

# Arteriosclerosis, Thrombosis, and Vascular Biology



JOURNAL OF THE AMERICAN HEART ASSOCIATION

## Sequence Variation in Vitamin K Epoxide Reductase Gene Is Associated With Survival and Progressive Coronary Calcification in Chronic Kidney Disease

Rachel M. Holden, Sarah L. Booth, Angie Tuttle, Paula D. James, Alexander R. Morton, Wilma M. Hopman, Robert Louis Nolan and Jocelyn S. Garland

*Arterioscler Thromb Vasc Biol.* published online May 22, 2014;  
*Arteriosclerosis, Thrombosis, and Vascular Biology* is published by the American Heart Association, 7272  
Greenville Avenue, Dallas, TX 75231

Copyright © 2014 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/early/2014/05/22/ATVBAHA.114.303211>

Data Supplement (unedited) at:

<http://atvb.ahajournals.org/content/suppl/2014/05/22/ATVBAHA.114.303211.DC1.html>

**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/>

# Sequence Variation in Vitamin K Epoxide Reductase Gene Is Associated With Survival and Progressive Coronary Calcification in Chronic Kidney Disease

Rachel M. Holden, Sarah L. Booth, Angie Tuttle, Paula D. James, Alexander R. Morton, Wilma M. Hopman, Robert Louis Nolan, Jocelyn S. Garland

**Objective**—Sequence variations in the gene(s) encoding vitamin K epoxide reductase complex subunit 1 (VKORC1), the enzyme target of warfarin, have been associated with increased cardiovascular disease in the general population. Coronary artery calcification (CAC) is a prevalent form of cardiovascular disease in chronic kidney disease. We tested the hypothesis that the VKORC1 rs8050894 CC genotype would be associated with mortality and progression of CAC  $\leq 4$  years.

**Approach and Results**—This study is an observational, prospective study of 167 individuals with stages 3 to 5 chronic kidney disease. Survival  $\leq 4$  years was assessed in all participants, and CAC progression was measured in a subset of 86 patients. Participants with the CG/GG genotype of VKORC1 had higher baseline CAC scores (median score, 112 versus 299;  $P=0.036$ ). Of those 86 patients who had a 4-year CAC score, those with the CG/GG genotype had an increased risk of progressive CAC (adjusted for age, diabetes mellitus, estimated glomerular filtration rate, and hypertension) compared with those with the CC genotype. Four-year mortality risk was 4 times higher for individuals with the CG/GG genotypes compared with individuals with the CC genotype (odds ratio, 3.8; 95% confidence interval, 1.2–12.5;  $P=0.02$ ), adjusted for age, sex, diabetes mellitus, estimated glomerular filtration rate, baseline CAC, and hypertension.

**Conclusions**—Patients with the CG/GG genotype of VKORC1 had a higher risk of CAC progression and a poorer survival. These data provide new perspectives on the potential extrahepatic role of VKORC1 in individuals with chronic kidney disease. (*Arterioscler Thromb Vasc Biol.* 2014;34:00-00.)

**Key Words:** mortality ■ renal insufficiency, chronic ■ vitamin K ■ vitamin K epoxide reductases

Cardiovascular disease is the leading cause of morbidity and mortality in patients with chronic kidney disease (CKD) and is attributable, in part, to atherosclerosis and vascular calcification. Platelet adhesion and thrombosis are integral to the development of atherosclerosis in humans. It is established that vitamin K-dependent proteins (VKDPs) play a critical role in the activation of the coagulation cascade. Some studies suggest that sequence variations in the gene encoding the enzyme vitamin K epoxide reductase complex subunit 1 (VKORC1) serve as novel genetic risk markers for the occurrence of cardiovascular and thrombotic events.<sup>1–3</sup> This effect is presumed to be based on VKORC1-dependent effects on enhancing the levels of carboxylated coagulation proteins that promote thrombosis.

Involvement of VKDPs in the regulation of extrasosseous calcification in humans is receiving increasing recognition. Matrix Gla protein, a VKDP that is synthesized by the vascular wall and cartilage, functions as an inhibitor of calcification and may be integral to the regulation of extrasosseous calcification in humans.<sup>4,5</sup> Calcification may occur in the intima of

the artery wall in association with atherosclerosis or within the media layer. Both forms occur in patients with CKD and contribute to the elevated risk of cardiovascular morbidity and mortality observed in this patient group.<sup>6</sup>

The calcium-binding activity of VKDPs is dependent on vitamin K-mediated  $\gamma$ -carboxylation of specific Glu residues.<sup>7</sup> The enzyme VKORC1 mediates the cyclic interconversion of vitamin K metabolites and is a rate-limiting step in the  $\gamma$ -carboxylation of VKDPs in the liver.<sup>8</sup> VKORC1, the target of warfarin, specifically recycles the vitamin K 2,3-epoxide to the vitamin K hydroquinone, and common polymorphisms in VKORC1 explain the clinically observed differences in warfarin dose requirements between various ethnic groups.<sup>9–12</sup> In experimental CKD, inhibiting the actions of VKORC1 with a therapeutic dosage warfarin markedly enhanced vascular calcification in rats with CKD.<sup>13</sup> However, human studies that have evaluated whether warfarin treatment accelerates cardiovascular disease and calcification are inconsistent.<sup>14–19</sup> New evidence suggests that VKDPs expressed in some extrahepatic tissues may be resistant to the effects of vitamin K antagonists

Received on: January 8, 2014; final version accepted on: May 7, 2014.

From the Medicine (R.M.H., A.T., P.D.J., A.R.M., J.S.G.), Community Health and Epidemiology, and Radiology (R.L.N.), Queen's University, Kingston, Ontario, Canada; Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, Boston, MA (S.L.B.); and Clinical Research Centre, Kingston General Hospital, Kingston, Ontario, Canada (W.M.H.).

