Diet-sensitive prognostic markers for cardiovascular and renal disease

Riphagen, Ineke Jowanna

Chapter 6

Prevalence and Effects of Functional Vitamin K Insufficiency: the PREVEND Study

Ineke J. Riphagen
Charlotte A. Keyzer
Nadja E.A. Drummen
Martin H. de Borst
Joline W.J. Beulens
Ron T. Gansevoort
Johanna M. Geleijnse
Frits A.J. Muskiet
Gerjan Navis
Sipke T. Visser
Cees Vermeer
Ido P. Kema
Stephan J.L. Bakker

Submitted

Abstract

Aims. Matrix Gla protein (MGP) is a strong vitamin K-dependent inhibitor of soft tissue calcification. In this study, we assessed the prevalence of functional vitamin K insufficiency, as derived from plasma desphospho-uncarboxylated MGP (dp-ucMGP), and investigated whether plasma dp-ucMGP is associated with all-cause and cardiovascular mortality in a large general population-based cohort.

Methods and Results. We included 4,275 subjects (age 53 ± 12 years, 46.0% male) participating in the prospective general population-based Prevention of Renal and Vascular End-Stage Disease (PREVEND) study. Median dp-ucMGP concentration was 372 [221-552] pmol/L and the prevalence of functional vitamin K insufficiency (i.e., dp-ucMGP >500 pmol/L) was 30.7% in the total study population. This prevalence was significantly higher among elderly (51.1%), subjects with hypertension (49.6%), type 2 diabetes (52.4%), chronic kidney disease (54.1%), and cardiovascular disease (54.2%). After 10 years of follow up, 279 subjects had died, with 74 deaths attributable to cardiovascular causes. Using Cox proportional hazard regression analyses, we found a significant J-shaped association of plasma dp-ucMGP concentrations with all-cause (linear term: HR [95% CI] = 0.20 [0.12-0.33], $P<0.001$; squared term: 1.14 [1.10-1.17], $P<0.001$) and cardiovascular mortality (linear term: 0.12 [0.05-0.27], $P<0.001$; squared term: 1.17 [1.11-1.23], $P<0.001$). These associations remained significant after adjustment for potential confounders.

Conclusion. In this study, we demonstrated that functional vitamin K insufficiency, as derived from plasma dp-ucMGP, is a common phenomenon associated with an increased all-cause and cardiovascular mortality risk. Whether correction of functional vitamin K insufficiency improves health outcomes needs to be addressed in future prospective intervention studies.

Introduction

Along with aging of the population and rising prevalence of lifestyle-related diseases like type 2 diabetes and hypertension, the prevalence of cardiovascular disease has steadily risen all over the world (1). Vascular calcification is common in patients with diabetes, hypertension, and chronic kidney disease (CKD) and results in a substantially increased risk for cardiovascular disease (CVD) and mortality (2).

Active matrix Gla protein (MGP) is a strong endogenous inhibitor of soft tissue calcification (3). Activation of MGP by γ-carboxylation is a vitamin K-dependent process (4). Vitamin K insufficiency therefore results in increased plasma levels of inactive uncarboxylated MGP proteins (5). Plasma desphospho-uncarboxylated MGP (dp-ucMGP) was found to be particularly sensitive for changes in vascular vitamin K status (6).

High plasma dp-ucMGP levels, indicative of functional vitamin K insufficiency, were found to be associated with an increased risk for CVD (7) and mortality (8-11) in patients with diabetes (7), CKD (8,9), and CVD (10,11). In the general population, high plasma dp-ucMGP levels were not associated with coronary heart disease (CHD) or stroke (12), but a recent study showed that high plasma dp-ucMGP concentrations were associated with an increased risk for mortality in the general population (13). However, data regarding the prevalence of functional vitamin K insufficiency, and thus its clinical impact, are incomplete. In the present study, we assessed the prevalence of functional vitamin K insufficiency, as derived from dp-ucMGP, in a large general population-based cohort. Furthermore, we investigated whether functional vitamin K status is associated with cardiovascular events and mortality and whether these associations are modified by comorbidities such as type 2 diabetes, hypertension, and CKD.

Methods

Study design and population

The PREVEND (Prevention of Renal and Vascular ENdstage Disease) study is a prospective cohort study designed to investigate the association of microalbuminuria with renal and cardiovascular disease in the general population. Details of the PREVEND study have been described elsewhere (14,15). In brief, from 1997 to 1998, all inhabitants of the city of Groningen, the Netherlands, aged 28-75 years (n=85,421) were sent a questionnaire and a vial to collect a first-morning void urine sample. Pregnant women and subjects with type 1 diabetes were excluded. The urinary albumin concentration was assessed in 40,856 responders. Subjects with a urinary albumin concentration ≥10 mg/L (n=7,768) were invited to participate in the cohort, of whom 6,000 were enrolled. In addition, a randomly selected group with a urinary albumin concentration of <10 mg/L (n=3,394) was invited to participate in the cohort, of whom 2,592 were enrolled. These 8,592 individuals form the PREVEND cohort. The PREVEND study has been approved by the medical ethics committee of the University Medical Center Groningen. All participants provided written informed consent.

The second examination round of the PREVEND study took place from 2001 to 2003, which included 6,894 subjects and was considered the 'baseline' for the present study. For the present study, we included subjects with available dp-ucMGP measurements and available data on the use of vitamin K antagonists at baseline, leaving 4,275 subjects for analyses.

