrations. Genotype frequencies did not deviate from the Hardy–Weinberg equilibrium.

25-OH-vitamin D concentrations were available from 3299 patients (99.5% of the entire study population). In a linear regression model adjusting for month of blood sampling, age, and sex, vitamin D concentrations were predicted by GC genotype ($P < 0.001$), CYP2R1 genotype ($P = 0.068$), and DHCR7 genotype ($P < 0.001$), with a coefficient of determination ($r^2$) of 0.175.

After a median follow-up of 9.9 years, 995 persons (30.0% of the study population at baseline) had died. Of these, 619 deaths were from cardiovascular causes and 355 were from noncardiovascular causes; 21 deaths could not be classified because of insufficient data about the cause of death.

In multivariate Cox regression models including age, sex, type 2 diabetes, CAD, smoking habit, 25-OH-vitamin D concentrations, GC genotype, CYP2R1 genotype, and DHCR7 genotype, none of the genotypes was significantly associated with all-cause mortality, cardiovascular mortality, or noncardiovascular mortality (Table 3).

### Table 1. Demographic data of the LURIC study.

<table>
<thead>
<tr>
<th>Age, years, mean (SD)</th>
<th>62.6 (10.6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex,</td>
<td>2310 (69.7)</td>
</tr>
<tr>
<td>Height, cm, mean (SD)</td>
<td>170 (8.7)</td>
</tr>
<tr>
<td>Weight, kg, mean (SD)</td>
<td>79.7 (14.1)</td>
</tr>
<tr>
<td>25-OH-vitamin D, ng/mL, mean (SD)</td>
<td>17.4 (9.7)</td>
</tr>
<tr>
<td>Vitamin D insufficiency, n (%)</td>
<td>2174 (65.6)</td>
</tr>
<tr>
<td>CAD, n (%)</td>
<td>2583 (77.9)</td>
</tr>
<tr>
<td>Current smoker, n (%)</td>
<td>654 (19.7)</td>
</tr>
<tr>
<td>Type 2 diabetes, n (%)</td>
<td>1064 (32.1)</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>2412 (72.7)</td>
</tr>
</tbody>
</table>

* Vitamin D insufficiency: 25-OH-vitamin D $\leq$ 20 ng/mL ($\leq$ 49.9 nmol/L).

### Table 2. 25-OH-vitamin-D–associated genotypes and 25-OH-vitamin-D concentrations.$^a$

<table>
<thead>
<tr>
<th>n (%)$^a$</th>
<th>25-OH-vitamin D, ng/mL$^b$, mean (SD)</th>
<th>$p^c$</th>
<th>$p^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC genotype TT</td>
<td>1622 (51.8)</td>
<td>18.05 (10.19)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GT</td>
<td>1247 (39.8)</td>
<td>16.86 (9.34)</td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>261 (8.3)</td>
<td>14.33 (7.76)</td>
<td></td>
</tr>
<tr>
<td>GC G allele frequency</td>
<td>0.283</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHCR7 genotype TT</td>
<td>1804 (58.0)</td>
<td>17.85 (9.54)</td>
<td></td>
</tr>
<tr>
<td>GT</td>
<td>1110 (35.7)</td>
<td>16.44 (10.05)</td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>261 (8.3)</td>
<td>16.56 (9.27)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DHCR7 G allele frequency</td>
<td>0.257</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2R1 genotype GG</td>
<td>1116 (37.4)</td>
<td>16.74 (9.75)</td>
<td></td>
</tr>
<tr>
<td>AG</td>
<td>1433 (48.1)</td>
<td>17.49 (9.37)</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>431 (14.5)</td>
<td>17.88 (10.26)</td>
<td>0.030</td>
</tr>
<tr>
<td>CYP2R1 A allele frequency</td>
<td>0.385</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ 25-OH-vitamin D concentrations according to GC (rs2282679), DHCR7 (rs12785878), and CYP2R1 (rs10741657) genotypes in the LURIC study.

$^b$ To convert 25-OH-vitamin D concentrations into nanomoles per liter, multiply by 2.496.

$^c$ Univariate linear regression.

$^d$ ANOVA.
We used a linear regression model including GC genotype, CYP2R1 genotype, and DHCR7 genotype to predict a genetically determined 25-OH-vitamin D concentration for each study participant. In that model, the genetically determined 25-OH vitamin D concentration (ng/mL) was 18.158 /H11002 (GC genotype /H11002 1.544) /H11002 (DHCR7 genotype /H11003 0.981) /H11001 (CYP2R1 genotype /H11003 0.588). The mortality hazard ratio per 1 ng/mL (2.5 nmol/L) genetically determined 25-OH-vitamin D concentration was 1.015 (95% CI, 0.962–1.070; P = 0.001). By contrast, the mortality hazard ratio per 1 ng/mL (2.5 nmol/L) serum 25-OH-vitamin concentration was 0.951 (95% CI, 0.943–0.959; P = 0.001). The point estimate of the effect of the genetically determined (i.e., causal) 25-OH-vitamin D concentrations was not within the 95% CI of the effect of the 25-OH-vitamin D serum concentration, suggesting that the 2 estimates are truly different.

**Discussion**

In the present study we aimed to test the hypothesis that genetically lowered concentrations of 25-OH-vitamin D causally increase mortality. Using a Mendelian randomization method, we analyzed SNPs associated with 25-OH-vitamin D concentrations and found that genetically determined differences in 25-OH-vitamin D concentrations were not associated with mortality rates. This lack of found association makes a causal role of 25-OH-vitamin D in mortality unlikely.

The biological basis of the predictive power of 25-OH-vitamin D for mortality as well as a variety of pathological conditions such as CVD and cancer remains unclear. A potential explanation might be a reverse causality, such that specific pathomechanisms involved in CVD or cancer could result in decreased 25-OH-vitamin D. Interestingly, similar results have been found for C-reactive protein, where C-reactive protein plasma concentrations were predictive for specific diseases, but Mendelian randomization studies found no evidence for a causal role of this biomarker (20, 21).

It should be mentioned that our data are limited by the fact that we investigated a cohort of patients referred to coronary angiography and our results may not be generalizable to patients with other diseases. Furthermore, in our survival analysis, the most important factors predicting mortality were age, sex, smoking, type 2 diabetes, hypertension, and coronary artery disease. Although these factors have been considered in the statistical analysis as confounders, we cannot rule out residual confounding by other factors.

Strengths of the present study are the in-depth clinical and biochemical characterization of all patients, the high number of participants, and the long time of follow-up, such that the primary end point death was reached in almost one-third of the study participants.

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It should be kept in mind that the present study was observational and not aimed to investigate the potential benefits of vitamin D supplementation. Interestingly, in a recent large metaanalysis of randomized clinical trials, only vitamin D3, but not 25-OH-vitamin D, decreased mortality significantly (22). Nevertheless, we would like to emphasize that the present results do not invalidate the predictive power of 25-OH-vitamin D concentrations for mortality. The biological pathways for the association between 25-OH-vitamin D
concentrations and mortality remain unclear and a causal role for 25-OH-vitamin D in mortality is unlikely.

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