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Associations between vitamin K status and haemostatic and inflammatory biomarkers in community-dwelling adults: The Multi-Ethnic Study of Atherosclerosis

M Kyla Shea^{*}, Mary Cushman[†], Sarah L Booth^{*}, Gregory L Burke[‡], Haiying Chen[‡], and Stephen B Kritchevsky[§]

^{*}USDA Human Nutrition Research Center on Aging at Tufts University, Boston MA

[†]Dept of Medicine, University of Vermont College of Medicine, Burlington VT

[‡]Division of Public Health Sciences, Wake Forest School of Medicine, Winston-Salem NC

[§]Sticht Center on Aging, Wake Forest School of Medicine, Winston-Salem NC

SUMMARY

Vitamin K is integral to haemostatic function, and in vitro and animal experiments suggest that vitamin K can suppress production of inflammatory cytokines. To test the hypothesis that higher vitamin K status is associated with lower hemostatic activation and inflammation in community-dwelling adults, we analyzed the cross-sectional association between serum phylloquinone (vitamin K1) with haemostatic and inflammatory biomarkers in 662 participants in the Multi-Ethnic Study of Atherosclerosis (MESA) [mean(SD) age=62(10)y; 46% female; 37% Caucasian, 25% African-American, 25% Hispanic, 13% Chinese-American]. Following adjustment for demographic and lifestyle characteristics, medication use, triglycerides and BMI, those in the highest quartile of serum phylloquinone had significantly lower circulating interleukin-6 [adjusted mean(SEM) pmol/L: quartile 4 (Q4)=1.22(0.07), quartile 1(Q1)=1.45(0.07); p-trend<0.01], CRP [adjusted mean(SEM) mg/dl: Q4=1.57(0.11), Q1=2.08(0.18); p-trend=0.02], soluble intercellular adhesion molecule-1 [adjusted mean(SEM) ng/ml: Q4=247(11), Q1=288(11); p-trend=0.02], and plasmin-antiplasmin complex [adjusted mean(SEM) nmol/L: Q4=4.02(0.1), Q1=4.31(0.1), p-trend=0.04]. We detected an interaction between age and serum phylloquinone with respect to factor VIII and D-dimer (interaction p-values=0.03 and 0.09 respectively). Among participants 70y, serum phylloquinone was inversely associated with factor VIII activity (p-trend=0.06) and positively associated with D-dimer (p-trend=0.01), but was not associated with either marker among participants <70y (both p 0.38). In contrast, dietary phylloquinone intake was not associated with any inflammatory or haemostatic biomarker evaluated (all p-trend>0.11). These findings are consistent with laboratory-based studies that suggest a possible anti-inflammatory role for vitamin K. Whether or not these associations predict clinical outcomes linked to elevated inflammation or haemostatic activation remains to be determined.

Corresponding Author: Kyla Shea, PhD, USDA Human Nutrition Research Center on Aging at Tufts University; kyla.shea@tufts.edu.

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Systemic inflammation is characteristic of several chronic diseases, including cardiovascular disease (CVD) and diabetes (1;2). Inflammatory cytokines can affect coagulation (and vice versa), and disordered haemostasis is a manifestation of chronic disease (3;4). Nutritional factors are involved in inflammatory pathways (5), and some (6;7) (but not all (8;9)) intervention and epidemiologic studies suggest micronutrient status is inversely associated with inflammatory and haemostatic outcomes.

Phylloquinone (PK, vitamin K1) is a fat-soluble nutrient found in green leafy vegetables and vegetable oils that has been implicated in haemostasis, inflammation and CVD (10). Its primary role is as an enzymatic co-factor for the γ -carboxylation of vitamin K-dependent (VKD) proteins, including several involved in haemostasis (10;11). Independent of this enzymatic function, *in vitro* and animal experiments have shown vitamin K suppresses expression and production of interleukin-6 (IL-6) and other pro-inflammatory cytokines through the inhibition of nuclear factor kappaB (NFkB) (12–14). In the Framingham Offspring Study (FOS), plasma PK and PK intake were inversely associated with numerous inflammatory markers (including IL-6) (15). Although several haemostatic proteins are vitamin K-dependent, and inflammation and haemostasis are inter-related, the association between vitamin K nutritional status and haemostatic biomarkers has not been examined in population-based studies. It is also unclear if vitamin K status is associated with inflammation/haemostasis in non-Caucasian race/ethnic groups because participants in the studies reported to date were primarily Caucasian (15–17).