Data collection and measurements

The procedures at each examination in the PREVEND study have been described in detail previously (15). In brief, each examination included two visits to an outpatient clinic separated by 3 weeks. Before the first visit, all participants completed a self-administered questionnaire regarding demographics, cardiovascular and renal disease history, smoking habits, and medication use. Information on medication use was combined with information from IADB.nl (16), a database comprising pharmacy-dispensing data from all community pharmacies in the city of Groningen, the Netherlands, since 1999. During each examination and during each visit, blood pressure was measured on the right arm, every minute for 10 and 8 min, by an automatic Dinamap XL Model 9300 series device (Johnson-Johnson Medical, Tampa, Florida, USA). The mean of the last two recordings from each of the two visits was used. Fasting blood samples were provided and stored at -80 °C.

Functional vitamin K status was assessed by measuring dp-ucMGP in EDTA plasma

using a dual-antibody enzyme-linked immunoassay (Ina*K*tif MGP [IDS-iSYS] assay). Serum creatinine (SCr) was measured using an enzymatic method on a RocheModular analyzer (Roche Diagnostics, Mannheim, Germany). Serum cystatin C concentrations were determined by Gentian Cystatin C Immunoassay (Gentian AS, Moss, Norway) on a Roche Diagnostics Modular auto-analyzer. Cystatin C was calibrated using the standard supplied by the manufacturer (traceable to the International Federation of Clinical Chemistry Working Group for Standardization of Serum Cystatin C). The combined creatinine-and cystatin C-based Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation was used to obtain the estimated glomerular filtration rate (eGFR) (17). Urinary albumin concentration was measured by immunonephelometry (Dade Behring Diagnostic, Marburg, Germany).

Functional vitamin K insufficiency was defined as a dp-ucMGP concentration >500 pmol/L (6). Type 2 diabetes was defined as a fasting serum glucose level ≥7.0 mmol/L, a non-fasting plasma glucose level ≥11.1 mmol/L, self-report of a physician diagnosis or the use of glucose lowering drugs, retrieved from a central pharmacy registry. Hypertension was defined as systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg according to the 7th Joint National Committee (JNC 7) (18). Chronic kidney disease (CKD) was defined as an eGFR <60 mL/min/1.73 m^2 and/or an urinary albumin excretion (UAE) ≥30 mg/24 h.

Clinical end points

In the present study, we examined the association of functional vitamin K status, as derived from dp-ucMGP, with the development of a first cardiovascular event (CVE), cardiac event (CE), and mortality. CVE was defined as the combined end point of incident cardiovascular morbidity and mortality which includes the following events: acute myocardial infarction, acute and subacute ischaemic heart disease, occlusion or stenosis of the precerebral or cerebral arteries or the following procedures: coronary artery bypass grafting, percutaneous transluminal coronary angioplasty or other vascular interventions (i.e., percutaneous transluminal angioplasty or bypass grafting of the aorta and peripheral vessels). CE was defined as fatal/nonfatal myocardial infarction, ischemic heart disease, coronary artery bypass grafting and percutaneous transluminal coronary angioplasty. Information for cardiovascular morbidity was obtained from PRISMANT, the Dutch national registry of hospital discharge diagnoses. Data on mortality were obtained from the municipal register, and the cause of death was coded by a physician from the Central Bureau of Statistics. Cause of death was coded according to the Tenth Revision of the International Classification of Diseases (ICD-10).

Statistical analyses

Statistical analyses were performed using SPSS version 22.0 for Windows (IBM Corporation, Chicago, IL), STATA version 13.1 (StataCorp LP, TX, USA), and R version 3.0.1 (Vienna, Austria) (http://cran.r-project.org/). Results were expressed as mean ± standard deviation (SD) or median [interquartile range] for normally and non-normally distributed data, respectively. Nominal data were presented as the total number of patients (percentage). A two-sided $P<0.05$ was considered to indicate statistical significance.

Baseline characteristics are presented for the total study population and for tertiles of baseline dp-ucMGP concentrations. *P*-values for differences between tertiles were assessed using ANOVA for normally distributed data, Kruskal-Wallis test for skewed data, and the χ^2 test for nominal data. The χ^2 test was used to compare the prevalence of functional vitamin K insufficiency among subjects with and without comorbidities.

We used Cox regression analyses to investigate the prospective association of baseline dp-ucMGP concentrations with CVE, CE, and mortality. Subjects with a history of CVD at baseline (n=308) were excluded from the analyses of dp-ucMGP with CVE and CE. We applied $\log_2$ transformation of dp-ucMGP values so the hazard ratios derived from Cox regression analyses were expressed as an increase in risk per doubling of baseline dp-ucMGP values. We used fractional polynomials to test for potential non-linearity of the prospective associations of dp-ucMGP with CVE, CE, and mortality. Cox regression analyses with restricted cubic splines (RCS) with 3 knots were used to depict the J-shaped association of dp-ucMGP with mortality. Cox regression analyses and the STATA package mfpigen were used to test for potential interactions between continuous variables.

