Vitamin K–Dependent Protein Activity and Incident Ischemic Cardiovascular Disease
The Multi-Ethnic Study of Atherosclerosis

John Danziger, Rebekah L. Young, M. Kyla Shea, Russell P. Tracy, Joachim H. Ix, Nancy S. Jenny, Kenneth J. Mukamal

Objective—Vitamin K–dependent proteins (VKDPs), which require post-translational modification to achieve biological activity, seem to contribute to thrombus formation, vascular calcification, and vessel stiffness. Whether VKDP activity is prospectively associated with incident cardiovascular disease has not been studied.

Approach and Results—VKDP activity was determined by measuring circulating des-γ-carboxy prothrombin concentrations in a random sample of 709 multiethnic adults free of cardiovascular disease drawn from the Multi-Ethnic Study of Atherosclerosis (MESA). Lower des-γ-carboxy prothrombin concentrations reflect greater VKDP activity. Subjects were followed up for the risk of ischemic cardiovascular disease (coronary heart disease, stroke, and fatal cardiovascular disease) for 11.0 years of follow-up. A total of 75 first ischemic CVD events occurred during follow-up. The incidence of ischemic cardiovascular disease increased progressively across des-γ-carboxy prothrombin quartiles, with event rates of 5.9 and 11.7 per 1000 person-years in the lowest and highest quartiles. In analyses adjusted for traditional cardiovascular risk factors and measures of vitamin K intake, a doubling of des-γ-carboxy prothrombin concentration was associated with a 1.53 (95% confidence interval, 1.09–2.13; \( P = 0.008 \)) higher risk of incident ischemic cardiovascular disease. The association was consistent across strata of participants with diabetes mellitus, hypertension, renal impairment, and low vitamin K nutritional intake.

Conclusions—In this sample of middle-aged and older adults, VKDP activity was associated with incident ischemic cardiovascular events. Further studies to understand the role of this large class of proteins in cardiovascular disease are warranted. (Arterioscler Thromb Vasc Biol. 2016;36:1037-1042. DOI: 10.1161/ATVBAHA.116.307273.)

Key Words: cardiovascular diseases ■ prothrombin ■ risk factors ■ vitamin K
Materials and Methods

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

Results

Baseline characteristics stratified by DCP quartiles are presented in Table 1. Participants with higher DCP concentrations (ie, lower VKDP activity) tended to be older, with higher body mass index and cholesterol medication usage, with lower renal function, and with less overall physical activity. The rates of diabetes mellitus and hypertension were similar, but high-sensitivity C-reactive protein (hs-CRP) concentrations increased across the DCP quartiles. As expected, increased phylloquinone concentrations, reflecting greater dietary vitamin K intake, were associated with lower DCP concentrations, reflecting greater VKDP activity. The weighted correlation

<table>
<thead>
<tr>
<th>Table 1. Baseline Characteristics of the Case–Cohort Sample, Stratified by DCP Quartiles (n=709)</th>
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<tbody>
<tr>
<td>Variables</td>
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<tr>
<td>Age, y</td>
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<tr>
<td>Women, n (%)</td>
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<tr>
<td>Race/ethnicity, n (%)</td>
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<tr>
<td>White</td>
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<tr>
<td>Chinese</td>
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<td>Black</td>
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<td>Body mass index</td>
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<td>Cigarette smoking status, n (%)</td>
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<tr>
<td>Current</td>
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<td>Pack-years cigarette smoking</td>
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<td>Intentional physical activity total, MET-min/d</td>
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<td>Current alcohol use, n (%)</td>
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<tr>
<td>High-school graduate, n (%)</td>
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<tr>
<td>Diabetes mellitus</td>
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<tr>
<td>Antihypertension medication, n (%)</td>
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<td>Systolic BP, mmHg</td>
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<td>Lipid-lowering medication, n (%)</td>
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<td>Triglycerides, mg/dL, median (IQR)</td>
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<td>hs-CRP, median (IQR), mg/L</td>
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<tr>
<td>Estimated GFR</td>
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<tr>
<td>Urinary albumin/creatinine, mg/g</td>
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<tr>
<td>Phylloquinone, nmol/L</td>
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<td>Dihydrophylloquinone, nmol/L</td>
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Mean (SDs) are provided. Sample n=709, except cigarette smoking, n=706; pack-years of smoking, n=701; intentional physical activity, n=706; high-school graduate, n=706; diabetes mellitus, n=708; LDL-C, n=701; HDL-C, n=708; triglycerides, n=708; estimated GFR, n=708; urinary albumin/creatinine, n=708; phylloquinone, n=630; dihydrophylloquinone, n=630. Descriptive statistics are not weighted for case–cohort design. BP indicates blood pressure; DCP, des-γ-carboxy prothrombin; eGFR, estimated glomerular filtration rate based on creatinine measurement; HDL, high-density lipoprotein cholesterol; hs-CRP, high-sensitivity C-reactive protein; IQR, interquartile range; LDL-C, low-density lipoprotein cholesterol; MET, metabolic equivalent; and Q, quartile.
between DCP and phylloquinone concentrations was inverse, as expected, but modest ($r=-0.11; P=0.006$). A total of 84% of the cohort participants had a DCP > 2 ng/mL (considered the threshold for VKDP inactivity), whereas 52% had a phylloquinone concentration < 1 nmol/L (considered the threshold for inadequate vitamin K intake). In keeping with previous reports suggesting higher dietary vitamin K intake in those of Chinese descent,7 the majority of Chinese participants (55%) were in the lowest DCP quartile compared with only 15% of white participants (Figure 1).