The online-only Data Supplement is available with this article at <http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.114.303211/-/DC1>.

Correspondence to Rachel M. Holden, MD, 3048C Etherington Hall, Queen's University, Kingston, Ontario K7L 3V6, Canada. E-mail holdenr@kgh.kari.net

© 2014 American Heart Association, Inc.

*Arterioscler Thromb Vasc Biol* is available at <http://atvb.ahajournals.org>

DOI: 10.1161/ATVBAHA.114.303211

## Nonstandard Abbreviations and Acronyms

<b>AU</b>	Agatston unit
<b>CAC</b>	coronary artery calcification
<b>FVII</b>	factor VII
<b>SNP</b>	single-nucleotide polymorphism
<b>VKDP</b>	vitamin K–dependent protein
<b>VKORC1</b>	vitamin K epoxide reductase complex subunit 1

because of the recently identified VKORC1L1, which can still recycle vitamin K in the presence of warfarin.<sup>20</sup> These data suggest tissue-specific VKOR activity.

Sequencing efforts by Rieder et al<sup>10</sup> have yielded 5 common haplotypes of VKORC1 that can be grouped into 2 evolutionarily distant groups called A and B. The A haplotype group is associated with attenuated expression of VKORC1 mRNA in the liver and, as a consequence, lower warfarin requirements.<sup>10</sup> Conversely, the B haplotype group is associated with higher expression of VKORC1 mRNA in the liver and higher warfarin requirements. The focus of this study is the single-nucleotide polymorphism (SNP) *rs8050894*, which has been demonstrated to be in high linkage equilibrium and enables the distinction between these 2 groups.<sup>21</sup> To date, no study has examined the impact of sequence variations of VKORC1 in a CKD population.

In this prospective cohort study, performed in a sample of individuals with CKD, we investigated the impact of genetic variations in VKORC1 on survival  $\leq 4$  years in a cohort of 167 predialysis stage 3 to 5 CKD patients. In a subset of 86 patients who had follow-up coronary artery calcification (CAC) quantified 4 years later, we examined the associations of sequence variations of VKORC1 on CAC progression. Our hypothesis was that the CC genotype that is associated with decreased warfarin requirements is a nonmodifiable risk factor for CAC and its progression and mortality in patients with CKD are based on low lifetime activity of the vitamin K cycle.

## Materials and Methods

Materials and Methods are available in the online-only Supplement.

## Results

The distribution of VKORC1 genotypes was as follows: CC,  $n=66$  (39.5%); CG,  $n=81$  (48.5%), and GG:  $n=20$  (12.0%). The allele frequencies were similar to the allele frequencies reported by Crosier et al<sup>22</sup> in individuals aged  $>60$  years in the United States who were also primarily of European descent. Ninety-eight percent of the enrolled subjects were white.

All comparisons were made between the CC and the CG/GG groups (Table 1). Patients in the CG/GG group were older (62.4 [13.2] versus 57.9 [15.2];  $P=0.05$ ). There were no statistically significant differences between groups with respect to sex, diabetes mellitus, estimated glomerular filtration rate, or biomarkers of vitamin K status. Low phyloquinone concentrations consistent with subclinical vitamin K deficiency (normal range, 0.4–2.65 nmol/L) were identified in 10 patients (6.0%), whereas elevated proteins induced by vitamin K absence or antagonism-II (normal range  $<2$  nmol/L) were

identified in 97% of patients. Carriers of the CC genotype had lower alkaline phosphatase but no other significant differences with respect to other parameters of mineral metabolism.

## Coronary Artery Calcification

In 2005, at baseline, 86.8% of participants had evidence of CAC: 13.2% had no evidence of calcification, 14.4% had minimal calcification (score, 1–9.9), 16.8% had mild calcification (score, 10–99.9), 19.2% had moderate calcification (score, 100–399.9), and 36.5% had severe calcification (score  $>400$ ). Patients with the CC genotype were slightly younger than patients with the CG/GG genotype, but did not differ in other cardiovascular disease risk factors. However, patients possessing the CC genotype had significantly less (2-fold) baseline CAC severity versus CG/GG (Table 1).

We wished to explore the impact of sequence variations in VKORC1 on CAC progression during 4 years of follow-up. Table 2 demonstrates the adjusted multivariable model describing the risk factors for CAC progression, where CAC progression was defined as an increase in CAC score of  $>50$  Agatston units (AU). The model was adjusted for baseline CAC score, which was higher in the CG/GG genotype groups (Table 1). Accounting for baseline CAC and VKORC1 genotype, individuals with the CG/GG genotypes were 4 times more likely to have CAC progression versus the CC genotype (model 1; also adjusted for age, hypertension, estimated glomerular filtration rate, and diabetes mellitus status). There was a significant interaction between VKORC1 genotype group and baseline CAC, indicating that the risk of progressive CAC within the CG/GG group was higher in those with greater baseline CAC. To ensure consistency in our results indicating an increased risk of CAC progression ascribed to the CG/GG genotype, we examined the impact of VKORC1 genotype on CAC progression in several ways. Our findings remained robust using alternative definitions for progressive CAC as the dependent variable: progressive CAC of  $>1$  AU (12.3; 95% confidence interval, 2.9–52.3;  $P=0.002$ ), progressive CAC of  $>10$  AU (3.6; 95% confidence interval, 0.94–13.7;  $P=0.06$ ), or progressive CAC of  $>100$  AU (4.3; 95% confidence interval, 0.97–19.22;  $P=0.05$ ; data not shown).