Several subjects had missing values for one or more baseline variables (i.e., race, smoking, education, BMI, systolic and diastolic blood pressure, UAE [≤1%]; total cholesterol/HDL ratio [3.3%]; eGFR [5.4%]; and hs-CRP [6.0%]). Because excluding subjects with missing values could result in bias, multiple imputation (fully conditional specification [MCMC]) was used to obtain 5 imputed datasets (19,20). Rubin's rules were used to obtain pooled estimates of the regression coefficients and their standard errors across the imputed datasets (21).

Various Cox regression models were built to adjust for possible confounders. The first model depicts the univariable association of $\log_2$ dp-ucMGP with outcomes; model 2 was adjusted for age and sex; model 3 was additionally adjusted for race, smoking, education, BMI, systolic blood pressure, cholesterol-HDL ratio, ln hs-CRP, type 2 diabetes, use of antihypertensive drugs, and use of vitamin K antagonists; model 4 was additionally adjusted for eGFR and ln UAE. As sensitivity analyses,

we additionally adjusted for the presence or absence of an UAE >10 mg/L to test for a potential sampling effect. In further sensitivity analyses, we repeated the Cox regression analyses in subjects that did not use vitamin K antagonists at baseline.

Furthermore, we used the method proposed by Liu et al. (22) based on the relationship with the receiver operating characteristic (ROC) curve to determine an optimal cut-off value for dp-ucMGP to identify subjects at risk for mortality.

Results

Baseline characteristics

In the present study, we included 4,275 subjects (aged 53 ± 12 years, 46.0% male). Out of all included subjects, 927 (21.7%) had hypertension, 1,306 (30.5%) were ≥60 years, 84 (2.0%) had type 2 diabetes, 712 (16.7%) had CKD, and 308 (7.2%) had a history of CVD. Median dp-ucMGP concentration was 372 [221-552] pmol/L and 1,311 subjects (30.7%) had functional vitamin K insufficiency (i.e., dp-ucMGP >500 pmol/L). Baseline characteristics of the total study population and according to tertiles of baseline dp-ucMGP concentrations are presented in Table 1. The prevalence of functional vitamin K insufficiency was significantly higher among subjects with hypertension (49.6%), older subjects (51.1%), subjects with type 2 diabetes (52.4%), CKD (54.1%), and CVD (54.2%), and subjects that used vitamin K antagonists (89.6%; all *P*-values <0.001; Figure 1).

Figure 1. Prevalence of functional vitamin K insufficiency (i.e., dp-ucMGP >500 pmol/L) for the total study population and for subjects with hypertension (HT), ≥60 years, type 2 diabetes (DM2), chronic kidney disease (CKD), history of cardiovascular disease (CVD), and subjects using vitamin K antagonists (VKA).

Table 1. Baseline characteristics of the study population.

	All subjects (n=4,275)	Tertiles of dp-ucMGP			*P*-value
		Tertile 1 (n=1,425)	Tertile 2 (n=1,425)	Tertile 3 (n=1,425)	
dp-ucMGP (pmol/L)	372 (221-552)	<275	275-479	≥480	-
Demographics					
Male gender (n, %)	1,966 (46.0)	570 (40.0)	669 (46.9)	727 (51.0)	<0.001
Age (years)	53 ± 12	49 ± 11	52 ± 11	59 ± 12	<0.001
Race					0.03
Caucasian (n, %)	4,041 (94.5)	1,333 (93.5)	1,343 (94.2)	1,365 (95.8)	
Black (n, %)	42 (1.0)	21 (1.5)	13 (0.9)	8 (0.6)	
Asian (n, %)	100 (2.3)	36 (2.5)	36 (2.5)	28 (2.0)	
Other (n, %)	59 (1.4)	27 (1.9)	21 (1.5)	11 (0.8)	
Education					<0.001
High (n, %)	1,431 (33.5)	566 (39.7)	504 (35.4)	361 (25.3)	
Middle (n, %)	1,015 (23.7)	366 (25.7)	340 (23.9)	309 (21.7)	
Low (n, %)	1,814 (42,4)	489 (34.3)	576 (40.4)	749 (52.6)	
Smoking (n, %)	1,206 (28.2)	472 (33.1)	448 (31.4)	286 (20.1)	<0.001
Type 2 diabetes (n, %)	84 (2.0)	16 (1.1)	19 (1.3)	49 (3.4)	<0.001
History of CVD (n, %)	308 (7.2)	47 (3.3)	86 (6.0)	175 (12.3)	<0.001
Clinical measurements					
BMI (kg/m^2)	26.7 ± 4.3	25.5 ± 3.9	26.4 ± 4.0	28.1 ± 4.5	<0.001
SBP (mmHg)	126 ± 19	121 ± 17	124 ± 18	133 ± 21	<0.001
DBP (mmHg)	73 ± 9	71 ± 9	73 ± 9	75 ± 9	<0.001
Laboratory parameters					
Total cholesterol (mmol/L)	5.4 ± 1.1	5.3 ± 1.0	5.5 ± 1.1	5.5 ± 1.1	<0.001
HDL cholesterol (mmol/L)	1.3 ± 0.3	1.3 ± 0.3	1.3 ± 0.3	1.2 ± 0.3	<0.001
Total cholesterol-HDL ratio	4.5 ± 1.3	4.3 ± 1.3	4.5 ± 1.3	4.7 ± 1.2	<0.001
Triglycerides (mmol/L)	1.1 (0.8-1.6)	1.0 (0.8-1.5)	1.1 (0.8-1.5)	1.2 (0.9-1.7)	<0.001
hs-CRP (mg/L)	1.4 (0.6-3.1)	1.1 (0.5-2.9)	1.2 (0.6-2.7)	1.8 (0.9-3.6)	<0.001
UAE (mg/day)	8.1 (5.9-13.4)	7.6 (5.7-11.4)	7.8 (5.8-12.0)	9.3 (6.3-17.9)	<0.001
Serum creatinine (μmol/L)	85 ± 22	81 ± 13	83 ± 14	90 ± 31	<0.001
eGFR (mL/min/1.73m^2)	85 ± 16	90 ± 14	87 ± 14	76 ± 17	<0.001
Medication					
Vitamin K antagonists (n, %)	106 (2.5)	5 (0.4)	6 (0.4)	95 (6.7)	<0.001
Antihypertensive drugs (n, %)	990 (23.2)	228 (16.0)	252 (17.7)	510 (35.8)	<0.001
Lipid lowering drugs (n, %)	459 (10.7)	98 (6.9)	130 (9.1)	231 (16.2)	<0.001