The aim of this study was to test the overall hypothesis that vitamin K nutritional status is inversely associated with concentrations of haemostatic and inflammatory biomarkers in a multi-ethnic cohort. We determined the cross-sectional associations between serum PK with biomarkers of haemostasis and inflammation in the Multi-Ethnic Study of Atherosclerosis (MESA). The associations between PK intake and haemostatic and inflammatory biomarkers were also determined in a secondary analysis.

METHODS

The MESA study is a large, ongoing, observational study that began in 2000–2002 to examine the prevalence and determinants of sub-clinical cardiovascular disease in a multi-ethnic cohort. The cohort (n=6,814) was recruited from six communities in the United States: Forsyth County, NC; northern Manhattan and the Bronx, NY; Baltimore County, MD; St. Paul, MN; Chicago and Maywood, IL; Los Angeles County, CA. The cohort is 38% non-Hispanic white, 28% African American, 22% Hispanic, and 12% Chinese American, all of whom were free of clinically diagnosed CVD at baseline. The study design and methods have been described in detail (18). MESA was approved by the Institutional Review Board (IRB) at all six study sites (18) and this sub-study was additionally approved by the IRBs at Wake Forest University and Tufts University. All participants gave written informed consent.

A randomly chosen subgroup of 780 MESA participants who were not taking warfarin (a vitamin K antagonist) had serum PK measured from samples obtained at the baseline visit. These participants did not differ from the 6,034 participants who did not have serum PK

measured in terms of age, gender, race, BMI, triglycerides, total cholesterol, vitamin K intake, education, income, season (of blood draw), study site, and smoking status (all $p > 0.14$). Among participants whose serum PK was measured, inflammation/haemostasis biomarkers, anti-inflammatory medication use, dietary intake and pertinent covariate data were available on 662, who were included in the present analyses. The 116 participants excluded due to missing data more likely to smoke ($p = 0.02$) and be African American ($p < 0.001$), but were otherwise similar compared to those who were included. By design, soluble intercellular adhesion molecule-1 (ICAM-1) was measured in the first 1/3 of enrolled participants, so 223 participants with ICAM-1 and complete covariate data were available for ICAM-1 analysis. Those without ICAM-1 were older (mean $\pm$ SD age = 63 ± 10 y versus 58 ± 10 y; $p < 0.001$) and more likely to be African-American ($p < 0.001$). Otherwise the two groups were similar.

Biochemical Measures

At the baseline MESA examination, fasting blood was collected, processed, frozen at -70°C and shipped to the Laboratory for Clinical Biochemistry Research (University of Vermont, Burlington, VT, USA) using standard methods, where all inflammatory and haemostatic biomarker laboratory analyses were performed as described (19;20). Serum PK was measured using reversed-phase HPLC with post-column, solid phase chemical reduction of PK to its hydroquinone, followed by fluorometric detection at the Vitamin K Laboratory at the USDA Human Nutrition Research Center on Aging (HNRCA) at Tufts University (21). The lower limit of detection for PK with this assay is 0.1 nmol/L (21).

Dietary intake

At the baseline visit, participants' dietary intake over the previous 12 months was assessed using a modified 120-item Block Food Frequency Questionnaire (FFQ) as described (22;23). The Nutrition Data Systems for Research database (Nutrition Coordinating Center University of Minnesota; Minneapolis, MN) was used to estimate dietary PK intake by multiplying the number of servings of a particular food by the PK content per serving size of that food (24). A similar strategy was used to estimate fatty acid and energy intakes (25;26).