## Mortality

During a follow-up of  $\leq 4$  years, there were 27 deaths. Individuals with the CC genotype were less likely to die (6.1%) than those with CG/GG genotypes (22.8%;  $P=0.005$ ). The Figure presents the Kaplan–Meier curve for mortality in accordance to VKORC1 genotypes, demonstrating an increased risk of death for individuals with the CG/GG genotype. In the adjusted Cox proportional model presented in Table 3, individuals with VKORC1 CG/GG genotypes were 4 times more likely to have died compared with those with the CC genotype (hazard ratio, 4.1; 95% confidence interval, 1.24–13.58;  $P=0.02$ ), accounting for age and baseline burden of CAC.

Based on the increased risk of death associated with the VKORC1 genotype, we measured levels of the vitamin K–dependent coagulation factors and compared these in carriers of the CC versus the CG/GG genotype. The only

**Table 1. Baseline Characteristics, Laboratory Measures, Calcification, and Vitamin K and D Status According to VKORC1**

Variables	VKORC1 CC (n=66)	VKORC1 CG or GG (n=101)	P Value
Clinical characteristics (n=167)			
Age, y	57.9 (15.2)	62.4 (13.2)	0.05
Sex (male)	45 (68%)	56 (55%)	0.11
Diabetes mellitus	25 (38%)	47 (47%)	0.34
Hypertension	61 (92%)	101 (93%)	1.0
Urine ACR >3 (n=163)	51 (79%)	80 (82%)	0.69
Cardiovascular disease	8 (12%)	21 (21%)	0.21
Peripheral vascular disease	5 (8%)	14 (14%)	0.32
Smoking	43 (65%)	62 (61%)	0.7
Body mass index, kg/m <sup>2</sup>	31.0 (6.8)	31.7 (8.4)	0.6
Laboratory measures			
Phosphate, mmol/L	1.31 (0.3)	1.31 (0.3)	0.5
Total calcium, mmol/L (n=166)	2.33 (0.2)	2.34 (0.2)	0.8
Alkaline phosphatase, mmol/L (n=164)	92.2 (34.5)	80.3 (25.3)	0.01
Parathyroid hormone, pmol/L	9.7 (4.9–21.1)	8.9 (5.4–17.2)	0.6
Albumin, g/L	40.9 (4.9)	40.8 (4.9)	0.9
Total cholesterol, mmol/L	4.52 (1.20)	4.65 (1.47)	0.55
LDL cholesterol, mmol/L (n=162)	2.23 (0.97)	2.28 (0.93)	0.72
HDL cholesterol, mmol/L (n=166)	1.22 (0.43)	1.33 (0.40)	0.09
Triglycerides, mmol/L	2.22 (1.23)	2.37 (1.30)	0.45
C-reactive protein, mg/L (n=161)	3.0 (1.2–7.7)	3.5 (1.5–6.2)	0.6
Urinary ACR (n=180)	36 (5.3–94.8)	42 (8.1–161.7)	0.3
eGFR	29.5 (18.5)	27.1 (14.5)	0.3
Calcification measure			
Median (IQR) baseline CAC score	112 (1.8–495)	299 (13–975)	0.036
Vitamin K and D status			
Phylloquinone, nmol/L (n=160)	1.2 (0.6–2.5)	1.4 (0.7–3.0)	0.4
PIVKA-II, nmol/L (n=102)	3.50 (1.16)	3.68 (1.62)	0.6
%ucOC (n=163)	25.8 (21.1)	26.7 (22.3)	0.8
25(OH)D, nmol/L (n=161)	65.0 (23.9)	70.5 (28.2)	0.2

P values are based on the  $\chi^2$  tests (Pearson or Fisher exact as appropriate) and the independent samples *t* test (or Mann–Whitney in the event of non-normal distributions). %ucOC indicates percentage uncarboxylated osteocalcin; 25(OH)D, 25-hydroxy vitamin D; ACR, albumin:creatinine ratio; CAC, coronary artery calcification; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; IQR, interquartile range; LDL, low-density lipoprotein; PIVKA, prothrombin induced by vitamin K absence; and VKORC1, vitamin K epoxide reductase complex subunit 1.

coagulation factor to differ by VKORC1 was factor VII (FVII). Levels of FVII were significantly higher in carriers of the CG/GG genotype (Table 4).

## Discussion

The main finding of this study is the identification that individuals with stage 3 to 5 CKD who have 1 SNP of VKORC1 (CG/GG genotype) have an increased burden of baseline CAC, an increased risk of CAC progression, and death in  $\leq 4$  years of follow-up. The CC genotype (A haplotype) group was associated with lower unadjusted median CAC scores at baseline and less CAC progression over time, suggesting 1 plausible mechanistic link between VKORC1 variation and mortality in patients with CKD. However, VKORC1 is thought to mediate the activity of  $\gamma$ -carboxylase, and enzymatic activity would be

predicted to be lowest in the CC group. Therefore, the reduced risk of CAC and mortality may not relate to the activity of VKDPs that are involved in the inhibition of vascular calcification. Indeed, we found higher levels of fFVII activity in carriers of the CG/GG genotype, suggesting that links between cardiovascular disease and VKORC1 may be primarily mediated via prothrombotic, proatherosclerotic pathways.

The literature linking sequence variations in VKORC1 with cardiovascular disease is inconsistent, and therefore, at present, no clear link exists between VKORC1 and arterial disease. Although there is variation in the reference allele studied, the results of this study are in agreement with 2 other studies in the general population that have examined variations of VKORC1 as a candidate risk factor for arterial disease.<sup>1,2</sup> Taken together, the genotype associated with higher



**Table 2. VKORC1 CG/GG Genotype Increases the Risk of Coronary Calcification Progression (Logistic Regression; Model 1)**

Model 1: (n=86) CAC Progression—Increase in CAC Score >50 Agatston Units			
	$R^2=0.31$ ; $P<0.001$		
	Odds Ratio	95% Confidence Interval	P Value
VKORC1 CG/GG	4.0	1.0–15.5	0.04
VKORC1 $\times$ log baseline CAC	2.12	1.35–3.35	0.001
Diabetes mellitus	3.2	1.1–9.5	0.04
Age per decade	1.1	0.71–1.77	0.62
$\downarrow$ eGFR by 10 mL/min	0.82	0.53–1.3	0.4
Hypertension	0.46	0.48	0.48

CAC indicates coronary artery calcification; eGFR, estimated glomerular filtration rate; and VKORC1, vitamin K epoxide reductase complex subunit 1.