We documented 75 first ischemic CVD events during a median of 11.0 years of follow-up, 16 myocardial infarctions, 29 coronary revascularization procedures, 22 fatal and non-fatal strokes, and 8 other fatal coronary heart disease events. Unadjusted cumulative incidence rates per DCP quartile are presented in Figure 2. In general, ischemic CVD incidence rates were higher with greater concentration of DCP.

We next assessed the dose–response relationship of DCP with ischemic CVD (Figure 3), which suggested a log-linear association of DCP with CVD incidence. We subsequently performed analyses of log-transformed DCP concentrations with risk, adjusting sequentially for demographics, cardiovascular risk factors, and measures of vitamin K intake. In these analyses (Table 2), a doubling of circulating DCP concentration was associated with ≈50% higher risk of ischemic CVD. The magnitude of this association was little changed with adjustment for traditional risk factors or phylloquinone concentrations. Given the previously recommended threshold of 2 ng/mL in DCP concentrations to indicate VKDP inactivity, we examined this cut point in fully adjusted models, with an adjusted hazard ratio of 3.42 (95% confidence interval, 0.97–12.09; $P=0.06$).

The association of DCP concentrations and ischemic CVD seemed to be consistent across a broad range of subgroups. We found no significant interaction of DCP with diabetes mellitus, hypertension, vitamin K intake, or hs-CRP (all multiplicative interaction terms, $P>0.5$).

Finally, redefining ischemic CVD to include only cardiovascular-related events, DCP concentrations were associated with 1.46 (95% confidence interval, 1.00–1.96; $P=0.047$), 1.59 (95% confidence interval, 1.03–2.44; $P=0.035$), and 1.53 (95% confidence interval, 1.00–2.35; $P=0.054$) odds of incident disease using models I, II, and III, respectively.

**Discussion**

In this population of adults followed up for 11 years, baseline measures of VKDP activity were associated with incident ischemic cardiovascular disease. Our findings raise awareness of this important class of proteins as a potential contributor to cardiovascular disease.

A large body of evidence relates VKDPs to cardiovascular disease. Early animal studies suggested that warfarin administration, a potent inhibitor of vitamin K epoxide reductase recycling, induces widespread medial vascular calcification,8,9 otherwise known as Monckeberg calcification. Medial calcification has been associated with vascular stiffness10 and increased mortality 11,12 and is prevalent among individuals with diabetes mellitus and chronic kidney disease. MGP has received considerable attention as the candidate VKDP protein responsible for the vascular calcification phenotype.13 MGP null mice have widespread vascular calcification,1 and the circulating form of uncarboxylated MGP has been associated with vascular calcification in clinical studies14–17.
Because dietary vitamin K may reduce the inactive form of MGP, the dietary vitamin K intake has been hypothesized as a determinant of vascular calcification. Animal models have shown regression of vascular calcification with high vitamin K diets, but clinical data have not been uniform, with some studies suggesting a potential benefit of dietary vitamin K, but not all, studies. Gas6-deficient mice have defective platelet signaling but also seem to have a paradoxical protection against thromboembolic disease. Thus, it is plausible that a generalized state of VKDP inactivity, as measured by increasing DCP concentrations, might also reflect a state of Gas6 inactivity and a consequent proatherosclerotic state. Periostin is a newly described VKDP, named because of its localization in cortical bone periosteuem and the periodontal ligament, with a role in embryonic cardiac development and cardiac remodeling. Periostin knockout (Pn−/−) mice have an increased rate of ventricular rupture after myocardial infarction, but increased periostin expression is also seen in ventricular hypertrophy and fibrosis. In addition, prothrombin itself has been linked to CVD, associated with an increased risk of venothrombotic disease but not arterial disease.