Covariates

Height and weight were measured without shoes, and BMI calculated as weight in kg/(height in meters)². Triglycerides were measured from fasting plasma samples (27). Physical activity was measured using a physical activity survey, described previously (28). Total intentional physical activity included walking for exercise, sports, dancing, and conditioning exercises (28). Medical history and medication use, smoking status and demographic information were collected using standardized questionnaires. Highest level of education was categorized as <high school, high school graduate, or college graduate. Race/ethnicity was self-reported as white, Chinese, African-American, or Hispanic.

Statistical analyses

A natural log (ln) transformation was applied to IL-6, CRP, D-dimer, PAP complex, serum PK, and PK intake. In descriptive analyses, differences in demographic and clinical

characteristics, medication use, and dietary intakes were examined across quartiles of serum PK using general linear models for continuous outcomes and Chi-square tests for categorical outcomes. The crude and adjusted associations between serum PK quartiles and each inflammatory/haemostatic biomarker were determined using a general linear model (SAS version 9.2, Cary NC). Because PK is transported on triglyceride-rich lipoproteins (29), triglycerides were adjusted for first. Additional covariates included age, gender, race/ethnicity, BMI, type 2 diabetes, education, aspirin use, non-steroidal anti-inflammatory medication use, oral steroid use, lipid-lowering medication use, alcohol intake, ratio of polyunsaturated (PUFA) to saturated fat intake, monounsaturated fatty acid (MUFA) intake, energy intake, smoking status, weekly intentional physical activity, and study site. We tested for interactions between serum PK and race/ethnicity, gender, and age with respect to each biomarker using product terms (ie. serum PK*race/ethnicity) in the fully-adjusted models. Because the distribution of serum PK differed according to race/ethnicity in MESA (30), we conducted a sensitivity analysis that examined the difference in inflammation/haemostatic biomarkers across race/ethnic specific quartiles of serum PK using a general linear model. The associations between dietary PK intake with each biomarker were determined following the same analytical approach. A p-value <0.05 was considered statistically significant for main effects and p<0.10 was considered statistically significant for interaction terms.

RESULTS

Participant characteristics according to quartile of serum PK are shown in Table 1. The mean±SD age and gender distribution were similar across quartiles. There were more Caucasian, African-American, and Hispanic participants in the lowest quartile and more Chinese-Americans in the highest quartile of serum PK (p<0.01). Plasma triglycerides and PK intake increased (both p-trend<0.01), while BMI and energy intake decreased (p-trend=0.04 and <0.01 respectively) across serum PK quartiles. Use of NSAIDs (non-steroidal anti-inflammatory medication) was higher among those in the lowest quartile of serum PK (p-trend=0.01), but use of other medications that influence inflammation did not significantly differ (all p-trend 0.08) (Table 1).

Serum PK was significantly associated with IL-6, CRP, ICAM-1, and PAP complex, such that those in the highest quartile of serum PK had significantly lower circulating biomarker concentrations (all fully-adjusted p-trend 0.04) (Figure 1). Fibrinogen did not differ across serum PK quartiles (fully-adjusted p-trend=0.34). Adjustment for osteo- and rheumatoid arthritis and recent cold or flu did not appreciably change our results. (Data not shown.)

We did not detect any significant interactions between serum PK quartile and gender (all interaction p-values>0.16) or race/ethnicity (all interaction p-values>0.16) with respect to any biomarker analyzed. The results of our sensitivity analyses indicate the associations across race/ethnic groups are consistent with our overall findings (although statistical significance was attenuated because sample sizes were reduced for individual race/ethnic groups) (Supplementary Figure 1). The association between serum PK and D-dimer and factor VIII appeared to differ by age (p-for interaction=0.02 and 0.09, respectively). Serum PK was positively associated with D-dimer and inversely associated with factor VIII among

older participants (≥ 70 y), but neither measure was associated with serum PK among participants <70 y (Table 2).

Dietary PK intake was not associated with any biomarker we examined (Supplementary Table 1). While there was an interaction between race/ethnicity and PK intake with respect to IL-6 (p -for-interaction=0.08), when the model was stratified by race/ethnicity the association was not significant in any race/ethnic group (all $p>0.10$). We did not detect any significant interactions between PK intake and age, gender or race/ethnicity with respect to any other biomarker (all interaction $p>0.13$).