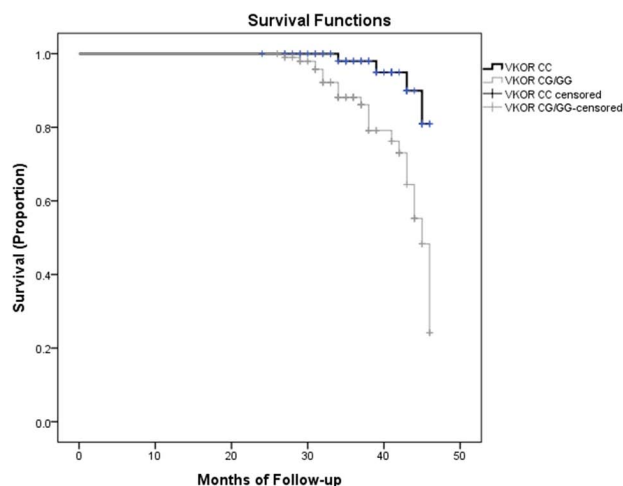
expression of VKOR mRNA in the liver has been linked, in these observational studies, to increased cardiovascular events and increased severity of CAC. The current study further links this genotype with progression of CAC and mortality but specifically in a sample of patients with reduced kidney function.

Three other studies in different ethnic groups have not shown any relationship between sequence variation in VKORC1 and risk of cardiovascular disease.<sup>23–25</sup> None of the 3 VKORC1 SNPs studied were significantly associated with any of the outcomes. These studies suggest that the impact of VKORC1 genotype and cardiovascular disease is not certain. Only 1 study has linked the T-allele of the 1173C>T SNP, which is associated with reduced activity of VKORC1, with a small, but significant, increased risk of aortic calcification measured by plain radiography.<sup>26</sup>

We hypothesized that the potential for enhanced  $\gamma$ -carboxylation of VKDPs associated with the B haplotype would theoretically translate to greater activity of VKDPs that inhibit calcification (eg, MGP). However, our data from a small group of patients with CKD do not support this hypothesis. Our observations may reflect VKORC1-mediated enhancement of those VKDPs involved in the coagulation cascade and atherosclerosis, such that a higher activity of VKORC1 could simultaneously increase the activity

of vitamin K–dependent procoagulant factors such as FII, FVII, FIX, and FX.<sup>3</sup> That is, if VKORC1 modified all vitamin K–dependent activities, the final outcome regarding the balance between procoagulant and anticalcification factors might be unpredictable. Indeed, new evidence supports a key role for VKORC1L1, an enzyme that can rescue VKOR activity in some extrahepatic tissues during anticoagulation therapy, suggesting that there could be important tissue-specific differences in the activity of VKORC1 that could help explain our findings.<sup>20</sup> To address the limitations of previous studies, we also measured the activity of vitamin K–dependent coagulation factors and found higher levels of fFVII activity in CG/GG carriers. Clinically, high levels of fFVII have been linked to carotid thickness<sup>27</sup> and risk of cardiovascular events in some<sup>28–30</sup> but not all studies.<sup>31</sup> Enhanced functional activity of vitamin K–dependent coagulation proteins could potentially facilitate atherosclerotic plaque progression. However, it is uncertain whether the higher levels of fFVII activity in these patients indicate increased carboxylation of this VKDP or simply reflect enhanced expression. A CAC score provides a quantitative assessment of coronary calcification, but it does not distinguish between calcium deposits which are localized to the intima (eg, associated with atherosclerosis) versus those that are in the medial layer. We previously identified in this cohort of patients with CKD that traditional Framingham risk factors for atherosclerosis were better predictors of the overall CAC score than nontraditional risk factors linked to kidney disease such as severity of kidney failure and abnormalities in mineral metabolism.<sup>32</sup> It is therefore unknown whether the increase in CAC score in this cohort is attributable to intimal progression related to atherosclerosis, medial progression primarily related to calcification, or a combination of both, and as such, we cannot draw any definitive conclusions.

Most previous studies evaluating the association between sequence variations in VKORC1 and vascular disease have not included measures of vitamin K status or activity of coagulation factors in the analyses. Whereas no significant heritability was reported in 2 biomarkers of vitamin K status in a cross-sectional study of almost 2000 subjects,<sup>33</sup> the minor allele G of the identical SNP was associated with higher concentrations



**Figure.** Kaplan–Meier survival curve for all-cause mortality. VKOR indicates vitamin K epoxide reductase.

**Table 3. VKORC1 CG/GG Genotype Increases the Risk of Death (Cox Proportional Hazards Model) During 4 Years of Follow-Up (Model 2)**

Model 2: (n=167) Mortality During 4 y of Follow-Up			
	$R^2=0.25$ ; $P<0.001$		
	Hazard Ratio	95% Confidence Interval	P Value
VKORC1 CG/GG	4.10	1.24–13.58	0.02
Age per decade	2.01	1.21–3.34	0.007
Hypertension	0.15	0.33–0.71	0.02
Diabetes mellitus status (no/yes)	1.14	0.44–2.96	0.80
Log baseline CAC score	1.20	0.74–1.95	0.46
$\uparrow$ eGFR by 10 mL/min	0.72	0.48–1.09	0.12

CAC indicates coronary artery calcification; eGFR, estimated glomerular filtration rate; and VKORC1, vitamin K epoxide reductase complex subunit 1.