In addition to the potential role of VKDPs in thrombus formation and vessel morphology, data suggest an association between VKDPs and inflammation. Administration of high-dose dietary vitamin K reduces inflammatory gene expression in animal models. In our cohort, hs-CRP concentrations increased with greater VKDP inactivity. Given data suggesting that prothrombin is an acute phase reactant, whether increasing DCP concentrations reflect prothrombin production or a carboxylation failure is uncertain although the association of DCP with ischemic cardiovascular disease was consistent across strata of hs-CRP. In our cohort, the weighted correlation for hs-CRP and DCP was r=0.10 (P=0.006).

Our findings add to clinical data linking VKDP activity and cardiovascular disease. In a community-based study of >4800 subjects, dietary vitamin K intake was inversely associated with incident cardiovascular disease. Because most American diets meet the recommended daily vitamin K allowances, there are likely dietary independent factors affecting VKDP activity. The function of the γ-carboxylase enzyme, responsible for converting a glutamic acid to glutamate residue, is impaired in kidney disease and diabetes mellitus, perhaps accounting for the unexpectedly high prevalence of VKDP inactivity in chronic kidney disease.

In our analysis, the overall correlation between serum phylloquinone and DCP concentrations was low, potentially supporting a role for dietary-independent factors, although phylloquinone concentrations may also be more sensitive to recent vitamin K intake than are DCP levels. Although high doses of dietary vitamin K can improve VKDP activity, whether such treatment will lead to an improvement of outcomes is speculative.

The strengths of our study included a well-characterized multiethnic population with adjudicated, prospectively measured end points and detailed phenotyping of cardiovascular risk factors. In addition, measurement of serum phylloquinone concentrations allowed the effect of VKDP
activity on cardiovascular disease to be adjusted for nutritional vitamin K. Our study also has important limitations. The correlation between the activity of circulating VKDPs, such as prothrombin, and organ-specific VKDPs, such as periostin or MGP, is not known, and the assumption that DCP concentrations reflect overall VKDP activity cannot be validated without further study. Although this is the largest prospective study of DCP and cardiovascular disease to be performed to our knowledge, we only measured DCP in a subcohort of Multi-Ethnic Study of Atherosclerosis (MESA) participants and had limited power to examine associations within subgroups or across components of our composite end point. Nevertheless, increasing DCP quartiles had incrementally higher risks of incident CVD that when examined continuously had sufficient power to detect a significant overall association between DCP and incident CVD. Further research to understand the role of VKDPs in cardiovascular disease is warranted, including a better understanding of the factors that affect post-translational carboxylation of VKDPs, the association between hepatic, platelet, and vascular smooth muscle cell VKDPs, and potential mechanisms of ischemia.

In summary, our analysis suggests that VKDP activity is associated with incident ischemic cardiovascular disease. Our results raise the possibility that dietary or pharmacological improvement of VKDP activity can reduce the incidence of CVD.

Acknowledgments

All authors have contributed to this article and have reviewed and agreed to its content.

Sources of Funding

This research was supported by a Normon S. Coplon grant from Satellite Health Care (J. Danziger), contracts N01-HC-95159, N01-HC-95160, N01-HC-95161, N01-HC-95162, N01-HC-95163, N01-HC-95164, N01-HC-95165, N01-HC-95166, N01-HC-95167, N01-HC-95168, and N01-HC-95169 from the National Heart, Lung, and Blood Institute, and by grants UL1-TR-000040 and UL1-TR-001079 from National Center for Research Resources. The authors thank the other investigators, the staff, and the participants of the Multi-Ethnic Study of Atherosclerosis (MESA) study for their valuable contributions.

Disclosures

None.

References

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Arterioscler Thromb Vasc Biol. 2016;36:1037-1042; originally published online March 31, 2016;
doi: 10.1161/ATVBAHA.116.307273

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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http://atvb.ahajournals.org/content/36/5/1037

Data Supplement (unedited) at:
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Methods

Participants
Details of the MESA study design and protocol have been published(1). In brief, between July 2000 and August 2002, 6,814 adults free from clinically apparent cardiovascular disease, between the ages of 45 to 84 years, were recruited from 6 representative communities and participated in the baseline examination. The cohort was comprised of 38% Caucasian (N=2,622), 28% African-American (N=1,893), 22% Hispanic (N=1,496) and 12% Chinese (N=803) participants. Individuals underwent extensive clinical, laboratory, and radiographic examination as part of the baseline examination. The institutional review boards at all participating centers approved the study, and all participants gave informed consent.