DISCUSSION

In this multi-ethnic community-based cohort, serum PK was inversely associated with several, but not all, inflammatory/haemostatic biomarkers. There is potential clinical relevance to the observed associations because haemostatic activation and inflammation contribute to development and progression of cardiovascular and other diseases (1;2). Vitamin K is one of several nutrients that have clinical potential to lower CVD risk partially through reducing inflammatory mediators (31).

Our finding that serum PK was inversely associated with IL-6, CRP, and ICAM is consistent with earlier cross-sectional analyses of primarily Caucasian adults (15;17). These epidemiological findings, however, are not supported by the results of the only known randomized placebo-controlled PK supplementation trial that evaluated inflammatory outcomes (17). Changes in circulating cytokine concentrations were small in this trial and the attenuated response may reflect a selection bias because the exclusion criteria (which included known heart disease) resulted in a healthy cohort (32). In contrast, high doses of dietary vitamin K were found to reduce inflammation in rats that had been injected with lipopolysaccharide to induce a pro-inflammatory state (12). It is plausible vitamin K may be more relevant to inflammatory outcomes in persons with a higher inflammatory burden, such as those with metabolic or cardiovascular diseases (1;2).

That the haemostatic/inflammatory biomarkers that were associated with serum PK in this study and others (15;17) are not carboxylated by vitamin K suggests vitamin K's role in these pathways is independent of its function as an enzymatic co-factor. *In vitro* and rodent experiments have shown vitamin K treatment suppressed production of IL-6 by inhibiting the phosphorylation of the I-kappaB kinase thereby inhibiting the nuclear translocation of NFkB and blocking gene expression (12–14). IL-6 is a pleiotropic cytokine that influences production of other inflammatory and haemostatic factors, including the acute phase reaction (33;34). It is plausible vitamin K may affect inflammatory mediators by influencing IL-6. ICAM-1 is also an NFkB-dependent gene (35), so vitamin K may influence ICAM-1 through this same pathway. Since novel roles for vitamin K independent of its carboxylation function are emerging (13;36), our findings may stimulate new hypotheses.

Most of the haemostatic biomarkers we analyzed – PAP complex, factor VIII, and D-dimer – were not evaluated previously (15;17), even though haemostatic activation and inflammation are related (33;37). We found higher serum PK was associated with lower

PAP complex overall. Elevated PAP is predictive of clinical CVD and mortality (19;38). In a sub-cohort of the Cardiovascular Health Study (CHS) (mean±SD age=77±7y), plasma CRP and factor VIII were identified as independent positive predictors of plasma PAP (39), and it was suggested that these three factors share common biological processes (40). There was a significant inverse association between serum PK and factor VIII among MESA participants ≥ 70 y (p-trend=0.03), but not among participants < 70 y (p-trend=0.84). Given the associations we found between serum PK with CRP overall and with factor VIII in MESA participants of comparable age, it is plausible the association between serum PK and PAP reflects a mechanism common to these measures that remains to be identified.

In contrast to our overall hypothesis, higher serum PK was associated with higher D-dimer among participants ≥ 70y. In a nested case-control analysis of CHS participants ≥ 65 y), elevated D-dimer was associated with a more than 3-fold greater risk of venous thromboembolism (41). It would follow that the positive association between serum PK and D-dimer would suggest that older adults with higher circulating PK may be at risk for thrombosis. However, until it is replicated in other cohorts and linked to clinical outcomes, the relevance is uncertain.

Unlike earlier studies (15;16), dietary PK intake was not associated with any biomarker we analyzed. This may be because the primary dietary sources of PK are green leafy and cruciferous vegetables, so higher PK intakes reflect healthier diet (42). We adjusted for potential confounders that could reflect healthier lifestyle), which were not completely accounted for in earlier studies (15;16). However, even prior to adjusting for potential diet/lifestyle confounders, the associations between dietary PK intake and most biomarkers were not significant in MESA. Alternatively, the FFQs used in the earlier studies were validated specifically for PK intake (15;16;42), while the MESA FFQ was not, as we have discussed previously (30).