**Table 4. Levels of Vitamin K-Dependent Coagulation Factors According to VKORC1**

Vitamin K-Dependent Coagulation Factors	VKORC1 CC (n=32)	VKORC1 CG or GG (n=61)	P Value
Factor II	0.96 (0.2)	1.00 (0.1)	0.2
Factor VII	1.50 (0.5)	1.71 (0.4)	0.03
Factor IX	1.56 (0.5)	1.57 (0.3)	0.9
Factor X	1.09 (0.2)	1.1 (0.3)	0.9
Protein S	1.14 (0.3)	1.14 (0.2)	0.9
Protein C	1.12 (0.4)	1.22 (0.4)	0.3

P values are based on the independent samples *t* test. VKORC1 indicates vitamin K epoxide reductase complex subunit 1.

of phyloquinone and lower concentrations of percentage uncarboxylated osteocalcin compared with those with the CC or GC genotypes in a smaller clinical trial.<sup>22</sup> Taken together, at the present time, there are insufficient data to support the contention that factors that influence VKORC1 activity potentially modify arterial disease via its chronic impact on peripheral vitamin K status.

Few studies have reported on CAC progression in patients with stages 3 to 5 CKD. A systematic review by McCullough et al<sup>34</sup> sought to determine the annualized rate of CAC progression in patients with CKD. However, the studies that were included in this review had few patients with CAC scores <30 AU, and mean CAC scores were used to calculate progression rates. Our CAC results were not normally distributed, and thus the annualized mean percent change in CAC used in the review by McCullough et al<sup>34</sup> could not be used for comparison purposes. Consequently, in evaluating CAC progression for this study, we considered multiple cut points of increase in CAC score. Our obtained results were consistent across all CAC categories. Approximately 30% of patients had zero or minimal CAC at baseline; therefore, our chosen outcome of an increase in CAC of  $\geq 50$  AU during 4 years would represent a clinically meaningful change, applicable to both lower and higher baseline CAC scores.

One limitation of this study is that the first series of CAC scans was completed on an earlier generation of scanner (4-slice helical) versus the second series of scans completed on a current generation of scanner (64-slice helical). The latter scanner is faster and results in decreased motion artifact, improved spatial resolution, and potentially more precise CAC results. The first series of scans could have resulted in an overestimation of CAC scores because of motion artifact, which would be less in the second series of scans. These differing measurement techniques would potentially bias results toward the null, and we are therefore confident that the change in CAC scores detected between the 2 series of scans is depicting an accurate reflection of CAC progression. Furthermore, the study design resulted in a healthy bias because individuals who died or went on to dialysis therapy were not included; however, this would similarly have biased the results toward the null. Further limitations include the small sample size for a candidate gene study and assessment of a single SNP. These findings therefore require replication in a larger cohort. In addition, there are other genetic components that have been

linked to warfarin dose requirements, including genetic variation in CYP2C9, apolipoprotein E, and  $\gamma$ -glutamyl carboxylase.  $\gamma$ -Glutamyl carboxylase is a critical enzyme responsible for the action of vitamin K in the  $\gamma$ -carboxylation of VKDPs, including those involved in coagulation pathways. Further studies should include other genetic influences related to vitamin K metabolism.

In summary, these data provide new perspectives on the potential role of VKORC1 in individuals with CKD. Larger, well-controlled studies are required to externally validate these findings.

## Acknowledgments

We gratefully acknowledge the assistance of Francis MacLeod, James Peterson, and Lisa Bartsch.

## Sources of Funding

This research was based on work supported by the Kidney Foundation of Canada and by the Agricultural Research Service, US Department of Agriculture (cooperative agreement 58-1950-7-707). Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the views of the US Department of Agriculture.

## Disclosures

R.M. Holden, A.R.H., and J.S. Garland have received funding support from Amgen for unrelated studies. The other authors report no conflicts.

## References

- Wang Y, Zhang W, Zhang Y, et al. VKORC1 haplotypes are associated with arterial vascular diseases (stroke, coronary heart disease, and aortic dissection). *Circulation*. 2006;113:1615–1621.
- Suh JW, Baek SH, Park JS, Kang HJ, Chae IH, Choi DJ, Park HJ, Kim PJ, Seung KB, Kim HS. Vitamin K epoxide reductase complex subunit 1 gene polymorphism is associated with atherothrombotic complication after drug-eluting stent implantation: 2-Center prospective cohort study. *Am Heart J*. 2009;157:908–912.
- Lacut K, Larramendy-Gozalo C, Le Gal G, Duchemin J, Mercier B, Gourhant L, Mottier D, Becquemont L, Oger E, Verstuyft C. Vitamin K epoxide reductase genetic polymorphism is associated with venous thromboembolism: results from the EDITH Study. *J Thromb Haemost*. 2007;5:2020–2024.
- Schurgers LJ, Spronk HM, Skepper JN, Hackeng TM, Shanahan CM, Vermeer C, Weissberg PL, Proudfoot D. Post-translational modifications regulate matrix Gla protein function: importance for inhibition of vascular smooth muscle cell calcification. *J Thromb Haemost*. 2007;5:2503–2511.
- Proudfoot D, Shanahan CM. Molecular mechanisms mediating vascular calcification: role of matrix Gla protein. *Nephrology (Carlton)*. 2006;11:455–461.
- Covic A, Kanbay M, Voroneanu L, Turgut F, Serban DN, Serban IL, Goldsmith DJ. Vascular calcification in chronic kidney disease. *Clin Sci (Lond)*. 2010;119:111–121.
- Viegas CS, Cavaco S, Neves PL, Ferreira A, João A, Williamson MK, Price PA, Cancela ML, Simes DC. Gla-rich protein is a novel vitamin K-dependent protein present in serum that accumulates at sites of pathological calcifications. *Am J Pathol*. 2009;175:2288–2298.
- Garcia AA, Reitsma PH. VKORC1 and the vitamin K cycle. *Vitam Horm*. 2008;78:23–33.
- Meckley LM, Wittkowsky AK, Rieder MJ, Rettie AE, Veenstra DL. An analysis of the relative effects of VKORC1 and CYP2C9 variants on anticoagulation related outcomes in warfarin-treated patients. *Thromb Haemost*. 2008;100:229–239.
- Rieder MJ, Reiner AP, Gage BF, Nickerson DA, Eby CS, McLeod HL, Blough DK, Thummel KE, Veenstra DL, Rettie AE. Effect of VKORC1 haplotypes on transcriptional regulation and warfarin dose. *N Engl J Med*. 2005;352:2285–2293.