Abbreviations: CVD, cardiovascular diseases; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; hs-CRP, high sensitive C-reactive protein; UAE, urinary albumin excretion.

Dp-ucMGP and cardiovascular events

After a median follow-up time of 8.5 [IQR 7.9-9.0] years, 263 (6.6%) cardiovascular events occurred, of which 195 were cardiac events (4.9%). Using multivariable fractional polynomials, we found no significant deviations from linear associations of dp-ucMGP with cardiovascular and cardiac events (*P*=0.7 and *P*=0.9, respectively). Log_2 dp-ucMGP was significantly associated with cardiovascular and cardiac events in the total study population, but these associations lost significance after adjustment for potential confounders (Table 2). Because we found a significant negative interaction between log_2 dp-ucMGP and systolic blood pressure (Table 2 and Supplementary Figure S1), we also performed analyses in subjects with and without hypertension. Log_2 dp-ucMGP was significantly associated with cardiovascular and cardiac events in subjects without hypertension, but these associations lost significance after adjustment (Table 2). We found a significant inverse association of log_2 dp-ucMGP with cardiac events in subjects with hypertension, which was materially unchanged after adjustment for potential confounders (Table 2). These results were materially unchanged after further adjustment for the presence or absence of an UAE >10 mg/L or after exclusion of subjects that used vitamin K antagonists (data not shown).

Dp-ucMGP and mortality

After a median follow-up time of 8.5 [IQR 8.0-9.1] years, 279 subjects (6.5%) had died, with 74 (1.7%) deaths attributable to cardiovascular causes. We found significant deviances from linear associations of dp-ucMGP with all-cause and cardiovascular mortality (both *P*-values <0.001). In line with the best fitting fractional polynomial model, we included a linear and quadratic term in the Cox regression models predicting mortality. We found a significant interaction between dp-ucMGP and systolic blood pressure (*P*=0.01 for both all-cause and cardiovascular mortality), but these interactions lost significance after adjustment for age and gender (*P*=0.7 for all-cause mortality and *P*=0.3 and cardiovascular mortality). We found no significant interactions of dp-ucMGP with BMI, diabetes, CKD, or CVD.

The J-shaped associations of dp-ucMGP with all-cause and cardiovascular mortality are shown in Figure 2 and Table 3. Plasma dp-ucMGP was significantly associated with all-cause and cardiovascular mortality. These associations remained significant after adjustment for potential confounders (Table 3). The associations of dp-ucMGP with mortality remained materially unchanged after further adjustment for the presence or absence of an UAE >10 mg/L (data not shown) or after exclusion of subjects that used vitamin K antagonists (Supplementary Table S1).

Finally, we specified a cut-off value for dp-ucMGP to identify subjects at risk for mortality. The optimal cut-off value was 414 pmol/L for all-cause mortality and 557 pmol/L for cardiovascular mortality.

Table 2. Associations of log_2 dp-ucMGP with cardiovascular and cardiac events in subjects without a history of cardiovascular diseases at baseline.

	All Subjects [a]		No Hypertension [b]		Hypertension [c]		*P* for interaction [d]
	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	
CVE							
Model 1	1.34 (1.19-1.50)	<0.001	1.41 (1.18-1.68)	<0.001	1.01 (0.89-1.16)	0.8	<0.001
Model 2	1.05 (0.94-1.16)	0.4	1.14 (0.97-1.34)	0.1	0.92 (0.81-1.06)	0.3	0.006
Model 3	1.02 (0.91-1.14)	0.8	1.09 (0.92-1.29)	0.3	0.95 (0.82-1.09)	0.5	0.008
Model 4	1.01 (0.90-1.12)	0.9	1.08 (0.91-1.28)	0.4	0.93 (0.81-1.08)	0.4	0.007
CE							
Model 1	1.21 (1.06-1.37)	0.004	1.36 (1.11-1.66)	0.003	0.91 (0.79-1.04)	0.2	<0.001
Model 2	0.98 (0.87-1.10)	0.7	1.11 (0.92-1.33)	0.3	0.84 (0.73-0.98)	0.02	0.005
Model 3	0.96 (0.85-1.09)	0.5	1.07 (0.88-1.29)	0.5	0.87 (0.74-1.01)	0.07	0.007
Model 4	0.95 (0.84-1.07)	0.4	1.06 (0.88-1.26)	0.6	0.86 (0.73-1.00)	0.05	0.005

Abbreviations: CE, cardiac events; CVE, cardiovascular events; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; hs-CRP, high sensitive C-reactive protein; UAE, urinary albumin excretion; VKA, vitamin K antagonists.