This study has several strengths and certain limitations to consider. We studied a race/ethnically diverse cohort. Serum PK, PK intake, and several biomarkers of haemostasis/inflammation are reported to differ by race/ethnicity in MESA (30;43). Although the association between serum PK and haemostasis/inflammation appeared to be consistent across race/ethnic groups (supplementary Figure 1), it is plausible we would have detected differences if the sample sizes within each race/ethnic group were larger. Considering the number of hypotheses tested, we cannot rule out chance as an explanation for some findings and the nominal p-value of 0.05 should be interpreted cautiously. The breadth of MESA data allowed us to control for multiple confounders, including use of medications that influence inflammation and indicators of healthy lifestyles (including diet, physical activity, and smoking). It is plausible anti-platelet medications may also influence the associations with hemostatic measures. Since only one participant in our sub-group reported taking anti-platelet medications our findings did not change when it was adjusted for. By design MESA participants were free of clinically apparent CVD at the baseline visit, when vitamin K status and haemostatic/inflammatory biomarkers were analyzed, so our findings may not be generalizable to less healthy persons. Due to our cross-sectional design, we do not know the temporal relationship between vitamin K status and hemostatic activation/inflammation. Although mechanistic studies demonstrated vitamin K reduces inflammation (12;13), it is

possible that a pro-inflammatory state may affect the metabolism and circulating concentrations of vitamin K. This can only be addressed by examining the association between vitamin K status and change in haemostatic activation/inflammation using prospective studies. Prospective cohort studies are needed to obtain foundational data to design randomized controlled trials. Should our findings be replicated, randomized controlled trials are needed to definitively test the hypothesis that vitamin K lowers haemostatic activation/inflammation among persons most likely to benefit clinically from additional vitamin K. Since vitamin K is safe, inexpensive and readily available, even a small effect on clinical outcomes related to hemostatic activation and inflammation could have a substantial public-health impact.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Associations between serum PK and haemostatic/inflammatory biomarkers in MESA*:

* Presented as LS means adjusted for age, gender, race/ethnicity, triglycerides, BMI, type 2 diabetes, education, aspirin use, nsaid use, oral steroid use, lipid lowering medication use, alcohol intake (drinks per week), PUFA:saturated fat intake, monounsaturated fat intake, energy intake, smoking status, intentional physical activity (MET-min/week), site; error bars represent SEMs, which were estimated using the delta method: [(geometric mean) $\times$ (SEM of ln-transformed outcome)].

[†] p-trend <0.05, based on general linear model using PK quartile as an ordinal exposure (values 1–4).

[‡] Outcome natural log-transformed to reduce skewness.

Table 1

Participant characteristics according to serum PK quartile. Data are median±interquartile range, unless otherwise indicated

Serum PK median (range)	Q1(n=174) <0.1mmol/L*	Q2(n=139) 0.7(0.1-0.9)mmol/L	Q3(n=166) 1.3(1.0-1.7)mmol/L	Q4(n=185) 2.6 (1.8-15.8)mmol/L	p-trend [†]
Age, y	61±20	62±16	63±17	62±13	0.71
Female, n(%)	76(44)	61(44)	86(52)	89(48)	0.23
Race/ethnicity, n(%) : Caucasian	61(35)	64(46)	61 (37)	59 (32)	<0.001 [‡]
African-American	50(29)	32(23)	42(16)	42(23)	
Hispanic	58(33)	39(28)	36(23)	31(17)	
Chinese-American	5(3)	4(4)	27(16)	53(29)	
Education, n(%) :					
High school graduate	74(43)	61(44)	83(50)	79(43)	0.45 [‡]
College graduate	57(33)	51(37)	57(34)	70(38)	
BMI, kg/m ²	28.0±7.1	28.6±6.9	27.4±5.8	27.2±6.7	0.04
Triglycerides, mg/dl	98±62	99±73	110±72	152±115	<0.01 [§]
Current smokers, n(%)	30(17)	22(16)	15 (9)	22(12)	0.05
Type 2 Diabetes n(%)	25 (14)	13 (9)	20 (12)	27 (15)	0.47
Alcohol intake, n(%)					
1-7 drinks/wk	51(29)	40(29)	52(32)	51(28)	0.33 [‡]
> 7 drinks/wk	14(8)	22(16)	15(9)	69(19)	
Medication use, n(%) :					
NSAIDS	43(25)	34(24)	18(11)	33(18)	0.01
Aspirin	35(20)	3 (27)	47(28)	50(30)	0.12
Oral steroids	1(0.6)	2(1)	1(0.6)	3(2)	0.47
Lipid lowering medications	20(11)	26(19)	37(23)	33(18)	0.08
PK intake, µg/day	79±93	84±102	96±132	108±101	<0.01 [§]
Energy intake, kcal/day	1507±871	1427±976	1353±904	1194±848	<0.01 [§]
PUFA:saturated fat intake, g/day:g/day	0.54±0.30	0.62±0.23	0.66±0.33	0.70±0.40	<0.01 [§]
MUFA intake (g/day)	20 (16)	18 (16)	19 (18)	15 (16)	<0.01 [§]
Intentional physical activity, MET-min/week	739±1778	630±1740	915±2003	923±2152	0.29 [§]

