Vitamin K Epoxide Reductase Complex Subunit 1 (VKORC1) Polymorphism and Aortic Calcification
The Rotterdam Study


Objective—Besides effects on hemostasis, vitamin K-dependent proteins play a role in bone mineralization and arterial calcification. We investigated the association between the VKORC1 1173C>T polymorphism and calcification of the aortic far wall in a large population-based cohort.

Methods and Results—Aortic calcification was diagnosed by radiographic detection of calcified deposits in the abdominal aorta. In all cohort members for whom DNA was available, the C1173T SNP of VKORC1 (rs9934438) was determined. With multivariable logistic regression analysis the association between this polymorphism and the risk of aortic calcification was calculated, adjusted for potential confounders. The T allele frequency of the VKORC1 1173C>T polymorphism was 38.8%. 1185 (37.2%) persons were homozygous CC, 1529 (48.0%) were heterozygous CT and 473 (14.8%) were homozygous TT. Persons with at least one T-allele had a statistically significant 19% (95% CI 2 to 40%) risk increase of calcification of the aortic far wall compared to CC homozygous persons, adjusted for age and gender.

Conclusion—The T-allele of the VKORC1 1173C>T polymorphism was associated with a significantly higher risk of aortic calcification in Whites. (Arterioscler Thromb Vasc Biol. 2008;28:771-776)

Key Words: aortic calcification ■ matrix Gla protein ■ vitamin K cycle ■ VKORC1 polymorphism

Vitamin K epoxide reductase (VKOR) mediates recycling of vitamin K 2,3 epoxide to vitamin K hydroquinone, an essential cosubstrate for modification of multiple glutamic acid residues to γ-carboxyglutamate in vitamin K–dependent proteins such as the coagulation factors II, VII, IX, and X, protein C, S, and Z, Matrix Gla protein (MGP), and osteocalcin.1 Recently, numerous single nucleotide polymorphisms (SNPs) were identified on chromosome 16 in the gene encoding the vitamin K epoxide reductase complex subunit 1 (VKORC1),2-3 of which several reflect 3 main natural haplotypes of VKORC1.4-6 Five SNPs (rs 9934438, rs 9923231, rs 8050894, rs 2359612, and rs 72924) were found to be in strong linkage disequilibrium (D’>0.9 and r²≥0.9), indicating that any of these could reflect VKORC1 haplotypes.5,7 One of these SNPs, rs 993448 or VKORC1 1173C>T, is as informative about coumarin sensitivity as 5 VKORC1 haplotypes which predicted warfarin dose requirement and together accounted for 96% to 99% of the total haplotypes in European-American White populations.5 The VKORC1 1173C>T SNP is likely to be one of the putative functional SNPs of the VKORC1 gene.8 The T-allele of this SNP modifies the effectiveness of coumarins, which reduce the activity of the VKORC1 enzyme.4-6,9-16 In carriers of the T-allele, additional inhibition by coumarins had a higher impact on hemostasis than in those with the 1173CC genotype. Beyond hemostatic effects, different studies suggest an influence of vitamin K–dependent proteins on bone mineralization and arterial calcification. The key function of MGP is to inhibit calcification in cartilage and arteries.17-19 Hereto, MGP has to be activated by γ-carboxylation of its 5 glutamic acid residues, which is mediated by vitamin K hydroquinone. During carboxylation, the hydroquinone becomes oxidized to vitamin K epoxide (Figure). Vitamin K hydroquinone is derived from dietary vitamin K intake or by recycling of the epoxide. First, the epoxide is reduced to vitamin K, catalyzed by the Vitamin K epoxide reductase (VKOR). Second, vitamin K is further reduced to the hydroquinone. This second reduction step differs between tissues.20 VKORC1 seems crucial for reduction of vitamin K in extra hepatic tissues, whereas in the liver also other enzymes such as DT diaphorase mediate further reduction of vitamin K into the hydrochinone.21,22 Inhibition of the VKORC1 with coumarins for coagulation factors could be antagonized by dietary vitamin K but not for MGP as extra hepatic protein. Price et al used the implication of this
information was obtained on several characteristics, including age, gender, smoking, blood pressure, diabetes mellitus, body mass index (BMI), medication use, measures of atherosclerosis such as vascular calcification, and a verified history of myocardial infarction and heart failure. During the first examination of the participants from 1990 to 1993, blood was taken and DNA was isolated, information on weight, height, morbidity, and blood variables was collected, and calcified deposits in the abdominal aorta were assessed by radiography.