[a] n_{CVE}/n_{total} = 263/3,967, n_{CE}/n_{total} = 195/3,967;
[b] n_{CVE}/n_{total} = 124/3,147, n_{CE}/n_{total} = 94/3,147;
[c] n_{CVE}/n_{total} = 139/818, n_{CE}/n_{total} = 101/818;
[d] *P*-value for interaction between log_2 dp-ucMGP and SBP.

Model 1: crude.
Model 2: adjusted for age and sex.
Model 3: as model 2 + race, education, smoking, BMI, SBP, cholesterol-HDL ratio, ln hs-CRP, type 2 diabetes, use of antihypertensive drugs, and use of VKA.
Model 4: as model 3 + eGFR and ln UAE.

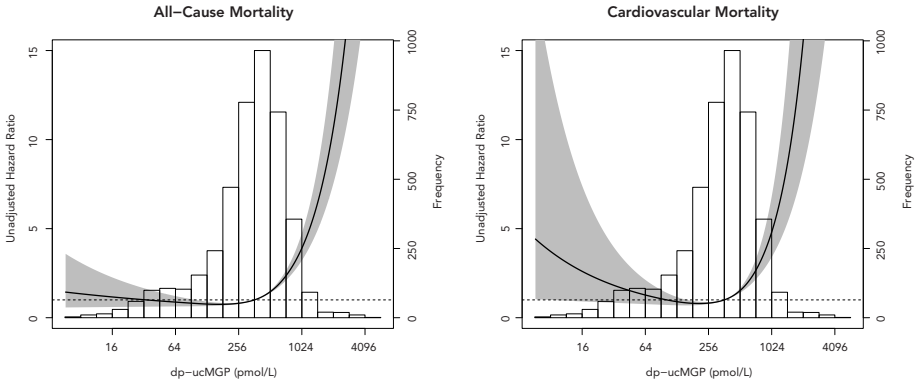


Figure 2. Restricted cubic spline depicting the J-shaped association of dp-ucMGP with all-cause and cardiovascular mortality. The line in the graph represents the risk for all-cause and cardiovascular mortality. The grey area represents the 95% CI of the HR.

Table 3. Associations of $\log_2$ dp-ucMGP with all-cause and cardiovascular mortality.

	All-Cause Mortality (n_{events}/n_{total} = 279/4,275)		Cardiovascular Mortality (n_{events}/n_{total} = 74/4,275)	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Model 1				
Linear term	0.20 (0.12-0.33)	<0.001	0.20 (0.12-0.33)	<0.001
Squared term	1.14 (1.10-1.17)	<0.001	1.14 (1.10-1.17)	<0.001
Model 2				
Linear term	0.27 (0.15-0.47)	<0.001	0.27 (0.15-0.47)	<0.001
Squared term	1.10 (1.06-1.13)	<0.001	1.10 (1.06-1.13)	<0.001
Model 3				
Linear term	0.36 (0.18-0.72)	0.004	0.36 (0.18-0.72)	0.004
Squared term	1.07 (1.03-1.12)	0.002	1.07 (1.03-1.12)	0.002
Model 4				
Linear term	0.33 (0.17-0.66)	0.002	0.33 (0.17-0.66)	0.002
Squared term	1.08 (1.03-1.13)	0.001	1.08 (1.03-1.13)	0.001

Abbreviations: CVD, cardiovascular diseases; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; hs-CRP, high sensitive C-reactive protein; UAE, urinary albumin excretion; VKA, vitamin K antagonists.

Model 1: crude.
Model 2: adjusted for age and sex.
Model 3: as model 2 + race, smoking, education level, BMI, SBP, cholesterol-HDL ratio, ln hs-CRP, type 2 diabetes, history of CVD, use of antihypertensive drugs, and use of VKA.
Model 4: as model 3 + eGFR and ln UAE.

Discussion

In the present study, we demonstrate that functional vitamin K insufficiency, as derived from the plasma dp-ucMGP concentration, is a common phenomenon associated with serious adverse health consequences. In this large general population-based cohort, 3 out of 10 subjects were vitamin K insufficient at baseline. Among elderly and subjects with hypertension, type 2 diabetes, CKD, and CVD, this prevalence was significantly higher (i.e., ~50%). Furthermore, we found J-shaped associations of plasma dp-ucMGP concentrations with all-cause and cardiovascular mortality. These associations remained significant after adjustment for potential confounders.

Previous studies have demonstrated that plasma dp-ucMGP concentrations are markedly increased in patients with cardiovascular and renal disease (5,8,9,11). In line with these findings, we found that the prevalence of functional vitamin K insufficiency was significantly higher among disease populations including hypertension, type 2 diabetes, CKD, and CVD. These high plasma dp-ucMGP levels may reflect a low dietary intake of vitamin K, which may contribute to functional vitamin K insufficiency. However, the composition of the intestinal flora (23) and impaired vitamin K recycling (24) may also contribute to vitamin K insufficiency. In a preclinical study, it was shown that the activity of γ-glutamyl carboxylase, the enzyme that catalyzes the posttranslational γ-carboxylation of vitamin K-dependent proteins such as MGP, was reduced in CKD (24).