11. Takahashi H, Wilkinson GR, Nutescu EA, Morita T, Ritchie MD, Scordo MG, Pengo V, Barban M, Padriani R, Ieiri I, Otsubo K, Kashima T, Kimura S, Kijima S, Echizen H. Different contributions of polymorphisms in VKORC1 and CYP2C9 to intra- and inter-population differences in maintenance dose of warfarin in Japanese, Caucasians and African-Americans. *Pharmacogenet Genomics*. 2006;16:101–110.
12. Vecsler M, Loebstein R, Almog S, Kurnik D, Goldman B, Halkin H, Gak E. Combined genetic profiles of components and regulators of the vitamin K-dependent gamma-carboxylation system affect individual sensitivity to warfarin. *Thromb Haemost*. 2006;95:205–211.
13. McCabe KM, Booth SL, Fu X, Shobeiri N, Pang JJ, Adams MA, Holden RM. Dietary vitamin K and therapeutic warfarin alter the susceptibility to vascular calcification in experimental chronic kidney disease. *Kidney Int*. 2013;83:835–844.
14. Ing SW, Mohler Iii ER, Putt ME, Torigian D, Leonard MB. Correlates of valvular ossification in patients with aortic valve stenosis. *Clin Transl Sci*. 2009;2:431–435.
15. Koos R, Krueger T, Westenfeld R, Kühl HP, Brandenburg V, Mahnen AH, Stanzel S, Vermeer C, Cranenburg EC, Floege J, Kelm M, Schurgers LJ. Relation of circulating Matrix Gla-Protein and anticoagulation status in patients with aortic valve calcification. *Thromb Haemost*. 2009;101:706–713.
16. Lerner RG, Aronow WS, Sekhri A, Palaniswamy C, Ahn C, Singh T, Sandhu R, McClung JA. Warfarin use and the risk of valvular calcification. *J Thromb Haemost*. 2009;7:2023–2027.
17. Palaniswamy C, Sekhri A, Aronow WS, Kalra A, Peterson SJ. Association of warfarin use with valvular and vascular calcification: a review. *Clin Cardiol*. 2011;34:74–81.
18. Villines TC, O'Malley PG, Feuerstein IM, Thomas S, Taylor AJ. Does prolonged warfarin exposure potentiate coronary calcification in humans? Results of the warfarin and coronary calcification study. *Calcif Tissue Int*. 2009;85:494–500.
19. Lim MA, Shafique S, See SY, Khan FN, Parikh CR, Peixoto AJ. Effects of warfarin on blood pressure in men with diabetes and hypertension—a longitudinal study. *J Clin Hypertens (Greenwich)*. 2007;9:256–258.
20. Hammed A, Matagrini B, Spohn G, Prouillac C, Benoit E, Lattard V. VKORC1L1, an enzyme rescuing the vitamin K 2,3-epoxide reductase activity in some extrahepatic tissues during anticoagulation therapy. *J Biol Chem*. 2013;288:28733–28742.
21. Reitsma PH. VKORC1 gene variation and venous thrombosis: 'another one bites the dust'? *J Thromb Haemost*. 2007;5:2017–2019.
22. Crosier MD, Peter I, Booth SL, Bennett G, Dawson-Hughes B, Ordovas JM. Association of sequence variations in vitamin K epoxide reductase and gamma-glutamyl carboxylase genes with biochemical measures of vitamin K status. *J Nutr Sci Vitaminol (Tokyo)*. 2009;55:112–119.
23. Watzka M, Nebel A, El Mokhtari NE, Ivandic B, Müller J, Schreiber S, Oldenburg J. Functional promoter polymorphism in the VKORC1 gene is no major genetic determinant for coronary heart disease in Northern Germans. *Thromb Haemost*. 2007;97:998–1002.
24. Beavan SR, Prentice A, Stirling DM, Dibba B, Yan L, Harrington DJ, Shearer MJ. Ethnic differences in osteocalcin gamma-carboxylation, plasma phylloquinone (vitamin K1) and apolipoprotein E genotype. *Eur J Clin Nutr*. 2005;59:72–81.
25. Hindorf LA, Heckbert SR, Smith N, Marcianti KD, Psaty BM. Common VKORC1 variants are not associated with arterial or venous thrombosis. *J Thromb Haemost*. 2007;5:2025–2027.
26. Teichert M, Visser LE, van Schaik RH, Hofman A, Uitterlinden AG, De Smet PA, Witteman JC, Stricker BH. Vitamin K epoxide reductase complex subunit 1 (VKORC1) polymorphism and aortic calcification: the Rotterdam Study. *Arterioscler Thromb Vasc Biol*. 2008;28:771–776.
27. Green D, Foiles N, Chan C, Kang J, Schreiner PJ, Liu K. An association between clotting factor VII and carotid intima-media thickness: the CARDIA study. *Stroke*. 2010;41:1417–1422.
28. Junker R, Heinrich J, Schulte H, van de Loo J, Assmann G. Coagulation factor VII and the risk of coronary heart disease in healthy men. *Arterioscler Thromb Vasc Biol*. 1997;17:1539–1544.
29. Heywood DM, Ossei-Gerning N, Grant PJ. Association of factor VII:C levels with environmental and genetic factors in patients with ischaemic heart disease and coronary atheroma characterised by angiography. *Thromb Haemost*. 1996;76:161–165.
30. Reiner AP, Carlson CS, Rieder MJ, Siscovick DS, Liu K, Chandler WL, Green D, Schwartz SM, Nickerson DA. Coagulation factor VII gene haplotypes, obesity-related traits, and cardiovascular risk in young women. *J Thromb Haemost*. 2007;5:42–49.
31. de Maat MP, Bladbjerg EM, Drivsholm T, Borch-Johnsen K, Møller L, Jespersen J. Inflammation, thrombosis and atherosclerosis: results of the Glostrup study. *J Thromb Haemost*. 2003;1:950–957.
32. Garland JS, Holden RM, Groome PA, Lam M, Nolan RL, Morton AR, Pickett W. Prevalence and associations of coronary artery calcification in patients with stages 3 to 5 CKD without cardiovascular disease. *Am J Kidney Dis*. 2008;52:849–858.
33. Shea MK, Benjamin EJ, Dupuis J, Massaro JM, Jacques PF, D'Agostino RB Sr, Ordovas JM, O'Donnell CJ, Dawson-Hughes B, Vasan RS, Booth SL. Genetic and non-genetic correlates of vitamins K and D. *Eur J Clin Nutr*. 2009;63:458–464.
34. McCullough PA, Chinnaiyan KM. Annual progression of coronary calcification in trials of preventive therapies: a systematic review. *Arch Intern Med*. 2009;169:2064–2070.