**Genotyping**

Genomic DNA was extracted from samples of peripheral venous blood according to standard procedures. 1 to 2 ng genomic DNA was dispensed into 384-wells plates using a Caliper Sciclone ALH3000 pipetting robot (Caliper LS). We chose the 1173C>T SNP at intron 1, dbSNP: rs9934438. Genotyping was performed using a Taqman allelic discrimination assay as previously described. To confirm the accuracy of genotyping results, 315 (5%) randomly selected samples were regenotyped with the same method. No inconsistencies were observed.

**Outcome**

In the present study, we used the measurements of calcified deposits in the aortic far wall taken at the baseline visits between 1990 and 1993. Aortic calcification was diagnosed by radiographic detection of calcified deposits in the abdominal aorta as described elsewhere. The interobserver agreement for absence versus presence of atherosclerotic plaques was 0.88, and the k statistic was 0.74. We defined moderate and severe extent of calcification with an area of the posterior aortic wall involved >2.5 cm as the outcome of interest and used persons with absent aortic calcification as the reference group.

Radiography of the aortic far wall only measured calcified plaques and could not detect plaques without calcification. To check whether the VKORC1 1173C>T allele was associated with calcification independently from existing plaques, we performed further analyses with the data available on carotid plaques. With the ultrasonography used for the carotid measurements, it was possible to detect plaques composed of calcified as well as of noncalcified components. In the Rotterdam Study from 1990 until October 1991, with ultrasonography calcified and noncalcified plaques were assessed separately in the carotid artery as described elsewhere. Assessment of calcified plaques was available from 3 locations in the carotid artery.

**Cofactors**

We adjusted for cardiovascular risk factors for atherosclerosis such as age, gender, present smoking, hypertension, hypercholesterolemia, and diabetes mellitus. We further adjusted for nutritional vitamin K intake, separately for vitamin K1 and vitamin K2. The daily intake of these 2 forms of vitamin K was determined using a food frequency questionnaire.

**Statistical Analyses**

Allele and genotype proportions were tested for deviations from Hardy-Weinberg equilibrium by a χ² test. For the 1173C>T SNP 3 different genotypes were present: CC, CT, and TT. With multivariable logistic regression analysis, adjusted for age and gender, we studied the association between the T allele and aortic calcification with an allele-dose-effect model (number of T-alleles with no T-alleles as a reference), genotype-effect model (genotype CT and TT separately with CC as a reference), a recessive model (TT versus CT plus CC), and with a dominant model (TT plus CT versus CC). We compared the risk of calcification of the aortic far wall to no detectable form of calcification as reference group. For each analysis an odds ratio (OR) and a 95% confidence interval (CI) was computed. Analyses were repeated for severe aortic calcification (area of plaque involved ≥5 cm) compared to no detectable form of calcification in the aortic far wall.

In multivariable logistic regression analyses we studied the association between the T-allele and calcification, adjusting for gender and age. Subsequently, we tested each risk factor for aortic calcifi-

**Methods**

**Setting**

Data were obtained from the Rotterdam Study, a prospective population-based cohort study, designed to study neurological, cardiovascular, locomotor, and ophthalmologic diseases. The rationale and design of this study have been described elsewhere.

Participants were visited at home for a standardized questionnaire and were subsequently examined at the research center. At baseline, a diminished functionality of the VKORC1 enzyme is therefore not likely to influence coagulation factors and hemostasis in persons with normal vitamin K1 intake and not using coumarins. A lifelong decreased activity of the VKORC1 enzyme, however, might impair MGP activity and by this increase the risk of vascular calcification. This could be further worsened by reduced intake of vitamin K2. The association between impaired carboxylation of MGP and intimal and medial vascular calcification in humans has been described before.

Calcification of the aortic far wall has shown to be a good indicator of vascular calcification. This implicates different effects of VKORC1 activity and of vitamin K1 and K2 intake on coagulation factors as hepatic proteins and on extrahepatic proteins such as MGP.

A vitamin K–dependent protein is activated in the presence of vitamin K. Coagulation and calcification processes need vitamin K–dependent proteins. Given the importance of vitamin K1 for normal function of VKORC1, we hypothesized that it is possible that the effects of VKORC1 variation on aortic calcification might be mediated by vitamin K1 and K2 intake.

**Cofactors**

We studied the association between VKORC1 polymorphism and aortic calcification. Genomic DNA was extracted from samples of peripheral venous blood according to standard procedures. 1 to 2 ng genomic DNA was dispensed into 384-wells plates using a Caliper Sciclone ALH3000 pipetting robot (Caliper LS). we chose the 1173C>T SNP at intron 1, dbSNP: rs9934438. Genotyping was performed using a Taqman allelic discrimination assay as previously described. To confirm the accuracy of genotyping results, 315 (5%) randomly selected samples were regenotyped with the same method. No inconsistencies were observed.