Furthermore, it has been shown that high plasma dp-ucMGP concentrations are associated with an increased all-cause and cardiovascular (11) mortality risk in several high-risk populations including patients with aortic stenosis (10), CVD (11), CKD (8), end-stage renal disease (ESRD) (9), and renal transplant recipients (RTR) (5). Recently, Liu et al. found that plasma dp-ucMGP levels were log-linearly associated with cardiovascular mortality and curvilinearly associated with all-cause mortality in a general population cohort (13). In line with these findings, we found curvilinear associations of plasma dp-ucMGP levels with all-cause and cardiovascular mortality in this large Dutch general population-based cohort.

The association of plasma dp-ucMGP with cardiovascular events has been studied less frequently and conflicting results have been reported. In type 2 diabetes (7) and elderly (25), plasma dp-ucMGP was found to be significantly associated with an increased risk for CVD. However, plasma dp-ucMGP levels were not associated with coronary heart disease (CHD) and stroke (7,12). In contrast, Liu et al. found that plasma dp-ucMGP levels were not associated with cardiovascular and cardiac events, but were inversely associated with coronary events (13). Complementing the results of previous

studies, we found a significant negative interaction of plasma dp-ucMGP levels with systolic blood pressure on both cardiovascular and cardiac events and found that plasma dp-ucMGP levels were inversely associated with cardiac events in subjects with hypertension. It may be hypothesized that these apparently conflicting results can be explained by the fact that there are two types of vascular calcification (i.e., arterial medial calcification and atherosclerotic intimal calcification) (12,13). Arterial medial calcification is highly prevalent in CKD and type 2 diabetes and is characterized by mineralization in the tunica media, resulting in stiffening of arterial walls and impaired hemodynamics (2). Medial calcification is a complex process involving both stimulators and inhibitors of calcification, such as MGP (26). Atherosclerotic calcification is seen at sites of atherosclerotic plaques, characterized by cholesterol and lipoprotein deposits and inflammation (12,27). MGP was found to be predominantly implicated in arterial medial calcification (12,28-30). Recent evidence suggests that another vitamin K-dependent protein (i.e., growth arrest-specific 6 [gas6]) plays a role in atherosclerotic calcification (31). Gas6 has proinflammatory and prothrombotic properties and may enhance plaque inflammation in atherosclerosis (31-33). Inhibition of gas6, as in circumstances of vitamin K insufficiency, may stabilize advanced plaque, thereby preventing rupture and subsequent thrombus formation (31). This may explain the inverse association of circulating dp-ucMGP concentrations with cardiac events in subjects with hypertension. However, further research is needed to test this hypothesis and clarify the mechanisms involved.

Some limitations of the present study need to be addressed. First, given the observational nature of this study it is impossible to draw a definite conclusion about the causality of the association of dp-ucMGP with cardiovascular events and mortality. However, a recent Mendelian randomization study suggests that the association of dp-ucMGP with coronary events and non-cancer mortality is causal (13). Second, the cut-off value for functional vitamin K insufficiency (i.e., >500 pmol/L) may be arbitrary. However, Liu et al. (13) found that the risk for all-cause mortality strongly increased when plasma dp-ucMGP levels were higher than 437 pmol/L. In good agreement with this previous finding, we found that the risk for all-cause mortality strongly increased when plasma dp-ucMGP levels were higher than 414 pmol/L. These findings, together with the fact that we found an optimal cut-off value of 557 pmol/L for cardiovascular mortality, support the use of a cut-off value of 500 pmol/L as clinically relevant. Finally, data regarding vitamin K intake or plasma vitamin K status were not available in this study population. However, plasma dp-ucMGP was found to be a sensitive marker for changes in vascular vitamin K status (6). Major strengths of the present study are the prospective design, the large sample size, and long-term follow-up.

In conclusion, we demonstrated that functional vitamin K insufficiency is a common phenomenon in the general population and occurs even more frequently among elderly and subjects with hypertension, type 2 diabetes, CKD, and CVD. Furthermore, we found that plasma dp-ucMGP was curvilinearly associated with an increased risk for all-cause and cardiovascular mortality._Importantly, functional vitamin K insufficiency is not only a clinically relevant risk factor for adverse health outcomes, but it may also be a modifiable risk factor. Given the availability of vitamin K supplements, vitamin K insufficiency seems an attractive target for preventive intervention. Further prospective clinical trials are needed to investigate whether correction of functional vitamin K insufficiency can indeed improve health outcomes.