### Significance

Coronary artery calcification is predictive of cardiovascular and all-cause mortality in patients with chronic kidney disease. Vitamin K is involved in the development and progression of vascular calcification via its impact on the  $\gamma$ -carboxylation of MGP, a key inhibitory protein that is present in vascular tissue. The enzyme vitamin K epoxide reductase complex subunit 1 mediates the cyclic interconversion of vitamin K metabolites and is a rate-limiting step in the  $\gamma$ -carboxylation of vitamin K-dependent proteins. Genotypes of vitamin K epoxide reductase complex subunit 1 that alter the activity of the vitamin K cycle could modify coronary artery calcification and its progression. We show that the genotype of vitamin K epoxide reductase complex subunit 1 that is linked to higher activity of the vitamin K cycle in the liver is associated with coronary artery calcification at baseline as well as coronary artery calcification progression and survival  $\leq 4$  years. Our observations may reflect vitamin K epoxide reductase complex subunit 1-mediated enhancement of vitamin K-dependent proteins involved in the coagulation cascade and atherosclerosis.



## Materials and Methods

**Participants:** Between December, 2005 and December, 2006, 185 patients presenting for care to the Kingston General Hospital's Chronic Kidney Disease clinic (Kingston, Ontario, Canada) were enrolled in a study evaluating the cross sectional association between vitamin K status and CAC. The full eligibility criteria are described elsewhere, but included: age > 18 years, stages 3 to 5 CKD (not requiring RRT), the absence of the use of a bisphosphonate and the absence of any lifetime exposure to warfarin <sup>1</sup>. Patients were followed until December, 2009 for survival status, which was available on 167 patients, and this established the cohort under study. Subjects were re-approached in 2009, and 86 subjects consented to a repeat multi-slice CT scan (MSCT) to determine their CAC score. Reasons for not repeating the CAC score in 2009: deceased (n=24), no longer attending the CKD clinic for follow-up (n=4), now requiring renal replacement therapy (n=24), unwillingness to participate (n=23), moved (n=2) and received a kidney transplant (n=4).

Clinical data were abstracted by patient interview and chart review. Weight and height were collected from each individual to calculate body mass index (BMI). Current smokers were defined as patients smoking at least 1 cigarette/day during the previous six months. All patients provided informed consent according to the Declaration of Helsinki. All procedures were in accordance with the ethical standards of Queen's University and approved by the Tufts-Medical Center Institutional Review Board (Boston, MA).

**Laboratory Measures:** Laboratory measures included ionized calcium (mmol/L), phosphate (mmol/L), intact parathyroid hormone (iPTH) (pmol/L), alkaline phosphatase (mmol/L), serum albumin (g/L) and 25(OH)D (nmol/L). iPTH was assessed by electrochemiluminescence (Roche, Basel, Switzerland) modular analytics E170 immunoassay. A Roche modular bromocresol green method was measured to measure serum albumin. In 2005, serum creatinine measured by the Roche Creatinine Plus Modular assay on the day of study enrollment was used in the 4-variable Modification of Diet in Renal Disease (MDRD) Study equation <sup>2</sup>, re-expressed for standardized creatinine <sup>3</sup>, to determine estimated glomerular filtration rate (eGFR) <sup>3</sup>. In 2009, serum creatinine was measured by Jaffe rate method (Beckman Coulter UniCel Dx C 800 SYNCHRON Clinical System assay, traceable to isotope dilution mass spectroscopy). All of these aforementioned analyses were performed in the laboratory at Kingston General Hospital to minimize inter-laboratory variability.