In the present study, we used the measurements of calcified deposits in the aortic far wall taken at the baseline visits between 1990 and 1993. Aortic calcification was diagnosed by radiographic detection of calcified deposits in the abdominal aorta as described elsewhere. The interobserver agreement for absence versus presence of atherosclerotic plaques was 0.88, and the k statistic was 0.74. We defined moderate and severe extent of calcification with an area of the posterior aortic wall involved >2.5 cm as the outcome of interest and used persons with absent aortic calcification as the reference group.

Radiography of the aortic far wall only measured calcified plaques and could not detect plaques without calcification. To check whether the VKORC1 1173C>T allele was associated with calcification independently from existing plaques, we performed further analyses with the data available on carotid plaques. With the ultrasonography used for the carotid measurements, it was possible to detect plaques composed of calcified as well as of noncalcified components. In the Rotterdam Study from 1990 until October 1991, with ultrasonography calcified and noncalcified plaques were assessed separately in the carotid artery as described elsewhere. Assessment of calcified plaques was available from 3 locations in the carotid artery.

**Cofactors**

We adjusted for cardiovascular risk factors for atherosclerosis such as age, gender, present smoking, hypertension, hypercholesterolemia, and diabetes mellitus. We further adjusted for nutritional vitamin K intake, separately for vitamin K1 and vitamin K2. The daily intake of these 2 forms of vitamin K was determined using a food frequency questionnaire.

**Statistical Analyses**

Allele and genotype proportions were tested for deviations from Hardy-Weinberg equilibrium by a χ² test. For the 1173C>T SNP 3 different genotypes were present: CC, CT, and TT. With multivariable logistic regression analysis, adjusted for age and gender, we studied the association between the T allele and aortic calcification with an allele-dose-effect model (number of T-alleles with no T-alleles as a reference), genotype-effect model (genotype CT and TT separately with CC as a reference), a recessive model (TT versus CT plus CC), and with a dominant model (TT plus CT versus CC). We compared the risk of calcification of the aortic far wall to no detectable form of calcification as reference group. For each analysis an odds ratio (OR) and a 95% confidence interval (CI) was computed. Analyses were repeated for severe aortic calcification (area of plaque involved ≥5 cm) compared to no detectable form of calcification in the aortic far wall.

In multivariable logistic regression analyses we studied the association between the T-allele and calcification, adjusting for gender and age. Subsequently, we tested each risk factor for aortic calcifi-
of aortic calcification and were excluded. Within the remaining 6161 patients, calcification of the aortic far wall was measured in 5123 persons during the first visit to the study center between 1990 and 1993. Measurements could not be evaluated for 123 persons, leaving 5000 persons. For 1667 persons no aortic calcification could be detected. In 1813 out of 3333 persons with plaques, the area of the aorta involved in the detected plaques was smaller than 2.5 cm whereas in the remaining 1520 persons the area of plaques involved a length of at least 2.5 cm. In the analyses we categorized persons without any detectable aortic calcification as calcification absent and those with plaques above 2.5 cm as calcification present. In total, our study population consisted of 3187 persons.

Characteristics of the study population are given in Table 1. Genotype proportions were similar to those in the whole population genotyped, and the population was in Hardy-Weinberg equilibrium ($P=0.58$). Within persons with aortic calcification the numbers of the VKORC1 CT genotype were higher than in persons without detectable calcification (Table 2). In the study population, the frequency of the T-1173 allele of the VKORC1 polymorphism was 38.8%. Persons with aortic calcification were found to have a higher T-allele frequency than persons with no detectable aortic calcification (39.8% versus 37.9%). From the models tested, only in the dominant model, persons with at least one T-allele had a statistically significant 1.19-increased risk (95% CI 1.02–1.40) for calcification of the aortic far wall compared to persons with the wild-type genotype of VKORC1 (Table 3). Systolic blood pressure, diabetes, present smoking, total serum cholesterol, BMI, and vitamin K1 and vitamin K2 intake did not change the point estimate by more than 10%, and for these variables no effect modification was observed for the association of the T-allele with aortic calcification. When adjusting for all these variables, the estimate for the T-allele slightly increased (1.21, 95% CI 1.02–1.43). Restricting the outcome to aortic calcification with an area of the posterior wall involved ≥5 cm instead of 2.5 cm, the risk in carriers of a T-allele was increased to 1.27 (95% CI 1.02–1.58).