For this analysis, we used a weighted cohort design drawing from two previous MESA studies, as seen in Figure 1. In a previous study, serum phyloquinones were measured in a random subcohort of MESA (n=780), after excluding warfarin users (n=24) and a random subcohort due to high sample usage (n=995)(2). In a second case-cohort study, DCP concentrations were measured in 104 participants (cases) defined as an ankle brachial index >=1.4, while the cohort (n=624) was a random sampling of the phyloquinone subcohort. Our cohort includes the cases and a random sampling of the phyloquinone cohort. To account for the oversampling of cases in the study design, Barlow weights were used in the Kaplan-Meier estimates and Cox regression models(3). Since the random cohort was drawn from a previously established random cohort, the subcohort sampling fraction of 11% was calculated as a proportion of the original cohort after exclusions. Sensitivity analysis showed little impact of using this value in the Barlow weighting method. Six extreme outliers were excluded from analysis, and two patients were missing follow up data, leaving a total weighted cohort of 709 participants for primary analysis; results that winsorized these individuals at the 99th percentile yielded essentially identical results.

Plasma VKDP activity
The primary exposure was DCP concentrations measured at the baseline MESA examination, using a commercially available ELISA kit (Asserachrom DCP-II, Stago, France) that uses murine monoclonal antibodies against the under-carboxylated form of prothrombin. The detectable range for this assay was 0.335 - 207 ng/mL. Based on 4 controls, the intra-assay CVs were 6.5%, 16.1%, 5.2%, and 12.2%, and the inter-assay CVs were 10.2%, 32.3%, 9.1%, and 12.5%. Higher DCP concentrations suggest lower VKDP activity.

Ischemic cardiovascular disease
Follow-up took place through December 2011, for a median of 11.0 years of follow-up. CVD events were adjudicated by the MESA mortality and morbidity review committee. A full description of MESA event ascertainment is available at http://www.mesa-hhli.org. We defined incident ischemic cardiovascular disease as definite and probable myocardial infarction, coronary percutaneous angioplasty, coronary artery bypass grafting surgery, fatal or nonfatal stroke, or coronary related death. Details of CVD ascertainment have been published(4).
Covariates
Age, gender, race/ethnicity (white, Chinese, African-American, or Hispanic), level of education, income, smoking history, physical activity, and medication usage were self-reported through standard questionnaires. Body mass index was calculated from height and weight measurements obtained without shoes. Diabetes was defined by either a fasting glucose \( \geq 126 \) mg/dl or the use of either oral hypoglycemic medications or insulin. Fasting blood was collected and stored at \(-70\) until needed for the appropriate assays. High density lipoprotein (HDL) cholesterol was measured using the cholesterol oxidase cholesterol method (Roche Diagnostics), and low density lipoprotein was calculated using the Friedewald equation. The estimated glomerular filtration rate (eGFR) was calculated by using the Chronic Kidney Disease Epidemiology Collaboration formula based on serum creatinine measurements. Urine albumin and creatinine were measured in a single morning sample by nephelometry and the rate Jaffé equations, and expressed as albumin-creatinine ratio (ACR) in mg/g. Serum phylloquinone was measured by reversed-phase HPLC followed by fluorometric detection.

Statistical analysis

We describe participants’ baseline characteristics according to quartiles of DCP. We calculated incident event rates per quartile of DCP accounting for the case-cohort study design by using Barlow weights, and describe the log-rank p-value across the 4 strata using the Kaplan-Meier product-limit estimator. Weighted Cox proportional hazard models using robust standard errors were used to describe the association between baseline DCP concentrations and incident ischemic CVD. Model I included adjustment for age (years), gender and race/ethnicity (Black, Hispanic, Chinese, white). Model II added adjustment for body mass index (BMI), cigarette smoking (never, former, current), intentional physical activity (MET-min/day), current alcohol use, high school graduation, diabetes, systolic blood pressure (SBP), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglycerides, high-sensitivity C-reactive protein (hs-CRP), lipid-lowering medication, hypertension medication, estimated glomerular filtration rate (eGFR), and albumin/creatinine ratio. Model III added adjustment for serum phylloquinone and dihydrophylloquinone concentrations. The proportional hazards assumption for Cox regression models was tested using Stata’s ‘ptest’ command to evaluate whether the log hazard ratio function was constant over time, and showed no evidence of non-proportionality in the global test of the full model.

To determine the dose-response relationship, we applied locally weighted scatterplot smoothing (LOWESS) relating DCP concentrations with cumulative incidence. Based on these results, and because DCP concentrations were skewed, we also present hazard ratios using a log base 2 transformation; hazard ratios from this model can be interpreted as the risk per doubling of DCP concentrations. In addition, given that DCP>2 ng/ml has been considered as a threshold of VKDP inactivity(S), we also investigated it as a binary variable.

To examine the robustness of our findings within strata, we tested multiplicative interaction terms between log base 2 DCP concentrations and diabetes, hypertension (anti-hypertensive medication usage
or SBP > 140 mm Hg), renal function (continuous eGFR and eGFR<60 ml/min), cohort median hs-CRP,
and serum phyloquinone < 1 nmol/L in adjusted analyses (Model III).

Finally, we also examined the association of DCP concentrations with incident ischemic cardiac disease,
excluding stroke events from the primary endpoint.