Serum PK median (range)	Q1(n=174) <0.1mmol/L*	Q2(n=139) 0.7(0.1–0.9)mmol/L	Q3(n=166) 1.3(1.0–1.7)mmol/L	Q4(n=185) 2.6 (1.8–15.8)mmol/L	p-trend [†]
Hemostatic and Inflammatory measures					
IL-6, pmol/L	1.43±1.18	1.27±0.89	1.19±0.96	1.16±1.11	<0.01
CRP, mg/dl	2.20±3.47	2.18±3.92	1.78±3.03	1.44±2.61	<0.01
Fibrinogen, mg/dl	347±103	337±92	330±72	333±84	0.04
D-dimer, µg/dl	0.20±0.20	0.23±0.24	0.20±0.22	0.20±0.17	0.03
PAP complex, nmol/L	4.47±2.31	4.46±2.29	4.11±2.45	3.83±1.77	<0.01
Factor VIII activity (%)	96±49	97±48	94±52	91±37	0.47
ICAM-1, ng/ml	(n=61)	(n=55)	(n=44)	(n=63)	
	285±79	292±109	262±67	231±54	<0.01

* the lower detectable limit for this assay; tied values resulted in unequal n's across quartiles.

[†] p-trend based on Cochran-Armitage trend test (categorical outcomes) or on general linear model using serum PK quartile as an ordinal exposure (continuous outcomes).

[‡] p-value for overall Chi-square test

[§] outcome was natural-log transformed for trend test

Table 2

Least-square mean ± SEM circulating D-dimer and factor VIII concentrations across serum PK quartiles in MESA, stratified by age*

	< 70 yrs (n=489)				70 yrs (n=174)				p-trend [†]	p-trend [†]
	Q1 (n=120)	Q2 (n=104)	Q3 (n=120)	Q4 (n=146)	Q1 (n=54)	Q2 (n=35)	Q3 (n=46)	Q4 (n=39)		
D-dimer (µg/ml) [‡]	0.20±0.02	0.19±0.02	0.18±0.02	0.18±0.01	0.38	0.28±0.05 [§]	0.35±0.06 [§]	0.34±0.06 [§]	0.43±0.07 [¶]	0.01
Factor VIII (%)	96±4	102±4	95±4	96±4	0.70	98±8 [§]	99±8 [§]	98±9 [§]	79±9 [¶]	0.06

* adjusted for age, gender, race/ethnicity, triglycerides, BMI, type 2 diabetes, education, aspirin use, nsaid use, oral steroid use, lipid lowering medication use, alcohol intake (drinks per week), polyunsaturated:saturated fat intake, monounsaturated fat intake (MUFA), smoking status, weekly intentional physical activity (MET-min/week), study site

[†] trend test based on general linear model using serum PK quartile as an ordinal exposure (values 1–4)

[‡] outcome natural log-transformed to reduce skewness; means presented are geometric least-square means±SEM

^{§¶} across rows, quartiles with different superscripts are statistically different from one another, p<0.05