Within the study population, there were 1084 persons with measurements of carotid calcification, 471 had no calcification detectable in the carotid artery and 75 persons had 4 to 6 calcified plaques in the carotid artery compared to no calcification detectable, adjusted for age and gender. To evaluate the independent risk estimate of the T-allele with the presence of 4 to 6 calcified plaques in the carotid artery, we further checked for multiplicative interactions between the T-allele and each other variable. When adjusting for missing values in present smoking, diabetes, and body mass index, the estimate for the T-allele slightly increased (1.21, 95% CI 1.02–1.43). Restricting the outcome to aortic calcification with an area of the posterior wall involved ≥5 cm instead of 2.5 cm, the risk in carriers of a T-allele was increased to 1.27 (95% CI 1.02–1.58).

Results
For 6547 of the 7983 persons in the Rotterdam Study a blood sample was available for genotyping of VKORC1 1173C>T. For 153 persons (2%) genotyping failed, leaving 6394 persons with a genotype assessed. During the study period, 233 of the 6394 persons had used coumarins before measurement
Calcification in the media is very diffuse and was not detectable by radiography or ultrasonography. Radiography, which was used to measure the calcification in the aorta, could not detect noncalcified plaques. Sonography, however, could distinguish calcified plaques in the intima from plaques consisting of lipids or polysaccharides only. Such measurements were taken in our study population in the carotid artery. Repeating the analyses for carotid plaques in the whole population doubled the number of persons with available carotid measurements (884 with no carotid plaques detectable and 140 persons with 4 to 6 calcified carotid plaques) but did not change our results. A previous study within the Rotterdam Study had detected graded associations between the T-allele and aortic calcification in our study population. persons with aortic calcification were not associated with a risk of 4 to 6 calcified plaques compared to no calcified plaques detectable (OR = 0.68, 95% CI 0.44–1.07). When including the numbers of calcified plaques and noncalcified plaques together in analyses to evaluate the independent risk estimate of calcification given the total number of plaques, also no association was found for the T-allele with 4 to 6 calcified carotid plaques compared to no calcified plaques (OR = 0.60, 95% CI 0.13–2.91).

### Discussion

In our study, the presence of the T-allele of the VKORC1 1173C>T was associated with a small but significantly increased risk of aortic calcification. To our knowledge this is the first study analyzing the association between the T-allele and aortic calcification. Persons with a T-allele have a lifelong reduced activity of VKORC1. Effects from this are expected in extrahepatic proteins such as MGP because γ-carboxylation here fully depends on VKORC.1 The estimated risk of aortic calcification for persons with the CT genotype (1 copy of the risk allele T) was higher than for those with the CC genotype (no risk allele). The risk of aortic calcification for homozygous persons with TT compared to CC individuals was not significantly increased, possibly because this group was much smaller with a less precise estimate and a wider 95% confidence interval. However, the point estimate for CT individuals was within the adjusted confidence interval of the TT individuals. This may be in line with a dominant effect of the T-allele, as already stated in another study.7 Restricting the outcome to aortic calcification with an area ≥5 cm² involved and its lower chance of misclassification, this was associated with a significant risk increase of 27% in the dominant model. So far as we know, there are few similar studies but with conflicting results.7,24

Dietary vitamin K2 intake could have modified the association between the T-allele and aortic calcification. Previously, menopausal women in the Rotterdam Study with low dietary vitamin K intake had an increased risk of aortic calcification. In our study population, persons with aortic calcification had a significantly lower daily intake of vitamin K2 than persons without detectable aortic calcification (27.3 mcg/d versus 29.5 mcg/d). However, there was no difference in dietary vitamin K intake in persons with or without a T-allele and further no interaction between a T-allele and dietary vitamin K2 intake was found. We also did not detect any confounding or multiplicative effect modification on this association by any of the known cardiovascular risk factors.

It is unlikely that our results can be explained by bias or confounding. Selection bias in this population-based setting is improbable, especially as the population was in Hardy-Weinberg equilibrium. In our study population we found a 38.9% allele frequency of the risk allele. The frequency of the T-allele in Whites has been reported so far between 39.8% and 42%,4,6,10,11 The absence of significant deviation from the HWE is also an indicator of good SNP genotyping quality in our population. As our data were collected prospectively and independently from our research hypothesis, information bias is also unlikely. We adjusted for known cardiovascular risk factors as potential confounders, and these furthermore did not substantially change our risk estimates.