Acknowledgments

Sources of Funding: The research has been funded by TI Food and Nutrition (grant no CH001), a public-private partnership on pre-competitive research in food and nutrition. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

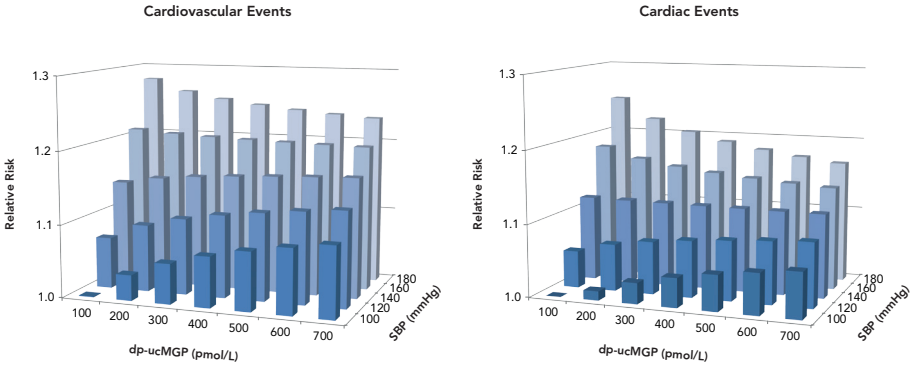
References

1. Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, et al. Heart disease and stroke statistics--2015 update: a report from the American Heart Association. *Circulation* 2015; 131(4): e29-322.
2. Demer LL, Tintut Y. Vascular calcification: pathobiology of a multifaceted disease. *Circulation* 2008; 117(22): 2938-48.
3. Luo G, Ducy P, McKee MD, Pinero GJ, Loyer E, Behringer RR, et al. Spontaneous calcification of arteries and cartilage in mice lacking matrix GLA protein. *Nature* 1997; 386(6620): 78-81.
4. Schurgers LJ, Uitto J, Reutelingsperger CP. Vitamin K-dependent carboxylation of matrix Gla-protein: a crucial switch to control ectopic mineralization. *Trends Mol Med* 2013; 19(4): 217-26.
5. Keyzer CA, Vermeer C, Joosten MM, Knapen MH, Drummen NE, Navis G, et al. Vitamin K status and mortality after kidney transplantation: a cohort study. *Am J Kidney Dis* 2015; 65(3): 474-83.
6. Cranenburg EC, Koos R, Schurgers LJ, Magdeleyns EJ, Schoonbrood TH, Landewe RB, et al. Characterisation and potential diagnostic value of circulating matrix Gla protein (MGP) species. *Thromb Haemost* 2010; 104(4): 811-22.
7. Dalmeijer GW, van der Schouw YT, Magdeleyns EJ, Vermeer C, Verschuren WM, Boer JM, et al. Matrix Gla protein species and risk of cardiovascular events in type 2 diabetic patients. *Diabetes Care* 2013; 36(11): 3766-71.
8. Schurgers LJ, Barreto DV, Barreto FC, Liabeuf S, Renard C, Magdeleyns EJ, et al. The circulating inactive form of matrix gla protein is a surrogate marker for vascular calcification in chronic kidney disease: a preliminary report. *Clin J Am Soc Nephrol* 2010; 5(4): 568-75.
9. Schlieper G, Westenfeld R, Kruger T, Cranenburg EC, Magdeleyns EJ, Brandenburg VM, et al. Circulating nonphosphorylated carboxylated matrix gla protein predicts survival in ESRD. *J Am Soc Nephrol* 2011; 22(2): 387-95.
10. Ueland T, Gullestad L, Dahl CP, Aukrust P, Aakhus S, Solberg OG, et al. Undercarboxylated matrix Gla protein is associated with indices of heart failure and mortality in symptomatic aortic stenosis. *J Intern Med* 2010; 268(5): 483-92.
11. Mayer O,Jr, Seidlerova J, Bruthans J, Filipovsky J, Timoracka K, Vanek J, et al. Desphospho-uncarboxylated matrix Gla-protein is associated with mortality risk in patients with chronic stable vascular disease. *Atherosclerosis* 2014; 235(1): 162-8.
12. Dalmeijer GW, van der Schouw YT, Magdeleyns EJ, Vermeer C, Verschuren WM, Boer JM, et al. Circulating desphospho-uncarboxylated matrix gamma-carboxyglutamate protein and the risk of coronary heart disease and stroke. *J Thromb Haemost* 2014; 12(7): 1028-34.
13. Liu YP, Gu YM, Thijs L, Knapen MH, Salvi E, Citterio L, et al. Inactive matrix Gla protein is causally related to adverse health outcomes: a Mendelian randomization study in a Flemish population. *Hypertension* 2015; 65(2): 463-70.
14. Hillege HL, Janssen WM, Bak AA, Diercks GF, Grobbee DE, Crijns HJ, et al. Microalbuminuria is common, also in a nondiabetic, nonhypertensive population, and an independent indicator of cardiovascular risk factors and cardiovascular morbidity. *J Intern Med* 2001; 249(6): 519-26.
15. Koning SH, Gansevoort RT, Mukamal KJ, Rimm EB, Bakker SJ, Joosten MM, et al. Alcohol consumption is inversely associated with the risk of developing chronic kidney disease. *Kidney Int* 2015; 87(5): 1009-16.
16. Visser ST, Schuiling-Veninga CC, Bos JH, de Jong-van den Berg LT, Postma MJ. The population-based prescription database IADB.nl: its development, usefulness in outcomes research and challenges. *Expert Rev Pharmacoecon Outcomes Res* 2013; 13(3): 285-92.