Genotyping of the Vitamin K Epoxide Reductase Complex Subunit (VKORC1) G1542C (rs8050894) single nucleotide polymorphism was performed by polymerase chain reaction (PCR) /restriction fragment length polymorphism analysis using real-time PCR using TaqMan-based assays implemented on an Applied Biosystems ABI Prism 7000 instrument (AMJ Laboratory Services Ltd., Millgrove, ON). The G<sub>1542</sub>C VKORC1 polymorphism is associated with decreased warfarin dose requirements in hetero- and homozygous mutant patients <sup>4</sup>. Plasma 25(OH)D was measured by radioimmunoassay (DiaSorin, MDS Laboratory Services, Toronto, ON, Canada). Fasting phylloquinone concentrations were measured using reversed-phase high-performance liquid chromatography <sup>5</sup>. PIVKA-II concentrations were determined by ELISA (Diagnostic Stago, Parsippany, NJ). Osteocalcin was measured in serum by using a radioimmunoassay that uses human OC for standard and tracer and a polyclonal antibody directed to intact OC as previously described (1)

F actor II, VII and X assays performed were based on prothrombin time (PT) reactions. Factor IX assays were based on partial thromboplastin time (PTT) reactions. Patient plasma was mixed with plasma deficient of the factor that was being measured. The amount of factor present was determined by comparing the clotting time



to a standard curve of known factor levels against clotting times generated using reference plasma. Functional protein C was measured based on the prolongation of the activated partial thromboplastin time (APTT). The presence of a specific activator extracted from *Agkistrodon c. contortrix* venom activates protein C which inhibits factors V and VIII resulting in a prolonged APTT. Functional protein S was measured based on the principle of factor Va inhibition. The cofactor activity of protein S enhances the anticoagulant activity of activated protein C resulting in a prolonged clotting time in the presence of factor Va. The protein C and protein S assays were performed using commercially available kits purchased from Diagnostica Stago Inc., Parsippany, NJ 07054 USA.

#### **Evaluation of coronary artery calcification:**

In 2005, CAC scores were evaluated using The Toshiba Aquillion computed tomography (CT) multislice scanner (4 sets of detectors) and VScore analytical software package. The scan thickness is 3 mm x 4 slices simultaneously over 12 mm per rotation, and the field of coverage is 12 cm. Images were acquired with prospective gating technique using a discrete algorithm<sup>6</sup>. In 2009, the CAC scores were evaluated using a General Electric VCT multislice CT (64 slice) helical scanner (Waukesha, Wisconsin, USA) and using Smartscore software (version 3.5, GE Medical Systems (Waukesha, Wisconsin, USA)). The slice thickness is 2.5 mm x 8 slices acquired over 2 cm using a step and shoot technique with no overlap. Images were acquired with prospective gating technique using a discrete algorithm. Total CAC score was generated as per the Agatston method<sup>7</sup>.

**Statistical Analysis:** All statistical tests (IBM SPSS for Windows version 21.0, Armonk, NY) were 2-sided and unadjusted P-values of 0.05 or less were considered significant. Chi-square tests (Pearson's or Fisher's Exact as appropriate) and independent samples t-tests (or Mann-Whitney U in the event of non-normal distributions) were used to compare those who did and did not undergo a repeat CT scan. VKOR CG and GG were combined for the analysis (CG/GG) and compared to the VKOR CC. The same analyses were used to assess the bivariate relationship between VKOR groups and *a priori* covariates of interest including biomarkers of vitamin K status. Multiple variable linear logistic regression modeling was performed to determine risk factors associated with CAC progression. A Kaplan Meier survival curve was constructed to examine the survival (months of follow-up) for VKOR CG/GG versus VKOR CC, followed by a Cox Proportional Hazards Model for time to death, with the surviving cohort censored at study end. *A priori* chosen risk factors for both the logistic regression and the Cox model included the following: age, diabetes, per 10 ml/min increase in eGFR and hypertension.

## Reference List

- (1) Holden RM, Morton AR, Garland JS, Pavlov A, Day AG, Booth SL. Vitamins K and D status in stages 3-5 chronic kidney disease. *Clinical Journal of The American Society of Nephrology: CJASN* 2010;5(4):590-597.
- (2) Levey AS, Bosch JP, Lewis JB, Greene T, Rogers N, Roth D. A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Modification of Diet in Renal Disease Study Group. *Annals of Internal Medicine* 1999;130:461-470.
- (3) Levey AS, Coresh J, Greene T, Stevens LA, Zhang YL, Hendriksen S, Kusek JW, Van LF, Chronic Kidney Disease Epidemiology Collaboration. Using standardized serum creatinine values in the modification of diet in renal disease study equation for estimating glomerular filtration rate. *Annals of Internal Medicine* 2006;145:247-254.
- (4) Vecsler M, Loebstein R, Almog S, Kurnik D, Goldman B, Halkin H, Gak E. Combined genetic profiles of components and regulators of the vitamin K-dependent gamma-carboxylation system affect individual sensitivity to warfarin. *Thrombosis & Haemostasis* 2006;95:205-211.
- (5) Davidson KW, Sadowski JA. Determination of vitamin K compounds in plasma or serum by high-performance liquid chromatography using postcolumn chemical reduction and fluorimetric detection. *Methods in Enzymology* 1997;282:408-421.
- (6) Wexler L, Brundage B, Crouse J, Detrano R, Fuster V, Maddahi J, Rumberger J, Stanford W, White R, Taubert K. Coronary artery calcification: pathophysiology, epidemiology, imaging methods, and clinical implications. A statement for health professionals from the American Heart Association. Writing Group. *Circulation* 1996;94:1175-1192.
- (7) Agatston AS, Janowitz WR, Hildner FJ, Zusmer NR, Viamonte M, Jr., Detrano R. Quantification of coronary artery calcium using ultrafast computed tomography. *Journal of the American College of Cardiology* 1990;15:827-832.