It is however possible that plaques already present in vessels might promote calcification and thus form an independent risk factor for aortic calcification. Vascular calcification can occur in the intima, always in the context of atherosclerosis, and in the media (Mönckeberg's sclerosis) where it is independent of atherosclerosis and almost exclusively associated with vascular smooth muscle cells.18,27 Calcification in the media is very diffuse and was not detectable by radiography or ultrasonography. Radiography, which was used to measure the calcification in the aorta, could not detect noncalcified plaques. Sonography, however, could distinguish calcified plaques in the intima from plaques consisting of lipids or polysaccharides only. Such measurements were taken in our study population in the carotid artery during 1990 and October 1991. In contrast to the association between the T-allele and aortic calcification in our study population, the T-allele was not associated with an independent higher risk of 4 to 6 calcified plaques in the carotid artery. Repeating the analyses for carotid plaques in the whole population doubled the number of persons with available carotid measurements (884 with no carotid plaques detectable and 140 persons with 4 to 6 calcified carotid plaques) but did not change our results. A previous study within the Rotterdam Study had detected graded associations for coronary calcification with aortic calcification as well as with calcified carotid plaques.28 In our study we found a high predictive value of calcified carotid plaques on aortic calcification (OR = 3.05, 95% CI 2.61–3.56). However, cooccurrence of calcification in both vessels was quite low (Cohen's kappa = 0.41), and this may explain why the association with aortic calcification was not seen in carotid arteries. Thus we

### Table 3. Association Between the VKORC1 1173C>T Genotypes and the Risk of Mild, Moderate and Severe Aortic Calcification

<table>
<thead>
<tr>
<th>Allele-dose-effect model</th>
<th>No. of T-alleles with 'no T-alleles' as a reference</th>
<th>Basic Model†</th>
<th>Adjusted Model†</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. n=3187 (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype effect model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>1185 (37.2)</td>
<td>1 (reference)</td>
<td>1 (reference)</td>
</tr>
<tr>
<td>CT</td>
<td>1529 (48.0)</td>
<td>1.25 (1.05–1.47)</td>
<td>1.26 (1.06–1.51)</td>
</tr>
<tr>
<td>TT</td>
<td>473 (14.8)</td>
<td>1.04 (0.82–1.32)</td>
<td>1.05 (0.82–1.35)</td>
</tr>
</tbody>
</table>

| Recessive model         |                                               |             |                |
| CC or CT               | 2714 (85.2)                                  | 1 (reference)| 1 (reference)  |
| TT                     | 473 (14.8)                                   | 0.92 (0.74–1.14)| 0.92 (0.74–1.16)|

| Dominant model          |                                               |             |                |
| CC                     | 1185 (37.2)                                  | 1 (reference)| 1 (reference)  |
| CT or TT               | 2002 (63.5)                                  | 1.19 (1.02–1.40)| 1.21 (1.02–1.43)|

No. n=3187 (100) 0.92 (0.74–1.14) 0.92 (0.74–1.16)

OR (95% CI)

Basic Model†

Adjusted Model†

**Note:** Adjusted for age and gender; †adjusted for age, gender, total serum cholesterol, systolic blood pressure, current smoking, diabetes mellitus, BMI, and vitamin K1 and vitamin K2 intake.
could not verify our hypothesis that the VKORC1 1173C>T polymorphism increases the risk of calcification independently from the number of preexisting plaques. Yet this seems plausible, as in the animal experiment vascular calcification occurred in newborn rats which were unlikely to suffer from plaques already present.17

Our results may be important as vascular calcification is regarded as one of the major complications of cardiovascular disease.36 It is now appreciated that the development of the atherosclerotic lesion ultimately causes plaque erosion and leads to rupture and to thrombus formation as one of the final events in atherosclerosis.18,37 On the other hand, in vitro studies for the impact of calcification on plaque stability showed protective rather than destabilizing influence on the atheromas.38 This was in contrast to lipid pools that dramatically destabilized the plaques. Whether intimal calcification stabilizes atherosclerotic plaques or promotes its rupture is therefore still a matter of debate.39

Acknowledgments
We thank Martin van Vliet for genotyping of VKORC1 in the ERGO population and Frank van Rooij for helping us with the vascular data.

Disclosures
None.

References
39. Spronk HM. Vitamin K epoxide reductase complex and vascular calcification: is this the important link between vitamin K and the arterial vessel wall? Circulation. 2006;113:1550–1552.
Vitamin K Epoxide Reductase Complex Subunit 1 (VKORC1) Polymorphism and Aortic Calcification: The Rotterdam Study

Arterioscler Thromb Vasc Biol. 2008;28:771-776; originally published online January 24, 2008;
doi: 10.1161/ATVBAHA.107.159913
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/28/4/771

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/