17. Inker LA, Schmid CH, Tighiouart H, Eckfeldt JH, Feldman HI, Greene T, et al. Estimating glomerular filtration rate from serum creatinine and cystatin C. *N Engl J Med* 2012; 367(1): 20-9.
18. Chobanian AV, Bakris GL, Black HR, Cushman WC, Green LA, Izzo JL,Jr, et al. The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure: the JNC 7 report. *JAMA* 2003; 289(19): 2560-72.
19. de Goeij MC, van Diepen M, Jager KJ, Tripepi G, Zoccali C, Dekker FW. Multiple imputation: dealing with missing data. *Nephrol Dial Transplant* 2013; .
20. Sterne JA, White IR, Carlin JB, Spratt M, Royston P, Kenward MG, et al. Multiple imputation for missing data in epidemiological and clinical research: potential and pitfalls. *BMJ* 2009; 338: b2393.
21. Harel O, Zhou XH. Multiple imputation: review of theory, implementation and software. *Stat Med* 2007; 26(16): 3057-77.
22. Liu X. Classification accuracy and cut point selection. *Stat Med* 2012; 31(23): 2676-86.
23. Ramotar K, Conly JM, Chubb H, Louie TJ. Production of menaquinones by intestinal anaerobes. *J Infect Dis* 1984; 150(2): 213-8.
24. Kaesler N, Magdeleyns E, Herfs M, Schettgen T, Brandenburg V, Fliser D, et al. Impaired vitamin K recycling in uremia is rescued by vitamin K supplementation. *Kidney Int* 2014; 86(2): 286-93.
25. van den Heuvel EG, van Schoor NM, Lips P, Magdeleyns EJ, Deeg DJ, Vermeer C, et al. Circulating uncarboxylated matrix Gla protein, a marker of vitamin K status, as a risk factor of cardiovascular disease. *Maturitas* 2014; 77(2): 137-41.
26. Johnson RC, Leopold JA, Loscalzo J. Vascular calcification: pathobiological mechanisms and clinical implications. *Circ Res* 2006; 99(10): 1044-59.
27. Thompson B, Towler DA. Arterial calcification and bone physiology: role of the bone-vascular axis. *Nat Rev Endocrinol* 2012; 8(9): 529-43.
28. Murshed M, Schinke T, McKee MD, Karsenty G. Extracellular matrix mineralization is regulated locally; different roles of two gla-containing proteins. *J Cell Biol* 2004; 165(5): 625-30.
29. Price PA, Faus SA, Williamson MK. Warfarin causes rapid calcification of the elastic lamellae in rat arteries and heart valves. *Arterioscler Thromb Vasc Biol* 1998; 18(9): 1400-7.
30. Schurgers LJ, Spronk HM, Soute BA, Schiffers PM, DeMey JG, Vermeer C. Regression of warfarin-induced medial elastocalcinosis by high intake of vitamin K in rats. *Blood* 2007; 109(7): 2823-31.
31. Laurance S, Lemarie CA, Blostein MD. Growth arrest-specific gene 6 (gas6) and vascular hemostasis. *Adv Nutr* 2012; 3(2): 196-203.
32. Tjwa M, Bellido-Martin L, Lin Y, Lutgens E, Plaisance S, Bono F, et al. Gas6 promotes inflammation by enhancing interactions between endothelial cells, platelets, and leukocytes. *Blood* 2008; 111(8): 4096-105.
33. Robins RS, Lemarie CA, Laurance S, Aghourian MN, Wu J, Blostein MD. Vascular Gas6 contributes to thrombogenesis and promotes tissue factor up-regulation after vessel injury in mice. *Blood* 2013; 121(4): 692-9.

Chapter
6

Supplementary Figure S1. Graphical presentation of the negative interaction of circulating dp-ucMGP with systolic blood pressure (SBP) on cardiovascular and cardiac events.



Supplementary Table S1. Associations of log_2 dp-ucMGP with all-cause and cardiovascular mortality after exclusion of subjects who used vitamin K antagonists.

	All-Cause Mortality (n_{events}/n_{total} = 244/4,169)		Cardiovascular Mortality (n_{events}/n_{total} = 65/4,169)	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Model 1				
Linear term	0.24 (0.13-0.44)	<0.001	0.24 (0.13-0.44)	<0.001
Squared term	1.12 (1.08-1.17)	<0.001	1.12 (1.08-1.17)	<0.001
Model 2				
Linear term	0.36 (0.16-0.79)	0.01	0.36 (0.16-0.79)	0.01
Squared term	1.07 (1.02-1.13)	0.008	1.07 (1.02-1.13)	0.008
Model 3				
Linear term	0.37 (0.16-0.81)	0.01	0.37 (0.16-0.81)	0.01
Squared term	1.07 (1.02-1.13)	0.01	1.07 (1.02-1.13)	0.01
Model 4				
Linear term	0.33 (0.15-0.72)	0.005	0.33 (0.15-0.72)	0.005
Squared term	1.08 (1.03-1.14)	0.003	1.08 (1.03-1.14)	0.003

Abbreviations: CVD, cardiovascular diseases; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; hs-CRP, high sensitive C-reactive protein; UAE, urinary albumin excretion

Model 1: crude.
Model 2: adjusted for age and sex.
Model 3: as model 2 + race, smoking, education level, BMI, SBP, cholesterol-HDL ratio, ln hs-CRP, type 2 diabetes, history of CVD, and use of antihypertensive drugs.
Model 4: as model 3 + eGFR and ln UAE.